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"UTILIZATION OF LOCAL ANIMAL BREEDS AND PRODUCTION SYSTEMS IN SUSTAINABLE PRODUCTION OF HIGH QUALITY ANIMAL PRODUCTS"

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SUPPORTS FOR LOCAL BREEDS IN THE EUROPEAN REGION – AN OVERVIEW

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Scientific review

SUMMARY

This study analysed the incentives for conservation of local breeds in 35 European countries, with the particular reference to the situation in Slovenia. In order to collect all necessary data in different countries, detailed questionnaire was developed and sent out to National coordinators for Animal Genetic Resources in the European Region. Data were edited and analysed using MS Excel program where basic descriptive statistics was performed to show differences among countries in incentive payments. Incentives for local breeds in Slovenia were paid from the Agri-environmental payments. The amount of payment for one livestock unit was 89.38 € per year. Subsidies for adult cattle and horses of local breeds were therefore 89.38 € per animal, while for pigs there were 44.69 € per animal and for sheep and goats 13.41 € per animal. Comparing data from different countries, the highest subsides were received for cattle ranging from 45 € to 520 € for bulls. From all 35 countries, 16 countries have subsidies for horses. Despite two breeds of sheep and one breed of goat in Slovenia highly endangered, the level of subsides for sheep and goats for local breeds included in the environmental payments were equal i.e., 13.41 € per animal. Compared to 21 countries reported the financial support for sheep, only two countries had lower support than in Slovenia. The EC Regulations can explain differences in payments where the Member States are free when determining the payments level. Another reason could be since out of 35 countries, eleven are not EU members. National coordinators from all countries agreed that financial support per head is very important tool for breed conservation and such a practice should be continued. However, the current level of support does not compensate loss of income due to lower productivity.

Key-words: local breeds, subsidies, incentives, conservation

INTRODUCTION

Direct payments are intended for farmers rearing a specific breed at extinction risk. Indirect payments encourage less intensive agriculture production that is more respectful to the environment and preserve agricultural biodiversity. Financial incentives are therefore provided for the local breed in order to compensate their lower productivity compared to highly productive ones (Environmental Performance…, 2008). In 1992 the European Union started with the financial support to the agriculture having benefits for the environment. Each Member State implemented their policies for agricultural development within the Rural Development Plan (RDP). RDPs provide direct and indirect incentives for the agrobiodiversity conservation. Today, incentive payments are provided by the Commission Regulation (EC) No 807/2014 for local breeds, being in loss danger and are genetically adapted to one or more traditional production systems or environments in the country (Commission…, 2015). The general view is that incentives are not contributed to the conservation of local breeds in a long term. Keeping local breeds is unprofitable for many farmers and the main reason is the difference in the profitability among local and highly productive breeds. Incentives are not high enough to maintain the current population of breeds at risk and farmers are not stimulated to switch from higher yielding breeds to less productive local breeds (Signorello and Pappalardo, 2003). Local breeds have economic values as well as

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social, cultural and environmental. They have ability to help sustain rural livelihoods, especially for peoples in remote areas (FAO, 2013). When countries are considered the level of supports all the values should be taken into account. The aim of this study was to analyse the incentives for conservation of local breeds in 35 countries members of the European Regional Focal Point (ERFP), with the particular reference to the situation in Slovenia.

MATERIAL AND METHODS

The survey was carried out in 35 countries members of the ERFP for Animal Genetic Resources (AnGR): Albania, Austria, Azerbaijan, Bosnia and Herzegovina, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, FYR Macedonia, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Montenegro, Norway, Poland, Portugal, Serbia, Slovak Republic, Slovenia, Spain, Sweden, Switzerland, The Netherlands, Turkey, Ukraine, United Kingdom.

In order to collect all necessary data on the level of subsidies in different countries, detailed questionnaire was developed. After revision of the questionnaire, involving stakeholders in the final stage, the questionnaire was sent out to all National coordinators for Animal Genetic Resources (AnGR) in the European Region by email. Data were collected individually and three workshops were organized for the final comments for all stakeholders included in the survey. Additional information on the questionnaire methodology was in detail presented elsewhere (Subsibreed, 2014). However, the questionnaire included thirteen chapters. Result of only one question, representing the level of subsidies included in this study. Data were edited and analysed using MS Excel program where basic descriptive statistics was performed to show differences among countries in incentive payments. The questionnaire involved different topics about legal arrangements in the field of AnGR, country programmes, definition of breed and level of endangerment, national budget allocated for the conservation of AnGR, methodologies for calculating subsidies and proposals for the improvement. In this study we discussed just one part of the questionnaire connected with the level of subsidies with the special emphasis on Slovenia.

RESULTS AND DISCUSSION

Although 35 countries were included in the project, only countries reporting level of subsidies for specific species or breeds are shown in Figures 1 to 4. Subsidies for species and breeds differed among countries evidently. A detailed list of payments for each country and level of subsidies is available upon request from the authors. No financial support for the specific species or breed was reported by three countries included in the project. Three countries reported that higher level of subsidies was allocated to the highly endangered breeds.

In accordance with the Commission Regulation (EC) No. 1974/2006 for the period from 2007 to 2014, incentives for local breeds in Slovenia were paid from the Agri-environmental payments (Commision…, 2015). The payments are allocated to the local breeds, genetically adapted to one or more traditional production systems or environments which are in danger of being lost. The highest amount of payment from agri-environmental measures for local breeds in that period have been defined for one livestock unit in the amount 89.38 € per year. Subsidies for adult cattle and horses of local breeds were 89.38 € per animal, while for pigs 44.69 € per animal and for sheep and goats 13.41 € per animal (Program…, 2007). One Livestock Unit (LU) refers to 500 kg of live weight.

Comparing data from different countries, the highest subsides are intended for the keeping of the cattle. Within 35 reported countries the level of subsides ranged from 45 € to 520 € for bulls. Only Macedonia and Montenegro, non EU members reported lower payments for local breeds compared to Slovenia. The highest payment for the cattle breed was reported by Croatia, Austria for the bulls and Greece. Hungary, Poland and Ukraine reported that payments were made only for cows, while Austria and Finland reported different level of subsides between genders (Figure 1).

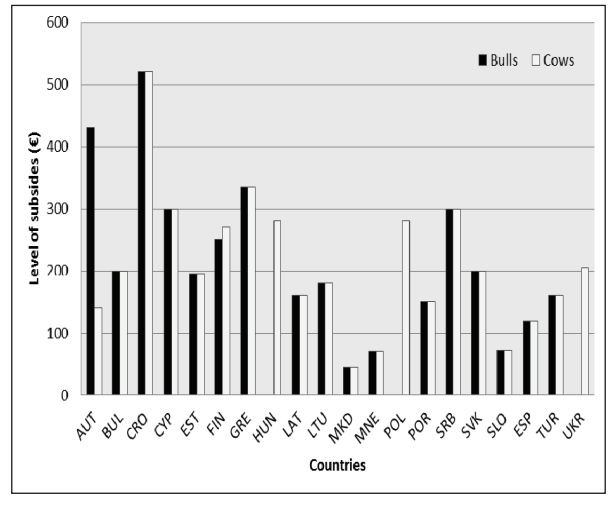

Figure 1. Level of financial support for cattle

Payments for local breeds of horses in Slovenia was 89.38 € per adult animal in the period from 2007 to 2014. From all 35 countries included in the project, only 16 countries reported supports for horses. Compared to supports in Slovenia subsidies for horses in all the reported countries were higher. Finland, Hungary and Poland allocated supports only to mares. In other countries, there were no differences between genders in the level of subsidies. The highest support for mares was reported from Austria in the amount of 430 € and the lowest in Slovenia in the amount of 72 € (Figure 2).

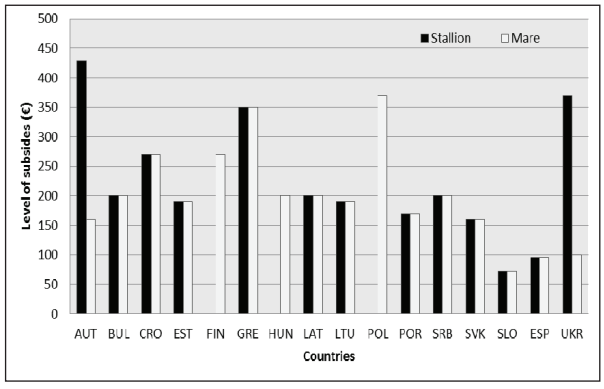

Figure 2. Level of financial support for horses

Despite two breeds of sheep and one breed of goat in Slovenia being highly endangered (Register…, 2014), the level of subsides for sheep and goats for local breeds included in the environmental payments are equal i.e., 13.41 € per animal. Compared to 21 countries reported the financial support for sheep, only two countries had lower support than in Slovenia.

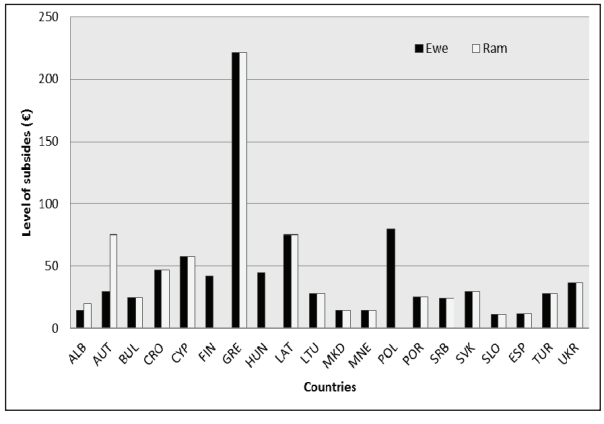

Figure 3. Level of financial support for sheep

When the amount of support is concerned, the Greece stands out with the highest level of support in the amount of 221 €. Poland, Latvia and Austria have the next highest support, Austria for rams, Poland for ewes and Latvia for both, ewes and rams. Slovenia, Spain, Macedonia and Montenegro have the lowest level of support for the sheep (Figure 3).

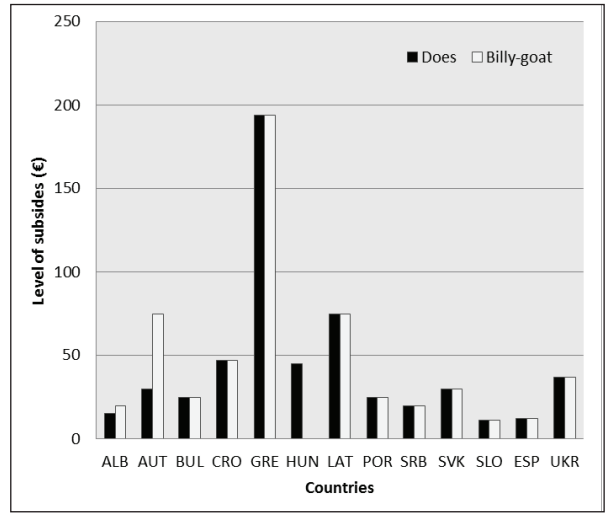

Figure 4. Level of financial support for goats

The levels of subsidies for goats were similar among the countries. However, only 13 countries reported that financial support was allocated to the particular breeds of goats. Slovenia and Spain fall in the category with the lowest level of support. Exceptions are Greece, Latvia and Austria which have the highest level of support. Greece stands out with the highest level of support in the amount of 194 € (Figure 4).

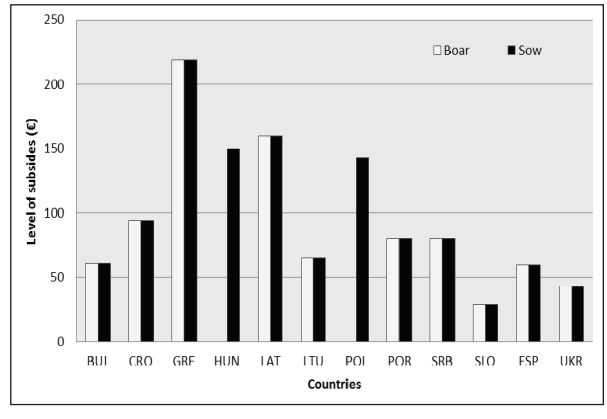

Figure 5. Level of financial support for pigs

The level of subsidies for pigs was again highest in Greece in the amount of 219 € and the next Latvia with 160 €. Slovenia again falls in the category with the lowest level of support. Hungary and Poland support only sows (Figure 5).

CONCLUSION

The countries included in this project report a diverse level of supports intended for the local breeds. The big differences in the level of support can be explained by the EC Regulations 1257/99 and 445/2002 where the Member States are free while determining the level of payments. The maximum level of payment is set on the 200 € per Livestock Unit (LU). These amounts

may be increased in the exceptional cases, member countries need to justify the higher level in the rural development programmes. Example of the successful countries, reaching the higher amount than 200 €/LU are Croatia, Austria and Greece. Another reason for the different supports is that eleven countries included in the project are not members of the EU. Despite that, few non EU countries like Serbia, Ukraine and Montenegro have the supports for local breeds included in their national programmes. In Slovenia, eleven local breeds are supported by the incentives. From 2003 when incentives started the critical population size of few breeds slightly increased. Experience shows that the population size of five local breeds in Slovenia remained stable in last few years while the population size of one breed decreased. The populations of another six breeds slightly increased. Comparing and looking at other countries data we can conclude, that even with the financial support, population size in some countries decreased or remain stable, taking long-term sustainability of such payments under the consideration. Nevertheless, without subsidies local breeds in some countries could be lost very quickly. National coordinators from all the included countries agreed that financial support per head is very important tool for breed conservation and such a practice should be continued. However, the current level of support does not compensate the loss of income resulting from the lower productivity of local breeds. For the long-term sustainable conservation of AnGR, some other ways of funding are proposed such as support to the national AnGR programmes, promoting and awareness raising, financing of the gene bank and marketing products of local breeds. In general, financial support should be intended to contribute to the long-term sustainability and self–sufficiency of specific local breed.

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RESEARCH ON DIVERSITY, UTILIZATION AND PRODUCTION QUALITY OF LOCAL BREEDS IN SLOVAKIA

Kasarda, R., Trakovická, A., Moravčíková, N., Šidlová, V., Kadlečík, O.

Scientific review

SUMMARY

State of the research in the area of utilization of local breeds in Slovakia provides sufficient information for the farmers and breeders associations either on level of diversity based even on pedigree or molecular information as well as the information on molecular composition of milk and meat for animals selection to produce specific quality products. With research and development applied we aim not only to protect something ancient, historical, but also provide progress to increase profitability with ensured safety and sustainability. Present research in area of local breeds utilization is oriented on study of functional traits in cattle as well as sport performance traits in warm blood horses.

Key-words: degree of endangerment, genetic diversity, livestock, local breeds

INTRODUCTION

In the last 40 years, production ability improvement of farm animals was mainly assigned (85%) to the genetic and animal breeding. The main challenge, pushing the research forward, is that the World needs more food. According to conclusion of the World food summit held in Rome, the agricultural production will increase by 70% till 2050. The farmers say that today's farm management isn't improving as fast as animal genetics. In front of us is the responsibility to show consumer future of the farms in form as it was made for example with computers. The responsibility on the other hand means that with afforded opportunities there are many expectations facing us, as to make agriculture attractive and to increase the profitability and ensure the sustainability. Using local breeds we try to tell our story that farm life is attractive, providing it to consumers as a brand. With research and development applied we aim not only to protect something ancient, historical, but also provide progress to increase profitability with ensured safety and sustainability.

With increased demand for animal origin food, some branches became very intensive, like milk production, pork production, poultry production using of specific breeds or hybrids even more. Traditionally bred local breeds became marginal. After 40 years of breeding for increased production those modern breeds miss the story, they lack adaptability, immunity, multi-purposity and robustness as known from formerly kept breeds. These are the opportunities provided by local breeds.

STATE OF THE BREEDS ENDANGERMENT IN SLOVAKIA

Breeds of horses, cattle, goat, sheep and poultry (Table 1) belong to the farm animal genetic resources in Slovakia (AnGR). Slovak spotted and Slovak Pinzgau whose origin is composite of autochthonous Carpathian Red (extinct) and Carpathian grey (extinct) from 17th to 18th century as well as Swiss Simmental and Austrian Pinzgau from 19th century common in Austro-Hungarian Empire belong to the main cattle breeds of national interest. Wallachian sheep and Hucul horse became present from Wallachian migration to Area of today's Slovakia. Even Original Wallachian sheep was replaced by Wallachian improved breed. Today there are activities to re-establish stock of original Wallachian sheep based on back-cross of negative variants. Besides Hucul, there are other horse breeds which were traditionally bred for purposes of Austro-Hungarian army: Furiosso, Nonius or aristocracy: Arab, Shagya-Arab, Lipizan, English thoroughbred. The heavy draught horse represents Norik of Muran. Experimental breeding of warm blood horses

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resulted in Slovak Sport Pony or Slovak Warm Blood horse. Breeder's interests re-started the Brown Short Hair goat breeding whereas national Suchovska goose or Oravka chicken hardly survives.

Table 1. Inventory of Farm Animal Genetic Resources in Slovakia (Hetényi et al., 2006)

Species	Breeds			Total
	Local	New import	Long time import	
Cattle	2	8	1	11
Sheep	4	4	4	12
Goat	2	1	-	3
Pig	4	-	4	8
Horse	9	-	5	14
Fur animals	10	-	1	11
Rabbit	12	-	39	51
Poultry chicken	7	-	24	31
Duck	2	-	3	5
Goose	4	-	2	6
Turkey	1	-	3	4
Quail	1	-	3	4

METHODS OF EVALUATION OF DEGREE OF ENDANGERMENT

Pedigree based evaluation

Kadlečík et al. (2007, 2011) evaluated complexity of pedigrees and equivalent parameters in purebred Slovak Pinzgau population and estimated intensity of inbreeding. From 1994 the population has been recognized as endangered. Hazuchová et al. (2010) evaluated the diversity of Pinzgau population based on pedigree information of active AI sires, estimated inbreeding levels and characteristics based on probability of gene origin. Lower pedigree completeness as well as unequal founder contribution was observed due to use of only several sires, but with positive tendency in inbreeding decrease. Hazuchová et al. (2012, 2013) evaluated genetic diversity of Slovak spotted bulls using parameters based on gene origin probability. Even with population not endangered signs of diversity loss were observed due to decrease in effective number of founders in reference population as well as overall decrease of genetic diversity as the consequence of unequal contribution of founders. Diversity of cattle breeds was also evaluated by Kadlečík et al. (2013). The whole analysed populations consisted of (reference populations in brackets) 274,756 (94,357) Holstein, 109,686 (36,949) Slovak spotted and 9,756 (2,501) Slovak Pinzgau cattle. Indices of pedigrees completeness differed by breeds but in the 5th generation their values were 18.4% in Slovak Pinzgau, 56% in Slovak Spotted breed and 63.8% in Holstein. Generation interval of Slovak Pinzgau was 7.2 years, tending to be shorter as the population size was bigger. There was 30% of inbred Slovak Pinzgau, 42.8% in Slovak Spotted and 83% in Holstein cows and sires in the reference populations. The average inbreeding coefficient ranged from 0.36% for the Slovak Spotted to 1.32% for the Holstein. The highest average individual increase of inbreeding ΔF_i=0.29% was found for the Holstein and the average individual relatedness coefficient AR=0.8% in Slovak Spotted cattle. Inbreeding trends in the whole reference populations as well as purebred animals were positive with increasing average values by animal birth years since 1990. Pavlík et al. (2014) performed diversity joint genealogical analysis evaluation of Pinzgau cattle populations in Slovakia and Austria and found minimum differences between respective populations. The same authors (2014) evaluated basic measures of diversity in Slovak Holstein (H) and Red-Holstein (R) populations by the pedigree analysis. Results presented higher inbreeding level in H than in R including higher increase in inbreeding. On the other hand diversity in R population is more significantly reduced by bottleneck and genetic drift.

Pjontek et al. (2010) analysed genetic diversity of endangered thoroughbred Arab horse population based on description probability of identity by gene descent and origin. They have observed that the high number of founders registered in the studbook would suggest the existence of a large genetic basis for a stallion's selection program. However, historical constraints and the breeding policy carried out by the breeders have reduced the available genetic variability as reflected in small values computed for parameters such as the effective number of founders and ancestors. Pjontek et al. (2012) conducted an analysis of the genetic diversity in four endangered horse populations bred in Slovakia, describing parameters on the probability of identity by descent and gene origin. The whole analysed populations consisted of (reference populations in brackets) 656 (158) Hucul horses, 2052 (162) Lipizan horses, 1951 (171) Shagya Arabian horses and 220 (42) Slovak Sport Ponies. The equivalent complete generations ranged from 4.93, for the Slovak Sport Pony, to 10.25, for the Lipizan horses. The average value of inbreeding ranged from 2.67%, for the Slovak Sport Pony, to 6.26%, for the Hucul. The average relationship coefficients were from 3.08%, for the Shagya Arabians, to 9.34%, for the Huculs. Individual increases in inbreeding ranged from 0.43%, for the Lipizans, to 1.06%, for the Huculs, while the realised effective sizes were from 117.14 to 47.67 animals. The evaluated populations derived from 80 to 499 founders. The effective number of founders ranged from 26 to 160, while the effective number of ancestors from 7 to 32. Kadlečík et al. (2012), Kadlečík and Kasarda (2014) assessed genetic diversity of Slovak Sport Pony nucleus based on pedigree information. Pavlík et al. (2014) evaluated the breeding population of Thoroughbreds in Slovakia using pedigree analysis. The examined population consisted of 123 mares and 10 stallions registered in Slovak Stud Book. The average inbreeding coefficient was 0.86% in the whole

reference population (133 individuals) and increase in inbreeding per generation was 0.17%, in stallions 0.25%. The number of founders in the investigated population was 949. The effective number of founders was 202, while the effective number of ancestors was 67. These results point out unbalanced contribution of founders and ancestors into reference population as well as the bottleneck effect occurrence.

MOLECULAR BASED EVALUATION

Židek and Kasarda (2010) provided information on the genetic structure in Pinzgau cattle in Slovakia based on polymorphism of 61 monovalent sera. Genetic distance between 777 animals has been computed. Obtained genetic distances were pooled according to relationship coefficient R_{XY}. Average genetic distance decreased from 0.225 (group with R_{XY} between 0 and 0.125) to 0.147 (group with R_{XY} between 0.375 and 0.5). Šidlová et al. (2013), Šidlová et al. (2014 a, b) evaluated genetic diversity of Slovak Pinzgau cattle using 8 microsatellite markers (TGLA122, CSSM66, TGLA227, ILST006, CSRM60, ETH3, BM1824, SPS115). Microsatellites were highly polymorphic with a mean number of 11 alleles (ranging from 9 to 16 per locus) and total number of 88 alleles. High level of polymorphism confirms also the average value of PIC (0.7662). The overall average of observed and expected heterozygosity has reached similar values (0.7927 and 0.7980), but the differences are noticeable at each locus separately. Židek et al. (2014) used molecular information on microsatellites to model case of missing pedigree information, when other methods can be used for traceability of animal´s origin. They concluded that genetic diversity written in genetic data is holding relatively useful information to identify animals originated from individual countries. Kasarda et al. (2014a, b), Šidlová et al. (2014a) evaluated the level of SNP polymorphisms and described the basic characteristic of the analysed population genotyped using the BovineSNP50 genotyping array. In total 19 purebred Pinzgau bulls were successfully genotyped with Illumina BovineSNP50 BeadChip (98.96% of SNPs) with average call rate 0.995. Genotyping results from 54,906 SNPs revealed that 43,120 SNPs (78.96%) were polymorphic with average minor allele frequency 0.273±0.133. Within 43,120 SNPs genotyped, 98.19% were autosomal, with 776 polymorphic SNP on chromosome X and only one on chromosome Y. The average values of the observed and expected heterozygosity across polymorphic loci were 0.375±0.157 and 0.362±0.126, respectively. Sufficient proportion of heterozygotes indicated the value of F_{IS} (0.037±0.031). Šidlová et al. (2014b, 2014c) derived inbreeding coefficient from runs of homozygosity. The highest level of autozygosity (F_{ROH1}=10.88%) as well as the longest ROH segments in total (271.84 Mb) has been observed in Slovak bull Norfolk having Austrian origin. Austrian bull Nero had the highest number of ROH (80) and the second top inbreeding (9.63%). Carlo, Slovak bull of Canadian origin had the lowest number of ROH, length of ROH segments and inbreeding level. Performing analyses with ROH of different lengths here allows estimation of the distance of the current population from the base population, hence provides information on inbreeding age. Previous results reported from other study on Pinzgau breed from Austria (F_{ROH1}=0.069) shows higher inbreeding levels than those found in this study on Slovak Pinzgau (F_{ROH1}=0.0519). It is also noticeable that bulls with Austrian origin have overall higher F_{ROH} levels.

DIVERSITY OF PRODUCTION TRAITS

Trakovická et al. (2013a) and Moravčíková et al. (2013) analysed in total 296 blood samples of Slovak Spotted and 85 hair roots samples of Pinzgau cows to verify the associations of polymorphisms in bovine *LEP* and *LEPR* genes with production and reproduction traits in Slovak Spotted and Pinzgau cows. Long–life production: milk, protein, and fat yield and reproduction traits: age at first calving, calving interval, days open, and insemination interval were evaluated. Trakovická et al. (2013b) analysed genetic diversity in population of Slovak Spotted cattle based on Pit-1/*Hin*fI polymorphism. Moravčíková et al. (2012a) analysed association of bovine growth hormone gene polymorphism with milk performance traits in Slovak Spotted cows and Moravčíková et al. (2012b) studied polymorphism in the intron region of the leptin gene on bovine chromosome in relation to evaluation of genetic diversity. Moravčíková et al. (2013) identified SNPs of leptin (*LEP*), leptin receptor (*LEPR*) and growth hormone (*GH*) genes in order to analyse genetic diversity of Slovak Spotted cattle and evaluate their effect on production traits. Meluš et al. (2008a,b) analysed *SCD1* gene polymorphism (T878C) in the Slovak Pinzgau steers in relation to the haematological parameters. Miluchová et al. (2013) identified A1 variant of bovine beta casein which involves ischemic heart disease and diabetes mellitus in human. The digestion of A1 beta casein can result in the production of bioactive beta casomorphin-7 (BCM-7) whereas this is not the case with A2. In the total population of cattle homozygotes A2A2 (0.5405) were the most frequent, while homozygotes A1A1 (0.1261) were the least frequent ones. This suggests a superiority of allele A2 (0.7072) which does not produce BCM-7, and thus is safe for human consumption. The expected homozygosity for gene *CSN2* was in the population stating a slight increase in homozygosity (0.5858). This caused a slight decrease in the level of possible variability realization (41.80%), which corresponds to the effective number of alleles (1.7071). Miluchová et al. (2014) studied genetic structure of five candidate genes for milk production in Slovak Pinzgau breed. A total of 86 mothers of bulls of Slovak Pinzgau cattle were used in this study. Slovak Pinzgau cattle exhibited the high values of heterozygosity, polymorphism information content, effective number of alleles and level of possible variability realization for genes *CSN2*, *CSN3* and *LALBA*.

In opposite, high values of homozygosity were observed for genes *CSN1S1* and *LGB*. Meat tenderness is one of the major characteristic qualities of beef not only for consumers but for breeders of beef cattle too. Selection of cattle focussed on an increment of meat tenderness is complicated because this trait has large variability not only between different breeds but between individuals of equal breed too. Similarly a measurement of meat tenderness is expensive because it is done after slaughter of animal and ageing of meat *post mortem*. Therefore a several methods are developed and made possible to increase meat tenderness. However variance still exists in values of meat tenderness caused by distinctness genetic base of animal. The most significant candidate genes (*CAPN1*, *CAST*) coding formation of the calpains – calpastatin proteolytic system, exercising an influence on tenderness was described by using molecular genetics methods. The single nucleotide polymorphisms (SNPs) in these genes were used by Gábor et al. (2010) to design genetic marker panels applying commercially available test. Gábor et al. (2012) analysed the population of 113 animals of Slovak Simmental (42 bulls and 71 cows) for the missense mutation resulting in SNP polymorphism in exon 3. The SNP in *CAST* gene (c.283 C>T) was detected by PCR-RFLP method with restriction endonuclease *Msp*I. The following frequency of alleles and genotypes for the SNP c.283 C>T of the CAST gene were detected in the analysed population of Slovak Simmental cattle. Frequencies of favourable C allele were 0.6460 whereas of genotypes were 0.4336 (genotype CC), 0.4248 (genotype CT) and 0.1416 (genotype TT).

CONCLUSION

State of the research in the utilization area of local breeds in Slovakia provides sufficient information for the farmers and breeders associations either on level of diversity based even on pedigree or molecular information as well as the information on molecular composition of milk and meat for selection of animals to produce products of specific quality. Provided information present missing innovation necessary for farmers to stay sustainable. The studied populations were evaluated in complex not only quantitative but also qualitative measures allowing global view on future performance of local breeds and populations. The present research in area of local breeds utilization is oriented on study of functional traits in cattle as well as sport performance traits in warm blood horses. Further research in area on genetic diversity will be molecularly based to get detailed view on genetic structures.

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LOCAL PIG BREEDS AND PORK PRODUCTS IN CROATIA AND SLOVENIA – UNEXPLOITED TREASURE

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Scientific review

SUMMARY

The rationale, the concept and key challenges of the H2020 project TREASURE dealing with local pig breeds is presented and discussed. The action addresses the phenotypic and genetic characterisation, performance of local pig breeds in diverse production systems and their environmental impact, specific quality of their products and market potential. The goal is to build up the capacities to develop sustainable pork chains based on local pig breeds. A special emphasis is given to describe the workplan for Black Slavonian and Turopolje local pig breeds from Croatia and Slovenian Krškopolje pig.

Key-words: sustainable pig production, local pig breeds, product quality

INTRODUCTION

Pig meat is the main product of meat sector in the European Union. In 2013 almost 22 million tonnes of pork was produced within EU-28 which is three times higher than the production of beef/veal (Eurostat, 2014). Such production needs highly effective pig production. Pork production is concentrated in few EU regions; in 2013 a quarter of its production was recorded in Germany (24.9 % or almost 5.5 million tonnes) followed by Spain (15.6%) and France (8.8%). Notable production of pork was recorded also in Poland and Denmark with the shares of 7.7% and 7.2%, respectively (Eurostat, 2014). Owing to that, EU has a self-sufficiency of about 110% and exports about 12% of its total production. These results make currently EU the world's second biggest producer of pig meat after China and also the biggest exporter. In relation to the production system most of the pigs in EU are fattened in intensive systems, i.e. on large farms with three quarters of pigs produced by just 1.5% of the largest farms (Marquer et al., 2014). The trend of concentration of the pig production on large farms is due to economic reasons (farms fixed costs are divided by a larger number of animals which consequently increases productivity and reduces the average cost of production). Such efficiency would not be achievable without technological advances. Modern pig production needs a variety of experts to handle the issues of herd health, production management, waste management, reproduction, genetics, nutrition, meat quality and safety, business management and more. It is clear that technological advances could not be achieved without growing knowledge and innovative ideas founded on scientific research. Indeed, the most advanced tools are today in everyday use for the improvement of economically important traits of pigs. For example, the genetic gain on body composition of breeding pigs is extensively studied by means of computed tomography (CT) as it is used in Norway as a part of Norsvin breeding system where 3500 boars are scanned annually (Kongsro, 2014). The same author reported an estimated 30% increase of genetic gain on lean meat percentage by the use of this non-invasive technology for collecting the data for phenotyping *in vivo*.

The development in pig production worldwide followed exactly the same scenario during the last 40 years. This situation caused spreading of genetically improved, productive pig breeds on a global scale and a reduction of population size in many local breeds which are less performing and some of them are nowadays close to extinction. However, the above described trends in a modern pig production, resulted in lower quality of their products, lower resilience and

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robustness, larger farms were often not able to solve problems related with handling of animal wastes causing pollution and animal welfare issues which are more and more important for the consumer. One of the first reactions to this development were different initiatives for preservation of small local breeds from extinction and how to make them competitive in modern animal production. Some governments tried to achieve this task with financing in-situ conservational farms e.g. direct payments to the breeders of local breeds but this was a relatively expensive solution and cannot give a warranty for preservation of a large number of local breeds.

To demonstrate an alternative a new paradigm of pig production was proposed in a Horizon 2020 project TREASURE aiming to improve the knowledge, skills and competences necessary to develop existing and create new sustainable pork chains based on European local pig genetic resources (local breeds).

To achieve this goal, we will try to find good answers to the following questions:

- What is the value of traditional local breeds and production systems they are held in?
- Are local breeds and their diversity valuable for the food chain?
- How can we bring biodiversity to the market and ensure that the consumer will recognize this value and appreciate it?

In this project the breeds from Croatia and Slovenia are also included, namely the Black Slavonian (Crna slavonska svinja), Turopolje (Turopoljska svinja) from Croatia and Krškopolje (Krškopoljski prašič) from Slovenia. In the past years due to many efforts these breeds have been preserved from extinction. However, they remain untapped, with their potential little explored and exploited. This denotes a special interest of the stakeholders to benefit as much as possible from the activities and network of the TREASURE project. Following is the description of the project activities scheduled for these breeds.

Figure 1. Croatian local pig breeds Black Slavonian (left), Turopolje (middle) and Slovenian local pig breed Krškopolje breed (right) will be studied in H2020 project TREASURE

TREASURE

Diversity of local pig breeds and production systems for high quality traditional products and sustainable pork chains

Funded by European Union
Horizon 2020
Grant agreement No 634476

Start of the action: 1 April 2015

Duration: 48 months

Budget: 3,395,986.75 EUR

25 partners from 9 countries

Coordinator: Kmetijski inštitut Slovenije = Agricultural Institute of Slovenia

www.treasure.kis.si

List of local pig breeds involved in the project

Country	Native name for pig/breed	Alternative name (found in the literature
DE	Schwäbisch-Hällisches	Schwäbisch-Hällisches
ES	Ibérico	Iberian
ES	Negre Mallorquí	Black Majorcan
FR	Gascon	Gascon
FR	Basque	Basque
HR	Crna slavonska	Black Slavonian
HR	Turopoljska	Turopolje
IT	Cinta Senese	Cinta Senese
IT	Mora Romagnola	Mora Romagnola
IT	Sarda	Sarda
IT	Apulo Calabrese	Apulo Calabrese
IT	Casertana	Casertana
IT	Nero Siciliano	Nero Siciliano
LT	Lietuvos vietinės	Lithuanian indigenous wattle
LT	Senojo tipo Lietuvos baltosios	Old type Lithuanian White
PT	Bisaro/Bisara	Bisaro
PT	Alentejano/ Alentejana	Alentejano
RS	Mangulica	Mangalitsa
RS	Moravka	Moravka
SI	Krškopoljski/ Krškopoljska	Krškopolje

Figure 1. Basic data about the project, partners and local pig breeds involved

PHENOTYPIC AND GENETIC CHARACTERIZATION OF BLACK SLAVONIAN AND TUROPOLJE BREEDS FROM CROATIA AND KRŠKOPOLJE BREED FROM SLOVENIA

In the project more than 20 local breeds (60 animals per breed) shall be studied which differ considerably in regard to their exploitation. Also the amount of information available for certain breeds is very different. In some breeds, as is the case of Black Slavonian, Turopolje and Krškopolje breeds, only scarce phenotype and population data are available but in other breeds, mainly those commercially more exploited (e.g. Iberian) even some molecular data are available. The basic phenotypic characterisation and establishing of population census will be the first step in our analysis. Our goal will be to use already existing information and to complete it with new data which will be collected during the project. We will try to collect also production data reflecting various production traits (growth performance, reproductive traits, body composition, and product quality). In addition to these standard traits we will establish also collection of some new types of data as adaptability, gut microbiota and some metabolic parameters. To get the basic information about the genetic richness of the population we will perform high throughput genotyping from each breed using high density SNP chips. Also, we will collect already existing information about STR allele frequencies in some breeds. In order to discover genetic background for production traits we will estimate allele frequencies of the known major genes (IGF2, RYR1, MC4R, PRKAG3, LEPR, MC1R, KIT, CALP, FTO). Since the adaptive traits are more and more of interest for pig breeders, we will try to compare genomic information of local, well adapted breeds with modern breeds using genome sequencing strategies. We will sequence several animals from each breed in order to identify genomic regions involved in shaping of adaptive traits. Based on these data we will develop a panel of DNA markers which will be useful for breed authentication and traceability of animal products. Our ambition is also to develop a custom DNA chip which will allow cheap genotyping of loci informative for breed identification and for characterisation of genetic base for important production and adaptive traits.

In order to better understand the physiological base of production traits which are also reflected in the quality of products we will perform some pilot functional studies on gene expression in selected pig populations with the best developed characterisation of specific products. This will allow us to identify population specific physiological processes affecting product quality. In addition, we will also investigate changes in gene expression related to diets, immunocastration and some technological parameters using RNA sequencing. The most promising effects will be validated using quantitative PCR. Finally, as an important factor determining pro-

duction parameters, the intestinal microbiota as a part of complex biological system will be analysed. Using massive parallel sequencing technology (e.g. Illumina's MySeq or Roche's 454) we will characterise 16S rRNA sequences in gut microbiota in order to establish proportions of main microbial taxa in different combinations of host genotype and specific diet.

PERFORMANCE AND MANAGEMENT OF BLACK SLAVONIAN, TUROPOLJE AND KRŠKOPOLJE BREED IN THEIR PRODUCTION SYSTEMS

Along with genetics, production system is the most important factor that affects all traits of interest for pig breeders. Unlike in modern breeds, there is a general lack of information about housing and nutritional requirements of local pig breeds. In particular, such information is lacking in Black Slavonian, Turopolje and Krškopolje breed. In Europe, rather wide variety of productive systems used in rearing of local pig breeds exists: indoor, outdoor housing, organic production, silvopastoral systems, deep litter; all of these are worth and will be investigated systematically. For small scale pig production farms or family farms it is reasonable to assume that a simpler, less capital-intensive systems are more suitable, and if their products have higher quality (as expected by consumers) the chances of profitability can be increased. Budimir et al. (2013) described the possibilities of silvopastoral keeping of the Black Slavonian pigs as cost effective, environmental and animal friendly system which can result in improved meat quality traits and nutritional characteristics of pork products. As the main shortcomings of such production system authors stressed the possibility of mating with wild boars and transmission of contagious diseases, which are common risks in outdoor pig farming systems, as recently reviewed by Salajpal et al. (2013). Also, the knowledge on growth and performance of local pig breeds is very limited. For example, the growth characteristics of pigs kept under ad libitum and restricted feeding regimes were studied by Kušec et al. (2007a); models for prediction of optimal slaughter weight/age were given and their accuracy was demonstrated on modern hybrids (Kušec et al., 2007b; Vincek et al. 2012). However, such investigations were never carried out on indigenous breeds. Within a scope of investigations to be carried out in Croatia during the project TREASURE growth characteristics, fattening traits, carcass traits and meat quality traits will be studied on Black Slavonian pigs kept in two different housing systems – outdoor and indoor (deep litter). It is expected that in this manner information crucial for decision making in the production of Black Slavonian pigs and pork products will be collected and shared with scientific and professional public.

Research on local Turopolje pigs will be directed at high quality meat products development, which may represent a new and effective model for recovery and long-term conservation of this still endangered breed on economically sustainable base. For development and market success of distinguishing, value-added pork products from local pig breeds it is, however very important to establish the link between the traditional production systems (e.g. outdoor pig farming) and its natural feed resources (e.g. forest, pasture) with the distinctive physical, sensory and/or nutritional attributes (e.g. more polyunsaturated fatty acid profile) of product. Hence, information about aspects benefiting to product quality can be used as a relevant differentiation tool in marketing of such products (Edwards, 2005). Hence, in the present project, the influence of traditional feeding resources of oak (Quercus robur) acorns in outdoor production system will be investigated in relation to various aspects of Turopolje breed performances and meat/product quality.

Similarly, an experiment will be performed comparing performance, carcass and products' quality from Slovenian Krškopolje pig fed different diets and raised in different production systems (conventional and organic, indoor, outdoor). Also, a collection of data will be performed for multicriteria evaluation (productivity, welfare and environment) of Krškopolje pig in different production systems.

HIGH QUALITY PORK PRODUCTS WITH REGIONAL IDENTITY FROM BLACK SLAVONIAN, TUROPOLJE AND KRŠKOPOLJE BREED AND THEIR MARKET VALUE

Purchasing decisions of consumers are nowadays increasingly influenced by factors such as animal breed, housing conditions and overall animal welfare which results in niche pork products of different kinds. In this light one should not be surprised that British consumers rated animal welfare as the most important food issue, even above safety and health concerns (IGD, 2011; DEFRA, 2011). Regarding the significance of breed, Warriss et al. (1996) reported better eating quality of meat originating from traditional breeds; the difference was explained by higher levels of intramuscular fat in traditional breeds that are genetically fatter and have finer muscle grain. Furthermore, pigs raised on organic farms or in outdoors systems are expected, by the consumers with certain ethical attitudes, to be more nutritious, tasty, healthy and safe (Edwards, 2005). European label "Protected designation of origin" promotes the use of indigenous pig breeds kept in traditional outdoor or free-range systems, even though some PDO pork products are issued from conventional breeds.

The description of intrinsic quality of pork originating from Black Slavonian pigs using the toolbox for sensory, healthy, technological and typical qualities is basic step to be performed within the TREASURE project. Moreover, database on carcass and meat quality traits will be formed in the aim of development of breeding programme for this breed. Influence of housing system (outdoor vs. deep litter) on carcass composition and quality of traditional pork products from Crna Slavonska

pig breed will be examined with special emphasis on Slavonian kulen, product with national PGI label.

Similarly, different data with regard to carcass traits and quality of fresh and processed meat of Turopolje pigs will be collected according to the common quality indicators and related to animal diet and traditional feeding resources, such as acorn. Additionally, in line with current trends in traditional food sector (Vanhonacker et al., 2013), the acceptability and preference tests of health related innovations in traditional meat products will be conducted with local consumers in Zagreb metropolitan area using the prototypes of Turopolje pig meat products (e.g. products with healthier FA composition and/or less smoked products) and harmonised protocols for consumer sensory studies developed within the framework of TREASURE project.

In Slovenia, no specific product is associated with Krškopolje breed therefore carcass traits and quality of fresh and processed meat of Krškopolje pigs will be evaluated using a common toolbox developed in TREASURE. Also, Krškopolje breed shall be involved in building up the database on carcass and meat quality and breeding programmes adapted to local pig breeds. The products from Krškopolje breed will also be included in consumer preferences tests and studies on marketing strategies with local products.

CLOSING REMARKS

Due to the development of pig sector in the last century and expansion of modern genetically improved pig genotypes, many local pig breeds were abandoned. Biodiversity preservation is one of the main concerns of modern society. Preservation of small local breeds from extinction by direct payments from governments can help but is not sustainable therefore efforts should be made to make them sustainable through marketing. TREASURE aims to acquire the knowledge and build up the skills and competences necessary to develop existing and create new sustainable pork chains based on the European local pig breeds, in line with the highest consumer demands for quality and healthiness of pork products, and social demands regarding animal welfare, environment and rural development. The project will deal with 20 local pig breeds in 9 countries, among them also Black Slavonian, Turopolje and Krškopolje breed which shall be involved in activities addressing genetic characterization, performance of breeds in various production systems, their product quality and market value. Besides research, activities of knowledge transfer and networking of academia, professional and public sectors is expected to build up the capacities. One of the biggest challenges and ambitions of the project is to have an umbrella trademark of all the breeds and their products expected to increase their market recognition.

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SUSTAINABLE MILK PRODUCTION IN DIFFERENT DAIRY CATTLE SYSTEMS AND VALORISATION OF ENVIRONMENTAL CHAIN ON THE BASIS OF ADDED VALUE

Cassandro, M.

Scientific review

SUMMARY

Aim of this review is to estimate milk yield and predicted methane emissions added values in local and cosmopolitan cow breeds reared in Italian circumstances. Nowadays it is well known that over the next 50 years, the world's farmers will be asked to produce more food than has been produced in the past thousand years, and in this concern it will be in environmentally sustainable way. The review will higlight the differences between intensive and extensive agricultural systems and this will be discussed and evaluated in dairy cattle production system context. In conclusion, animal genetic resources need to be evaluated not only per unit of output but for other direct and indirect output units related to social and human returns supporting different animal production systems, intensive or extensive ones. The intensive and extensive farming systems are not replaceable to each other, but they should be combined in order to respond to different social and environmental needs, so, to define the best sustainable production system. Moreover, both systems should also consider the modern demands that nowadays agriculture requires as, guarantee for food security. Therefore each system, intensive or extensive, should improve the animal products technological characteristics and at the same time reduce the carbon footprint.

Key-words: substainable systems, animal production, cattle breeds, added values, production and environmental chains

INTRODUCTION

Animal production has been practised for thousands years since the first animal domestication. Humans keep livestocks because they provide food and revenues. Animal's most universal and significant productivity is milk, meat and/or eggs for direct animal owners consumption or for selling to others. Important, but frequently overlooked contributions include transportation, manure, fibre, hides, other by-products, environmental protection and several historical and social traditions. The major factors impacting the classification of animal production systems are based on climate, level of technology, infrastructure, production incentives, political constraints and human resources. For semplicity, two can be the classification of agricultural systems: the Intensive Agricultural Systems (IAS, based mainly on double cropping, crop rotation, crop residue management, erosion control) and the Extensive Agricultural Systems (EAS, based mainly on broad, much variation, inter cropping, strip cropping, involving several different different crops or livestock species).

Over the next 50 years, farmers will be called upon to produce more food than has been produced in the past 10,000 years, and to do so in environmentally sustainable ways (FAO, 2009). An important strategy to increase added value for animal products, to preserve the environment and biodiversity, and to orientate tourism and food consumptions, would be the promotion of connections among the three key factors: breed, product and agricultural system.

Aim of this review is to explore the different effects in which it is possibile to see which contribuition the livestock sector, intensive and extensive, might have in the world modern concept.

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INTENSIVE AGRICULTURAL SYSTEM

This intensive agricultural system (IAS) has produced a large amount of food using green-revolution first generation techniques. Since 1950, increase in global food production has come from increased yield per unit of animal reared or area of crop land, using highly-selected breeds and high-input monoculture, using selectively bred or genetically-engineered crops; high productions using high levels of fertilizer, extensive use of pesticides and high amounts of water and finally a multiple cropping in order to increase the number of crops grown per year in a plot of land.

On the contrary, this intensive system produced an increase of outputs per profit, determined an increase of land and environment pollutions, increase waste runoff that increased nutrients and pathogens in streams, high soil erosion and pesticides concentration, reduced, therefore, animal welfare, food security and overall sustainability of animal production. The intensive agriculture showed that several human intensive agriculture practices can alter native habitats and reduce native biodiversity. However, it has guaranteed a large food security in developed countries.

FAO (2007) reported that animal production in the future needs for urgent action because of the wise management of the world's animal genetic resources, in order to guarantee the food security and the environmental protection. The world's population is expected to increase in the next forty years from 6.2 billion to 9 billion people. It is clear that more people will require more meat, milk, eggs and other livestock products. A wide portfolio of animal genetic resources will be crucial in adapting and developing the world's agricultural production systems so increasing the resilience of our food supply.

In particular, nearly all of this population increase will occur in developing countries, the urbanization will continue at an accelerated pace and about 70 percent of the world's population will be urban (compared to 49 percent today). Income levels will be many multiples of what they are now. In order to feed this larger, more urban and richer population, food production (net of food used for biofuels) has to increase by 70 percent and annual cereal production will need to rise to about 3 billion tonnes from 2.1 billion today. Annual meat production will need to rise by over 200 million tonnes to reach 470 million tonnes.

Summarizing, the IAS is an agricultural production system characterized by high use of input such as capital, labour and chemicalfertilizers relative to land area. Agricultural intensification has been the dominant response to population growth, as it allows producing more food on the same amount of land. Intensive animal farming practices can involve very large numbers of animals raised on limited land requireing large amounts of food, water and medical inputs. Intensive livestock farming provides opportunity to capture methane emissions which would otherwise contribute to global warming. Once captured, these emissions can be used to generate heat or electrical energy, thereby reduce local demand for fossil fuels. Factory farming is the process of raising livestock in confinement at high stocking density, where a farm operates as a factory being a practice typical in industrial farming by agribusinesses. The main products of this industry are meat, milk and eggs for human consumption.

EXTENSIVE AGRICULTURAL SYSTEM

The extensive agricultural system (EAS) is a system of farming carried out on very large holdings with a high reliance on technologies and local biodiversity. Moreover, the EAS is based on relatively low input and low yields compensated by the very large area cultivaed. Decisions taken by the farmer, or the corporation, are of great importance. The EAS can define a better system than IAS because it pursue the conservation of a state of armony between human and land (Knight and Riedel, 2002). The idea of Leopold, reported by Knight and Riedel (2002) is to describe the land, as it is not merely soil; it is a source of energy flowing through a pyramidal circuit with he soils at the base t and above their plants and animals. Food chains are the living channels which conduct energy upward; death and decay return it to the soil.

Summarizing, the EAS is an agricultural production system that uses small inputs of labour and capital restriceted to the land area farmed or grazed. Nomadic herding is an extreme example of extensive farming where herders move their animals to use feed from occasional rainfalls. Animal welfare is generally improved because animals are not kept in confined conditions. Moreover, extensive livestock farming provides opportunity to produce low methane emissions per unit of metabolic body weight or per hectar, and the main strategies of extensive system is to valorize local genetic diversity and make profit with a reduction of costs of production and with an increment of added values of its products (Cassandro, 2013).

INTENSIVE AND EXTENSIVE ANIMAL PRODUCTION SYSTEMS

In the specific sector of animal production the intensive and extensive systems can be defined as follows:

- Intensive systems are based on smaller acreage, fewer animals, cosmopolitan breeds, more input costs per individual animal, more labor, more often, sell for higher prices (often purebred/seedstock operations).
- Estensive systems are based on larger acreage, more animals, local breeds, fewer input costs, less labor, less often, sell for lower prices (often crossbred commercial herds).

Differences in how we assess the impact of intensive and extensive systems might be evaluated based on:

- added values for dairy chain by intensive and extensive systems;
- added values for environmental chain comparing greenhouse gas emissions generated by intensive and extensive systems.

A comparison of added values for milk yield and predicted methane emissions from local cows and intensive systems reared in Italian circumstances could be used as an example of how we can assess the impact of the intensive and extensive systems in different circumstances. Market-oriented strategies to payment systems that include milk yield added values of could enhance profitability and interest in rearing and safeguarding of extensive systems based on local animal genetic resources; but, not all countries can apply these market strategies. Therefore, other strategies to enrich milk production added values of might be based on the differences in greenhouse gases emissions among the production systems and breeds. Indeed, local animal genetic resources are expected to reduce the greenhouse gases emissions because of their lowest metabolic body weight, respect to high selected animals, or because their larger use of pasture providing a carbon sink effect. The impact of livestock species reared in different production systems is showed in Table 1 in terms of greenhouse gas emissions.

Table 1. Impact of livestock species reared in different production systems on greenhouse gas emissions (Cassandro et al, 2013)

	Ruminant Species		Monogastrics species	
Greenhouse gas emissions	Extensive grazing	Intensive systems	Traditional systems	Industrial systems
CO_2 emissions from land-use change for grazing and feed-crop production	---	-	ns	--
CO_2 emissions from Energy and input use	ns	--	ns	--
Carbon sequestretion in rangelands	++	ns	ns	ns
Methane emissions from digestion	---	--	ns	--

Legend: - = negative effect; + = positive effect; ns=not signficant effect; the number of minuses are proportional to the efffect on the greenhouse gas emissions

ADDED VALUE FOR DAIRY CHAIN

The definition, in a broad sense, of the added value (AV), can be the difference between the final selling price of a product and the direct and indirect inputs used to manufacture it. Therefore, the AV can be defined as the measurement of increment of gross value for a product made following a specific process. In dairy chain the AV can be calculated as difference between value V (the price value of final product, e.g. value of cheese produced by 1 kg of milk) and value K (the price value of input, e.g. value of 1 kg of milk used as fluid milk). Therfore, if the AV is positive, the product has added value, whereas if the AV is negative, the product has reduced value. In other terms, if the AV is greater than the cost of the process if is profitable, otherwise, the process is not profitable. Using a study of Cassandro (2013), the intensive systems (IS), namely Holstein Friesian, Brown Swiss, and Simmental, produced 9.2 kg/d ($P<0.05$) more milk compared with extensive systems (ES), namely Burlina, Rendena, Reggiana and Valdostana Red Pied. Fat percentage was significantly ($P<0.05$) higher for IS (3.89%) compared with ES (3.56%), whereas not significant differences were found for protein percentage and somatic cells count. Regarding body weight, IS were 213 kg ($P<0.001$) heavier than ES. Table 2 reports the added value per kg of milk yield estimated using standard milk and cheese prices adopted in Italy including cheese yield (Bozza, 2007). The average added value for milk yield was 0.15 ± 0.03 Euro/kg and it was lower for IS than ES (0.13 vs 0.17 Euro/kg; $P<0.05$). Hence, the milk yielded by ES is more suited to be destined to cheese production comparered with milk from IS. However, in terms of lactation yield the comparison between IS and ES can change due to the higher longevity of IS compared with ES. The added value for 305-d lactation yield showed that on the average, the added value was 813.7 ± 106.2 Euro, and it was higher, but not statistically significant, for IS than ES (893 vs 754 Euro; NS).

Table 2. Added value per kg of milk yield and per 305-d lactation of different livestock systems (Cassandro, 2013)

Livestock system	Value of cheese, €/kg	Value of milk yield, €/kg	Added value, €/kg	Added value, €/305d
Intensive:				
- Holstein Friesian	0.502	0.399	0.103	918
- Brown Swiss	0.569	0.423	0.146	950
- Simmental	0.553	0.425	0.128	810
Extensive:				
- Burlina	0.552	0.393	0.159	706
- Rendena	0.565	0.393	0.173	822
- Reggiana	0.574	0.411	0.162	835
- Valdostana Red Pied	0.576	0.387	0.189	654
Average ± SD	0.556 ± 0.025	0.404 ± 0.015	0.151 ± 0.029	813.7 ± 106.2
lsmeans of Intensive vs Extensive systems [1]			0.13 vs 0.17 P<0.05	893 vs 754 NS

[1] One way ANOVA using as fixed effect the livestock systems grouping in two levels (Extensive and Intensive); NS = not statistically significant

ADDED VALUES FOR ENVIRONMENTAL CHAIN COMPARING GREENHOUSE GAS EMISSIONS BY INTENSIVE AND EXTENSIVE SYSTEMS

In the environmental chain the AV may be defined as the minimum air pollution due to enteric methane emissions. Methane emissions contribute significantly to the greenhouse effect having many times the global warming potential of carbon dioxide (IPPC, 2001; Kebread et al., 2008). Among human activities, the FAO (2006) declared that the agriculture sector accounts for 22% of the total greenhouse gases (GHG) emissions and 3% is due to livestock sector (Cassandro et al., 2010). In Italy, cattle breeds account for 78% of the total GHG emissions from livestock species; 54% is produced by dairy cattle and 24% by beef cattle. Typically, 2 to 12% of the gross energy intake in cattle is lost through eructation of methane (Johnson and Johnson, 1995). As methane concentration in the atmosphere is increasing, there is a strong interest in developing strategies to reduce its emissions, particularly from the livestock sector. A mitigation action to reduce the emission might be possible by improving the breeds with the highest AV for environmental chain that can be defined as the GHG emission per 1 kg of milk yield or metabolic weight. In this case the AV is a measurement of an environmental mitigation and might be used as a new brand of the breed for a valorization project.

Cassandro et al. (2013), using an indirect method, predicted the methane emissions in different cattle breeds, that can be assumed as intensive and extensive systems. The predicted methane production of 16.28±3.24 MJ/d with a maximum value of 21.23 MJ/d for the IS based on Holstein Friesian and a minimum of 12.53 MJ/d for ES based on Valdostana Red Pied on the average are reported in Table 3. The average of ES showed better AV than average of IS for environmental chain, because of lower predicted methane production (13.90 vs 19.46 MJ/d; P<0.01). In terms of methane emission per kg of milk yield, the average value was 0.9059±0.1098 MJ/d with a maximum value of 1.1029 MJ/d for ES based on Valdostana Red Pied and a minimum of 0.7309 MJ/d for IS based on Holstein Friesian. Not significative differences were found between ES and IS for daily methane production per kg of milk yield (0.9627 vs 0.8301 MJ/kg/d; P>0.05). Moreover, in terms of methane emission per kg of metabolic weight, the average value was 0.1378 ± 0.0063 MJ/kg with a maximum value of 0.1488 MJ/kg for IS based on Holstein Friesian and a minimum value of 0.1283 MJ/kg for ES based on Valdostana Red Pied. The ES showed better AV than IS for environmental chain, because of lower predicted methane production (0.1339 vs 0.1424 MJ/kg; P<0.05).

Table 3. Added value (AV) for environmental chain, expressed as predicted methane emission (MJ/d) in absolute value, as predicted methane emission per kg of milk yield and as kg of metabolic weight (Cassandro, 2013)

Livestock system	Methane, MJ/d	Methane/Milk yield MJ/Kg/d	Methane/Metabolic Body Weight, MJ/Kg
Intensive:			
- Holstein Friesian	21.33	0.7309	0.1488
- Brown Swiss	18.22	0.8552	0.1416
- Simmental	18.82	0.9041	0.1383
Extensive:			
- Burlina	13.37	0.9185	0.1368
- Rendena	14.31	0.9174	0.1353
- Reggiana	15.38	0.9120	0.1354
- Valdostana Red Pied	12.53	1.1029	0.1283
Average ± SD	16.28 ± 3.24	0.9059 ± 0.1098	0.1378 ± 0.0063
Ismeans of Intensive vs Extensive systems [1]	19.47 vs 13.90 P<0.01	0.8301 vs 0.9627 NS	0.1424 vs 0.1339 P<0.05

[1] One way ANOVA using as fixed effect the livestock systems grouping in two levels (Extensive and Intensive); NS = not statistically significant

CONCLUSION

Animal Agriculture is an important aspect of human life since the beginning of the world. A major constraint to the adoption of improved innovations in animal agriculture is the land use system which should be based on modern extensive system in respect to intensive system. The present intensive system in agriculture of the developed countries has created an overexploitation and general mismanagement of resources. Land use and rangeland policies that guarantee a low environment impact will enable farmers to propagate fodders and control breeding of their animals. All these improved management practices will ensure sustainable animal agricultural development.

Analyses on added value for dairy chain was better on extensive systems than intensive systems, so, cheese yield is preferred for extensive systems to milk fluid production which is more appropriate to intensive systems. Similarly, analyses on added value for environmental chain, showed that added value is better with extensive systems in respect to intensive systems. Hence, extensive systems showed to cope better with mitigation of predicted (CH_4) emission in absolute value and per unit of metabolic weight than for unit of milk. Knowing that CH_4 emission per unit of metabolic weight might be considered as a measure at net of the selection effect, while the CH_4 emission per unit of milk yield is a measure at gross of the selection effect, this study showed that livestock systems have a dual role not only in food production, but also in the provision of public good objectives including, biodiversity and landscape values as well as diffuse pollution to environment. Therefore, the comparison of different livestock systems should be evaluated in terms of environmental efficiency and not only in term of economic efficiency. Livestock systems need to be evaluated not only per unit of output but for other direct and indirect units of output related to social and human returns, valorizing added values for cheese yield and environment mitigation including other social and public goods, as territory preservation, consumer habits, turists requests as well as history and cultural aspects of link between breed and food. In conclusion, the intensive and extensive farming systems are not alternatives to each other, but must be combined in order to respond to different social and environmental needs. Both systems must still be considered the modern demands that nowadays agriculture requires as guarantee for the food security, improving the technological characteristics of animal products and reducing the carbon footprint.

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POPULATION GENETIC STRUCTURE OF AUTOCHTHONOUS BLACK SLAVONIAN PIG

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Original scientific paper

SUMMARY

As Black Slavonian pig was forgotten and replaced by commercial hybrid pigs, after mid 1950s population declined drastically. Today's population was founded from 46 sows and 6 boars in 1996 while currently consist of 1064 sows and 163 boars. Aim of this study was to evaluate the genetic structure and genetic variability in autochthonous Black Slavonian pig breed using pedigree. The quality of the pedigree information was evaluated by percentage of known ancestors, which was about 35.16%. Increase in pedigree completeness over the years is evident. Number of inbred Black Slavonian animals was 1695. Average inbreeding was 3.2%, with evident growth observed by year. Effective number of founders was 50.8 for males and 51.1 for females. Effective number of ancestors was, 35.8 for male and 34.8 for female animals, respectively. The proportion of the genes contributed to the reference population of males and females by the most important ancestor was 7.1% and 9.1%. The first 15 ancestors for males and 12 for females explained around 50% variability in the gene pool. Average effective population size via number of parents for the last 10 years of Black Slavonian pig is 189.2. In order to perform proper selection decisions, additional work should be done to increase the quality of pedigree data affecting the reliability of estimated parameters and genetic structure of the population.

Key-words: pedigree analysis, Black Slavonian pig, inbreeding, genetic variability, effective population size

INTRODUCTION

In pig breeding during the last decades, highly productive breeds developed mainly by breeding companies have replaced the local breeds which are essential resources of genetic diversity (FAO, 2007). It is therefore crucial to preserve the genetic diversity of local autochthonous breeds for future breeding programs. In Croatia, the autochthonous Black Slavonian pig was created by earl Karl Leopold Pffeifer in the second half of the 19th century. For this purpose of creating a new breed with improved production performance, he crossed gilts of local breed Lasasta mangulica with Berkshire boars. Later from the 1880s till the 1910, he was crossing the best gilts with boars of American breed Poland China to further improve performance. The result of this breeding program was successful as Black Slavonian pig was the most widespread breed in Croatia till the middle of 20th century used for both fat and meat production. However, later due to no breeding program established, the breed was replaced by modern hybrids so the population declined drastically and suffered a narrow bottleneck especially in the mid-1990s.

In September of 1996, population consisted of 46 sows and 6 boars (Uremović, 2004) which were the first sows and boars of this breed registered in Croatian Agricultural Agency. Since that time, the pedigree was recorded.

Breeds as Black Slavonian pig usually have small population sizes resulting in high inbreeding coefficients often followed by reduced fitness. The high inbreeding coefficient should be reduced by choosing the proper strategy (Meuwissen and Woolliams, 1994) considering

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the genetic structure and genetic variability. Aim of this study was to evaluate genetic structure and genetic variability of Black Slavonian pig with using pedigree information.

MATERIAL AND METHODS

The reference population contained all pigs born between 1995 and 2014. Entire pedigree of Black Slavonian pig breed consisted of 3478 animals and was recorded by Croatian Agricultural Agency. Current population of Black Slavonian pig consists of 1064 active sows and 163 boars registered (Annual report 2014, Croatian Agricultural Agency).

Pedigree analysis and genetic variability parameters

Ancestors having both parents unknown were considered as founders, while in the case of one known parent, it was considered to be a founder. The quality of the pedigree was evaluated by the following parameters: percentage of known ancestors - expressed as percentage of known ancestors of the expected number in a generation; maximum generation traced back - expressed by tracing back the pedigree for each individual and average pedigree completeness per year. The first measure of genetic variability calculated was the effective number of founders f_e (Lacy, 1989). Secondly, effective number of ancestors f_a was calculated as the minimum number of ancestors described by Boichard et al. (1997). Individual inbreeding coefficient (F) was used to show probability of two individuals having genes identical by descent and was calculated according to Wright (1931). Average relatedness of individuals from a given pedigree was also analysed. Furthermore, effective population size (N_e) per year was calculated according to Wright's (1931) method and denoted as Ne_W and by Falconer and Mackay (1996) denoted as Ne_F. Number of breeding males and females by year and generation interval is shown.

Software used

Preparation of pedigree file and statistical analyses were carried out using SAS software (SAS/STAT^{TM}, 1999). Analysis were performed using CFC (Sargolzaei et al., 2006), PopRep 1.0 (Groeneveld et al., 2009) and PEDIG (Boichard et al., 1997) program packages.

RESULTS AND DISSCUSSION

Figure 1 shows the number of animals registered in Croatian Agricultural Agency per year starting from 1996. The figure clearly shows that the number of registered boars and sows was continuously on the rise during the last 18 years.

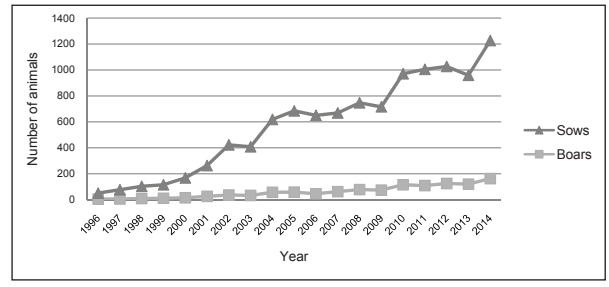

Figure 1. Number of animals registered in Croatian Agricultural Agency from 1996 to 2014

This positive trend could be explained by the several actions taken. In the early years, the important actions were probably joining the FAO initiative for preserving the autochthonous pig breeds in 1999 and designing the breeding program in 2000 aiming to breed Black Slavonian pigs on small family farms. Later on, small but important pig breeders associations were established and further encouraged the breeding of this pig breed. Additionally, state government supported the breeding of indigenous breeds by subsidizing production. Nowadays, producing the traditional high quality products with more added values offers the opportunity for small local farmers and in that way make these products more competitive on a global market.

Average pedigree completeness per year is shown in Table 1. The recording of the pedigree information for Black Slavonian pig began in 1996 and there was a maximum of 14 generations traced back, which was by the 2% of animals in the pedigree. Animals from the pedigree had 278 known sires and 945 dams. By comparing the animals born in 1996 with the newer generations in 2014, pedigree completeness was increased as expected since the pedigree completeness provides information of known ancestors within arbitrary generations. The highest completeness was in the last recorded year where almost 70% of the pedigree was known for 6 generation back. However, still a lot of effort should be done in order to provide higher quality of the pedigree data since the more completed information is available only in the last recorded years for the first 4 to 5 generations.

Table 1.The average pedigree completeness (%) for 1 to 6 generations deep by year

Year	Number of animals	Complete generation 1	Complete generation 2	Complete generation 3	Complete generation 4	Complete generation 5	Complete generation 6
1995	2	0.0	0.0	0.0	0.0	0.0	0.0
1996	7	0.0	0.0	0.0	0.0	0.0	0.0
1997	14	0.0	0.0	0.0	0.0	0.0	0.0
1998	12	16.7	10.4	6.9	5.2	4.2	3.5
1999	16	31.2	15.6	10.4	7.8	6.2	5.2
2000	43	39.5	23.4	15.6	11.7	9.4	7.8
2001	59	54.2	30.6	20.5	15.4	12.3	10.2
2002	128	41.4	26.2	18.3	13.7	11.0	9.1
2003	256	62.5	42.3	29.3	22.0	17.6	14.6
2004	201	68.7	50.9	37.7	28.6	22.9	19.1
2005	213	92.0	68.0	49.9	38.1	30.5	25.4
2006	325	92.6	74.2	57.6	45.1	36.2	30.2
2007	234	94.9	84.0	68.7	54.7	44.3	36.9
2008	285	99.3	84.0	70.0	56.9	46.5	38.8
2009	306	92.8	88.6	79.2	66.8	55.6	46.6
2010	258	99.6	97.1	90.3	79.4	67.4	57.0
2011	286	94.4	92.4	87.3	79.3	68.9	58.8
2012	197	100.0	99.1	95.3	86.6	75.3	64.5
2013	292	100.0	99.1	96.0	89.0	79.1	68.6
2014*	18	100.0	100.0	95.2	89.0	79.9	69.6

* data included until 31 March 2014

Total number of founders for this population was 452 while the effective number of founders was 50.8 and 51.1 for males and females, respectively. The total number of founders is much higher than the effective number of founders, probably because all animals were included at the initial years of the recording. Similar results were obtained in the study of Crovetti et al. (2013) on Italian local breeds. As these parameters were calculated using the formula of Lacy (1989) which considers only the founders contribution, information on more recent ancestors could be more relevant in expressing genetic diversity. Therefore, calculating the effective number of ancestors f_a using the formula of Boichard et al. (1997) was used to overcome this drawback and cases if the bottlenecks in the pedigree were present. The obtained f_a values were lower than effective number of founders, 35.8 for male and 34.8 for female animals, respectively. Moreover, those values were higher than the values of Italian (Crovetti et al., 2013) and French (Maignel et al., 2001) local breeds that could be the result of shallow pedigree in our situation compared to theirs.

The proportion of the genes contributed to the reference population of males and females by the most important ancestor is 7.1% and 9.1%. The first 15 ancestors for males and 12 for females explained around 50% variability in gene pool. The average age of parents indicates that animals reproduce young and stay relatively short in the reproduction (3.1 years was the mean age of reproducing sires and 2.7 years for dams).

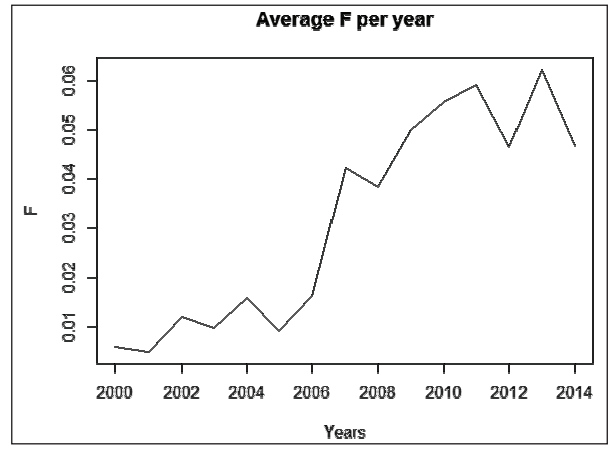

Figure 2. Average inbreeding (F) per year

Mean relatedness coefficient was 0.049. Number of inbred Black Slavonian animals is 1695. The mean coefficient of inbreeding for the population (inbred and non-inbred animals) was very high, 3.20% (with maximum value of 40.75%). Mean inbreeding coefficient for inbred animals was estimated to 6.56%. As the pedigree was getting more complete by years, it is easier to spot inbreeding. Therefore, highest inbreeding was present in the years 2011 and 2013. Average inbreeding by year is shown in Figure 2.

The calculated values of inbreeding were higher than the values from the studies of Crovetti et al., (2013) and Maignel et al., (2001) on similar type of local breeds, but were lower than the values calculated for the Bisaro local breed (Fernandes et al., 2010). Nevertheless, planned and carefully organized breeding is required in order to

prevent high inbreeding and loss of genetic variability. Effective population size (N_e) per year is presented in Table 2. As for the certain conditions like population substructure or pedigree completeness, different methods for calculating N_e should be considered. In this study, inbreeding Ne_W calculated by Wright (1931) was 132 in the year 2014. However, as the inbreeding was present in this study, it can be used to calculate more realistic effective population size of a population. When inbreeding rate was accounted, Ne_F (Falconer and Mackay, 1996) dropped significantly. On the other hand, this method is affected by the long term effects of selection and it is also very sensitive to incomplete pedigree information (Boichard et al., 1997).

Table 2. Effective population size by year via number of parents

Year	Number					
	Animals	Sires	Dams	Parents	Ne_W	Ne_F
1995	2	1	1	2	1	-
1996	9	3	1	4	2	-
1997	23	3	1	4	2	-
1998	33	6	3	9	6	-
1999	42	10	8	18	12	-
2000	71	16	22	38	26	142
2001	118	21	43	64	40	111
2002	230	33	83	116	66	121
2003	443	53	147	200	109	278
2004	585	69	184	253	141	124
2005	670	76	223	299	159	120
2006	739	89	253	342	184	69
2007	772	93	265	358	193	37
2008	844	106	283	389	216	25
2009	825	102	271	373	208	20
2010	849	101	296	397	211	25
2011	850	102	308	410	215	26
2012	741	92	281	373	194	62
2013	775	83	285	368	180	92
2014	507	62	197	259	132	-

Ne_W: Effective population size calculated by Wright (1931); Ne_F: Effective population size calculated by Falconer and Mackay (1996)

There are few recommendations for minimum N_e in domestic animals. Food and Agriculture Organization of the United Nations (FAO, 2000) suggested the N_e values higher than 50 being considered as critical. Meuwissen and Woolliams (1994) suggested that the values of N_e should range from 31 to 250 to prevent a decline in fitness. Values in this study are higher, however, they should be taken with caution as they depend on the quality of the pedigree data.

CONCLUSION

Although population of Black Slavonian pig showed positive trends in growth during last 20 years, it is still very vulnerable. Pedigree for the Black Slavonian pig population showed to be more complete by each year. Inbreeding coefficient of 3.2% for entire population is not alarming itself, but completeness of the pedigree should be taken into account as well as information that inbreeding is growing with the pedigree completeness. Effective population size presented here has to be considered with caution in regard to inbreeding coefficient. Future breeding strategies should rely more on the pedigree to avoid inbreeding.

ACKNOWLEDGMENT

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ESTIMATION OF GENOMIC VARIATION IN CERVIDS USING CROSS-SPECIES APPLICATION OF SNP ARRAYS

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Original scientific paper

SUMMARY

The aim of this study was to assess the utility of commercially developed genotyping array for cross-species genotyping in order to estimate the genetic variation across two species from family Cervidae. The genotyping of individuals was carried out using Illumina BovineSNP50 BeadChip. The cross-species application of bovine array was tested overall in 3 farmed and 5 free range Red deer (Cervus elaphus) and 2 free range Fallow deer (Dama dama). After applying data quality control 97.2% of SNPs localized on the chip were removed and only 1,530 autosomal markers showed polymorphism across all analysed individuals. Across all polymorphic SNPs the minor allele frequency reached the average value 0.23±0.16. The analysis based on Bayesian clustering approach clearly showed a partition of deer in two separate clusters in relation to their phylogenetical relationship. Moreover, the PCA analysis indicated that the genetic differences between farmed and free range Red deer caused the division of analysed individuals into the two subpopulations. But the results of cross-species genotyping should be present with caution, because the bovine chip developed primarily for taurine cattle breeds is not fully representative to the evolutionary changes in genome of cervids. Nevertheless, our results suggested that the utility of bovine array alongside microsatellite markers and mtDNA can be very perspective for genetic diversity estimation in deer populations.

Key-words: bovineSNP50 chip, fallow deer, genetic differentiation, polymorphism, red deer

INTRODUCTION

The patterns of genetic variation within wild animal populations may be influenced mainly by natural and anthropogenic factors. Human activity such as agriculture, deforestation, hunting, introduction of alien species or translocation of populations may greatly affect the level of genetic diversity and structure of natural population as well as and reduce their fitness and future adaptive potential (Rosvold et al., 2012). The microevolutionary consequences of human practices might have profound effects not only on threatened species living in small and isolated populations, but also on common and widespread species subject to strong management practices (Olano-Marin et al., 2014). In both present and ancient time deer (*Cervidae*) belong to the most important species representing usable models to assess the consequences of introduction events and breeding practises on genetic diversity. In addition, *Cervidae* is one of the few mammal family for which farmed and wild/feral populations may be found in sympatry (de Garine-Wichatitsky et al., 2009). However, due to heavy hunting and habitat alterations, many populations were severely reduced in numbers in previous centuries (Rosvold et al., 2012). Understanding of population genetic structure is important for management of species as genetically isolated populations with limited diversity are often associated with inbreeding and reduced reproductive fitness. The population bottleneck is one of the factors that can result in the genetic diversity loss. It may cause a reduction in number of alleles and heterozygosity, the fixation of deleterious alleles, and potentially the occurrence of inbreeding depression in populations (Webley et al., 2007; Ernst et al., 2012). The reduction of genetic diversity and the impact of bottleneck effect have been

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demonstrated with historical records often in accordance with molecular data in several deer populations (Broders et al., 1999; Webley et al., 2007).

Mainly two methods utilizing microsatellite markers and mitochondrial DNA (mtDNA) control region are preferred for the analysis of genetic diversity in deer species. The aim of this study was to assess the utility of cross-species genotyping in the genetic variation estimation within two species from the family *Cervidae*, red deer and fallow deer.

MATERIAL AND METHODS

Two species from family *Cervidae* were included in this study. The cross-species application of genotyping array was tested overall in three farmed and five free range Red deer (*Cervus elaphus*) and two free range Fallow deer (*Dama dama*). All the analysed individuals were originated from Slovakia. The farmed deer samples represented male progeny of sires from New Zealand and dams from Hungary, whereas the free range deer were trophy animals. The single nucleotide polymorphisms (SNPs) genotyping were carried out using Illumina BovineSNP50 BeadChip in commercial lab (Illumina, Inc. San Diego, USA). Firstly any markers with unknown chromosomal position and SNPs located on sex chromosome were removed. Secondly the quality control of genotyping data was applied to eliminate any SNPs with genotyping errors (loci with >10% missing genotypes), less informative markers (MAF<0.01) and markers deviating from Hardy-Weinberg equilibrium limit of 0.001. This resulted in total of 1,530 informative autosomal SNPs which passed the above criteria and were usable for genetic variability analysis within analysed cervids.

The genetic differentiation among the analysed deer was estimated using two approaches, the Bayesian clustering method and principal component analysis (PCA). The basic sample and marker statistic of genotyping data was calculated using SNP & Variation Suite (v7.6.8 Win 64, Golden Helix, Bozeman, MT, USA, www.goldenhelix.com). The Bayesian clustering implemented in STRUCTURE 2.3.4 (Pritchard et al., 2000) was used for estimation of groups number represented by all analysed individuals in relation to the individual admixture proportion. The STRUCTURE analysis was carried out using the default parameters of an admixture model and correlated allele frequencies across all the individuals based on burn-in period of 10,000 followed by 100,000 Markov chain Monte Carlo (MCMC) replications. Ten runs were performed from K=1 to K=10 a4nd the K with the highest likelihood was selected using the STRUCTURE HARVESTER (Earl and von Holdt, 2012) that evaluate the log probability of data (ΔK) according to Evanno et al. (2005). Subsequently, the genetic differences among the analysed deer in relation to the population structure pertaining to the individuals and species were estimated based on principal component analysis (PCA) performed using the SNP & Variation Suite (v7.6.8 Win 64, Golden Helix, Bozeman, MT, USA, www.goldenhelix.com).

RESULTS AND DISCUSSION

After applying the quality control of genotyping data from the total 54,609 SNPs localized on the chip 552 loci with unknown position and 1,171 SNPs related to the sex chromosomes were removed. Subsequently, in the control processes of remaining autosomal SNPs it was found that the 53.89% of markers were genotyped successfully at least 90% of individuals. However most of them were monomorphic and only 5.37% of markers showed polymorphism (2.8% from the total SNPs amount). The proportion of polymorphic SNPs varied across autosomes. The highest part of polymorphic loci was found on autosome 2 and the lowest on autosome 23 (Figure 1). The observed proportion of polymorphic markers was comparable with results published by Haynes and Latch (2012) that similarly tested the application of bovine genotyping array on species from family *Cervidae*.

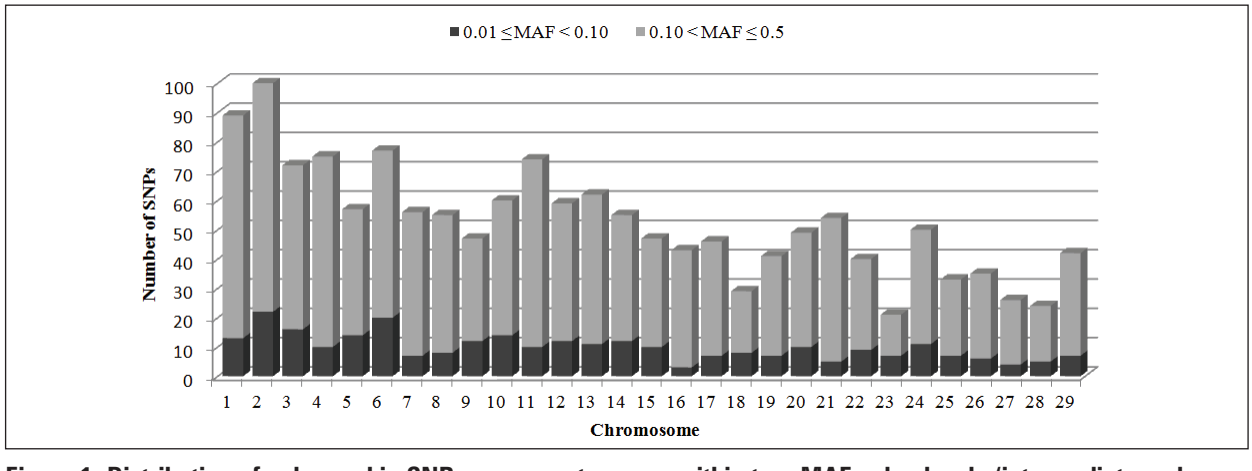

Figure 1. Distribution of polymorphic SNPs across autosomes within two MAF value levels (intermediate and common variants)

The power to detect genetic effects is dependent on minor allele frequency (MAF). In the previous studies it has been demonstrated that rare genotypes are more likely to result in spurious findings, and therefore SNPs with MAF<10% are mostly removed (Tabangin et al., 2009). This study tested only SNPs with MAF limit of 0.01. Across all the polymorphic SNPs the minor allele frequency reached the average value 0.23±0.16. The highest proportion of SNPs with common MAFs variants (0.10<MAF≤0.5) was found on chromosome 16. On the contrary the highest proportion of MAFs<0.10 was observed on autosome 18. Across the all analysed individuals and informative loci the heterozygosity was found at the level 0.28±0.007.

The Bayesian clustering approach revealed a partition of deer in to two separate clusters. However, based on the method assessing log probability of ΔK (Evanno et al., 2005) the local maxima at K=3 (Figure 2) was detected. The observed difference can be attributed to the genetic diversity in Red deer group also demonstrating the principal component analysis. However the distribution observed using STRUCTURE analysis clearly showed division of analysed individuals in relation to the species origin.

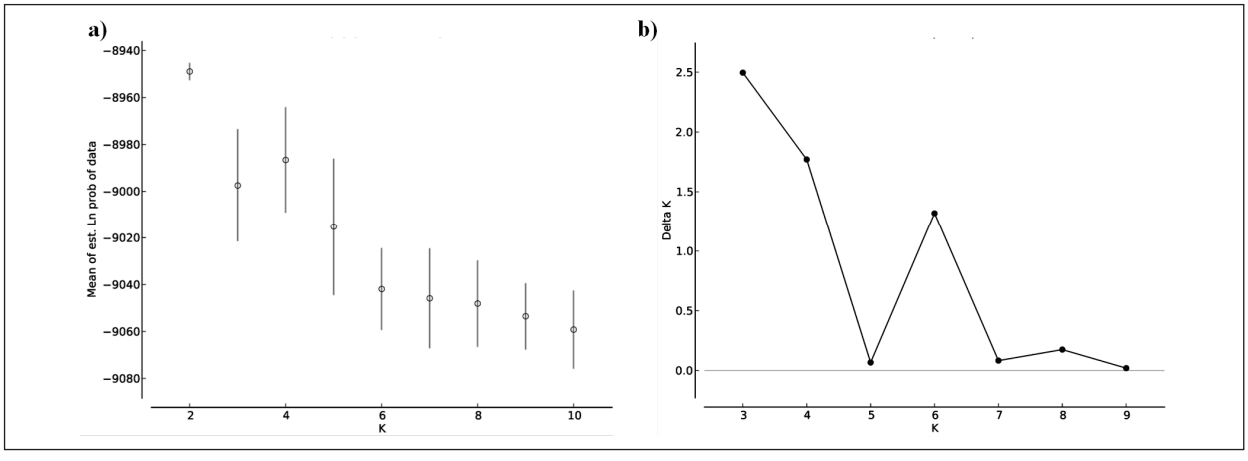

Figure 2. The results of STRUCTURE analysis showing the most likely number of subpopulations (a) mean ± SD of the log likelihood in relation to the different values of K and (b) values of ΔK evaluated according to Evanno et al. (2005)

The principal component analysis performed from the genetic covariance matrix using genotyping data and the correlation coefficient between sample and genotypes for each locus across analysed individuals showed similarly the clear differentiation of animals into the separate clusters related to each species (Figure 3.). Moreover, the PCA analysis indicated that the genetic differences between farmed and free range Red deer caused the division of the analysed individuals into the two subpopulations. The bovineSNP50 array was successfully used in cross-species genotyping in order to estimate genetic diversity in several phylogenetically related species (Michelizzi et al., 2011; Miller et al., 2012). However, the results of these studies should be interpreted with caution because the problem associated with the use of commercially developed array for discovery of novel SNPs in genetically related species is the occurrence of ascertainment bias, the systematic deviation from the expected allele frequency distribution (Haynes and Latch, 2012). This may occur if the SNPs are identified in a small sample of population from part of the species range and also by application to the evolutionary more distant species, because the loci cannot be representative to the evolutionary changes in both species.

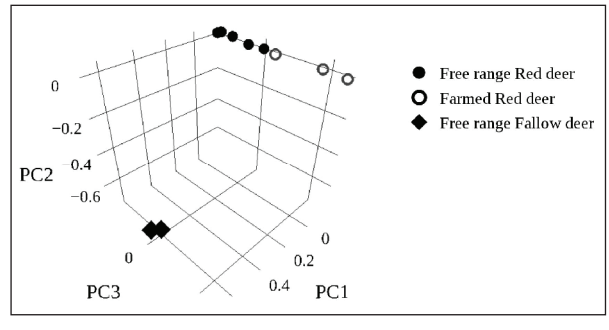

Figure 3. The PCA analysis of the genetic relationship among analysed cervids

CONCLUSION

One of the problems that mainly biased all analyses in relation to the cross-species genotyping is the fact that the applied array is not fully representative to the genome of genotyped species. Moreover, the cervid karyotype is very different as in bovids. However the results of our study indicated that the utility of commercially developed array for model animals can be very perspective to analysis of genetic differentiation within phylogenetically related non-model species. In the situation when the whole genome scan of cervids is not available use of bovine array is one of the ways that can provide usable tools alongside microsatellite

markers and mtDNA for genetic diversity estimation and phylogeny studies in deer populations.

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DEVELOPMENT OF AN APPROXIMATE MULTIVARIATE TWO-STEP APPROACH FOR THE JOINT GENETIC EVALUATION OF AUSTRIAN AND GERMAN DAIRY CATTLE

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Original scientific paper

SUMMARY

Multivariate genetic evaluation in modern dairy cattle breeding programs became important in the last decades. The simultaneous estimation of all production and functional traits is still demanding. Different meta-models are used to overcome several constraints. The aim of this study was to conduct an approximate multivariate two-step procedure applied to de-regressed breeding values and yield deviations of five fertility traits of Austrian Pinzgau cattle and to compare results with routinely estimated breeding values. The approximate two-step procedure applied to de-regressed breeding values performed better than the procedure applied to yield deviations. Spearman rank correlations for all animals, sires and cows were between 0.996 and 0.999 for the procedure applied to de-regressed breeding values and between 0.866 and 0.995 for the procedure applied to yield deviations. The results are encouraging to move from the currently used selection index in routine genetic evaluation towards an approximate two-step procedure applied to de-regressed breeding values.

Key-words: approximate multiple trait, de-regressed breeding values, yield deviation, fertility, cattle

INTRODUCTION

Due to the increased number of production and functional traits in modern dairy cattle breeding programs, multivariate genetic evaluation became increasingly interesting over the last decades. One of the major challenges is the simultaneous evaluation of all traits. Hence, different meta-models were proposed for national and international genetic evaluations, e.g. an approximate two-step approach using pseudo-phenotypes (Ducrocq et al., 2001) or Multiple trait Across Country Evaluation (MACE; Schaeffer, 1994; Schaeffer, 2001). At present, the joint genetic evaluation of Austria and Germany is optimised aiming at a multiple trait genetic evaluation. Currently, selection is based on a total merit index (TMI) derived by Miesenberger (1997). The TMI of the evaluated cattle breeds consists up to 30 production and functional traits.

Breeding values for the TMI as well as for several sub-indices are estimated either using univariate (e.g. protein yield) or multivariate (e.g. calving ease and stillbirth) methods by applying animal or sire-maternal-grandsire models (the latter for functional longevity only). Some of these models include repeated measures, such as somatic cell count (Fuerst et al., 2015). Subsequently, estimated breeding values (EBV) for individual traits are combined to form the TMI or other sub-indices by assuming that residual covariances between traits or groups of traits are equal to zero. Additionally, genetic correlations between many traits are assumed to be zero or were obtained from literature (Miesenberger, 1997). These constraints lead to an upwards biased TMI for animals with low reliabilities. This assumption was confirmed on simulated data by Pfeiffer et al. (2015). In fact, a full multivariate estimation based on phenotypic data would be the optimum methodology. However, it is usually not feasible (Mrode, 2014) due to the tremendous amount of data in genetic evaluations and restricted computer power (Lassen et

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al., 2007). Thus, an approximate multivariate model using a two-step procedure was proposed and validated using simulated data (Durcrocq et al., 2001; Lassen et al., 2007; Pfeiffer et al., 2015). Results of the simulation study by Pfeiffer et al. (2015) were encouraging, but the procedure has to be approved on field data. Therefore, the objective of this study was the comparison of routinely estimated breeding values (EBVr) for fertility traits with results of an approximate two-step procedure based on de-regressed breeding values (drEBV) and yield deviations (YD), respectively.

MATERIAL AND METHODS

All routinely evaluated fertility traits of Pinzgau cattle were chosen to test the approximate two-step procedure applied to drEBV (procedure PdrEBV) and YD (procedure PYD). These were non-return-rate 56 for heifers (NR-H) and cows (NR-C), days from first calving to first insemination (CFI) and days from first to last insemination for heifers (FLI-H) and cows (FLI-C). In total, 294,027 records of 104,866 cows and heifers inseminated between the years 1990 and 2014 were analysed. The pedigree consisted of 183,129 animals. In the first step for PdrEBV, a 5-trait genetic evaluation was applied to get EBV using the program package MiX99 (Lidauer et al., 2015). The following statistical model was used:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Za} + \mathbf{Wp} + \mathbf{e} \qquad (1)$$

where **y** is a vector of observations of the traits NR-H, NR-C, CFI, FLI-H and FLI-C; **b** is a vector of systematic effects, including fixed effect of region-year-month of insemination interaction, herd-year interaction, parity-age at calving/insemination interaction, inseminator-year (only for NR-H and NR-C) interaction and service sire (only for NR-H and NR-C); **a** is a vector of animal effects; **p** is a vector of permanent environmental effects (only for cows) and **e** is a vector of residuals; **X**, **Z** and **W** are the corresponding incidence matrices. Fertility EBV were then de-regressed by a multivariate de-regression approach (Schaeffer, 2001), implemented in the program package MiX99 (Lidauer et al., 2015). The de-regression procedure uses the estimated breeding values and their respective effective daughter contributions as weights only considering the general mean as fixed effect. For the second procedure PYD, YD were computed, again using the software MiX99 (Lidauer et al., 2015). The following model was applied:

$$\mathbf{y^*} = \mathbf{y} - \mathbf{Xb} - \mathbf{Zp} \qquad (2)$$

where **y*** is a vector of YD; **y** is a vector of phenotypic observations of the traits NR-H, NR-C, CFI, FLI-H and FLI-C; **b** indicates the vector of all fixed effects, already described for equation 1; **p** is a vector of permanent environmental effects (only for cows); **X** and **Z** are the corresponding incidence matrices.

After de-regressing EBV and computing YD, respectively, all five traits were analysed by means of the following multivariate animal model:

$$\mathbf{y^{\#}} = \boldsymbol{\mu} + \mathbf{Za} + \mathbf{e} \qquad (3)$$

where $\mathbf{y^{\#}}$ indicates either drEBV or YD of the respective trait; $\boldsymbol{\mu}$ is the general mean; **a** is a vector of random animal additive genetic effects and **e** denotes a vector of random residual effects. **X** and **Z** represent the corresponding incidence matrices. Based on approximate Interbull reliabilities (Strandén et al., 2000) effective own performances (Edel et al., 2009) were calculated and used as weighting factors for drEBV and YD in the multivariate genetic evaluation. Routine genetic parameters were used (Fuerst et al., 2015) for the multivariate genetic evaluation. Spearman rank correlations between EBV of routine genetic evaluation, PdrEBV and PYD were calculated using the program package SAS 9.2 (SAS, 2008). All EBV were standardised to relative breeding values with a mean of 100 and an additive genetic standard deviation of 12.

RESULTS AND DISCUSSION

Means and standard deviations of EBVr, PdrEBV and PYD for all animals and sires with reliabilities higher than 50% (n=318) are given in Table 1. Means for each trait and procedure were similar, also standard deviations of EBVr and PdrEBV were almost equal. Standard deviations for PYD were lower compared to EBVr and PdrEBV. Table 2 shows the rank correlations between routinely estimated breeding values and the two-step procedure applied to drEBV and YD, respectively, for all animals, sires with reliabilities higher than 50% and cows with reliabilities higher than 30%. Correlations between EBVr and PdrEBV were almost 1 for all traits and animal groups. Correlations between EBVr and PYD were lower. These results were in accordance with simulation study of Pfeiffer et al. (2015). Authors could show that outcomes of an approximate two-step procedure applied to drEBV were always closer to the reference method, which was a full multivariate animal model based on phenotypic data, than those of an approximate two-step procedure applied to YD.

Table 1. Means and standard deviations of the routinely estimated breeding values, breeding values derived from an approximate two-step procedure based on de-regressed breeding values and yield deviations for non-return-rate 56 heifer (NR-H) and cow (NR-C), days from first calving to first insemination (CFI) and days from first to last insemination for heifers (FLI-H) and cows (FLI-C)

	Estimated breeding values				De-regressed breeding values				Yield deviations			
	x_a	s_a	x_s	s_s	x_a	s_a	x_s	s_s	x_a	s_a	x_s	s_s
NR-H	99.4	4.2	99.8	6.2	100.0	4.1	99.8	6.2	99.7	3.0	99.7	5.7
NR-C	99.0	4.0	99.4	6.6	99.0	4.0	99.4	6.5	99.0	3.5	99.3	6.3
CFI	104.8	6.7	102.5	11.5	104.8	6.6	102.5	11.4	104.5	6.1	102.4	10.8
FLI-H	100.7	5.0	100.5	7.2	100.7	4.9	100.5	7.1	100.7	3.8	100.4	6.9
FLI-C	101.0	5.5	100.1	9.0	101.0	5.4	100.1	8.9	100.7	4.9	99.9	8.6

x_a = mean of all animals, s_a = standard deviation of all animals; x_s = means of sires with reliabilities >50%; s_s = standard deviation of sires with reliabilities >50%

Table 2. Rank correlations between routinely estimated breeding values (EBVr), breeding values derived from an approximate two-step procedure based on de-regressed breeding values and yield deviations for non-return-rate 56 heifer (NR-H) and cow (NR-C), days from first calving to first insemination (CFI) and days from first to last insemination for heifers (FLI-H) and cows (FLI-C) for all animals (N=183,129), sires with reliabilities >50% (N=318) and cows with reliabilities >30% (N=12,792)

Trait	De-regressed breeding values			Yield deviations		
	All	Sires	Cows	All	Sires	Cows
NR-H	0.997	0.999	0.998	0.866	0.967	0.970
NR-C	0.996	0.999	0.998	0.966	0.989	0.984
CFI	0.998	0.999	0.999	0.983	0.995	0.988
FLI-H	0.998	0.999	0.999	0.937	0.987	0.977
FLI-C	0.996	0.999	0.998	0.982	0.992	0.987

In accordance to the earlier studies (Sigurdsson and Banos, 1995; Thomsen et al., 2001), proposing drEBV to be reliable alternatives to daughter yield deviations, the approximate two-step procedure applied to drEBV is feasible. For the development of routine genetic evaluation, an approximate two-step procedure applied to drEBV is recommended as drEBV are easier available for all traits included in the TMI than YD and Interbull breeding values can be implemented straightforwardly. Unpublished results of Pfeiffer (2015) also showed that the estimation of genetic parameters using an approximate two-step procedure applied to drEBV was feasible. The entire procedure, including new genetic parameters is still under development.

CONCLUSION

An approximate two-step procedure applied to drEBV and YD based on field data is feasible. The results are encouraging for further work on their implementation in routine genetic evaluation. The results open up perspectives for the replacement of the current selection index method by an approximate two-step procedure based on drEBV.

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POPULATION STUDIES OF CZECH HUCUL HORSES

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Original scientific paper

SUMMARY

Population studies were carried out analysis Czech Hucul breed based on pedigree information of animals registered in the Studbook. Pedigree records collected from the year 1834 to 2013 comprised information on 9455 animals used in the analyses. The pedigree depth of the analysed individuals was up to 19 generations. The mean value of inbreeding coefficient was 5.35% (with maximum value 30%). The proportion of inbreed animals was high (98%). The average rate of inbreeding in the reference population was lower than 1%, and the respective estimates of effective population sizes were 54. The presented paper is indicating that genetic diversity in the Czech Hucul breeds is still relatively high and conservation programs should be continued.

Key-words: inbreeding, rate of inbreeding, effective populations

INTRODUCTION

Small populations such as Czech Hucul breeds bear the risk of inbreeding and reduction in genetic diversity within the population. The mating of related animals (inbreeding) was previously aimed at strengthening the required characteristics and traits in the population and at the concentration of appropriate genes in the population. Another aim was to increase offspring uniformity. However, it was observed at the same time that an increase in the inbreeding coefficient caused an increase in mortality, fertility and adaptability of farm animals (Falconer and Mackay, 1996). Preservation of endangered species is one of the most important goals for the present biological science. Conservation programs are needed to preserve breeds in which a significant part of given species' genetic diversity still presents negative effects of inbreeding. The populations of Czech Hucul horses are small sized, which has led to an increase in the population inbreeding coefficient of these breeds, especially in the 1990 when these populations were close to the import of genes from other populations. The increase in inbreeding coefficient may bring about undesirable inbreeding depression manifested in characteristics associated with fitness and reproduction and in other characteristics related.

The objective of the present paper was to estimate the inbreeding trend and the effective population size using pedigree data.

MATERIAL AND METHODS

Data

Data from pedigrees of the registered horses in the Czech Studbook of the Hucul contained information from the year 1823 to 31st June 2013. Data were provided by the Studbook Board of the Hucul (n=9455). The pedigree analysis was performed on one reference population. The reference population was defined as the whole active populations - individuals (stallions and mares) born in the years 1996-2010 (n=501).

Pedigree analysis

Pedigree completeness in the analysed breed was assessed with complete generations equivalent. The number of equivalent generations traced was computed as the sum over all the known ancestors of the terms according to $(1/2)^n$,where *n* is the ancestor's generation number, which is equal to one for the parents, two for the grandparents, etc. (Maignel et al., 1996). Pedigree completeness may influence the accuracy of inbreeding estimated values.

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Inbreeding coefficient (F_X) of each individual was estimated by a tabular method (Falconer and Mackay, 1996) based upon VanRaden's method (1992). The pedigree depth was 19 generations for the calculation of inbreeding coefficients.

Average relatedness coefficient of each individual (AR) is computed as the average coefficient integrating the row from the individual in the numerator relationship matrix. This coefficient indicates, with what probability, that a randomly selected allele in the population occurs in a selected individual or in a group of individuals (Goyache et al., 2003).

RESULTS AND DISCUSSION

The numbers of animals registered in the Studbook between years 1900 and 1966 were stable, between 1 to 7 individuals per year (54% stallions and 46% mares) (Figure 1). There was a moderate increase of the registered individuals between the 1966 and 1996. The maximum level of the registered animals in the Studbook was in 1996. In recent years, the numbers of the registered individual have been decreasing.

The mean number of stallions and mares registered in the Studbook were 47 and 81 in the period 1990-2013, respectively. The maximum level of the registered stallions (90) and mares (143) was in 1997.

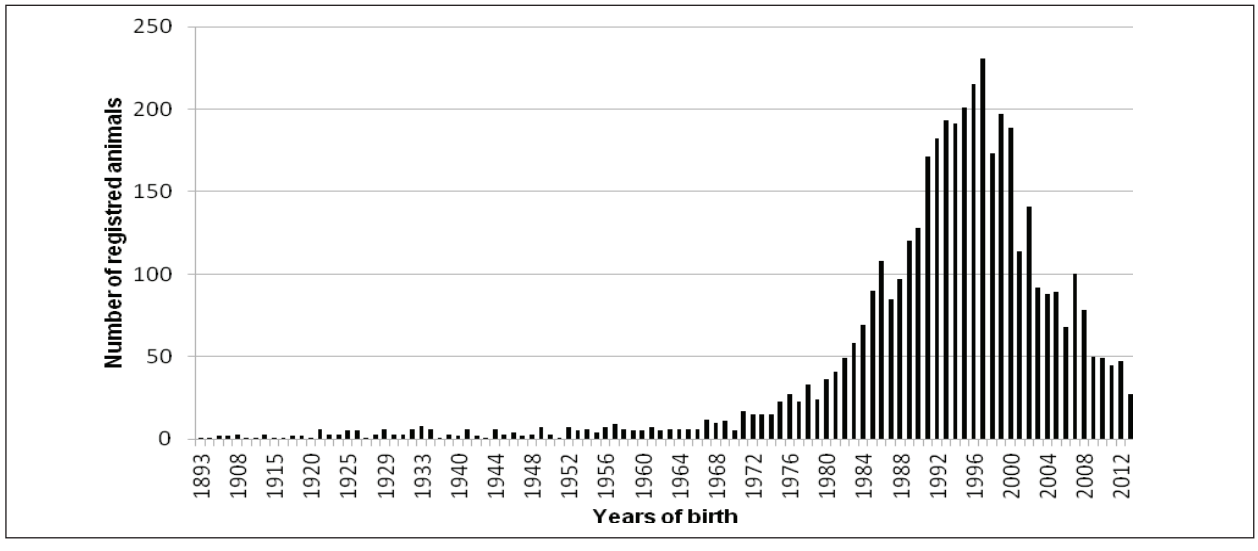

Figure 1. Number of animals registered in the Studbook per year of birth

The average complete generations equivalent was 6.83 and ranged from 0 to 9.02 being similar to the Polish Hucul horses where complete generations equivalent ranged from 3.8 to 7.0. The values computed by us are lower than the values determined for populations of Lipizzaner horse (15.2, Zechner et al., 2002) and Austrian Noriker (12.3, Druml et al., 2009). Pjontek et al. (2012) reported an equivalent number of ancestor generations to be 10.25 for Lipizzaner breed. Similar values were determined in Andalusian horse (8.3, Valera et al., 2005), Spanish Arabian horse (7.9, Cervantes et al., 2008) and German Paint horse (4.77, Siderits et al., 2013).

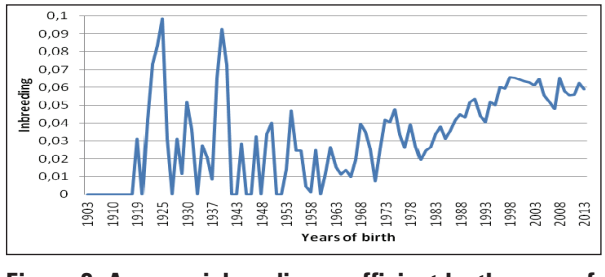

Figure 2. Average inbreeding coefficient by the year of birth

Average inbreeding coefficients in different years are presented in Figure. 2. The highest increase has been recorded since 1925. There was a random change decrease in the values of average inbreeding coefficient due to small number of the registered animals between years 1910 and 1968. Since 1988 a permanent increase has been observed when the 6.25% limit was exceeded in 1998. The average value of inbreeding coefficient in reference population was 5.35% while the maximum value was 30.63%. Average inbreeding coefficients published for Polish Hucul was similar (4.2%, Mackowski et al., 2015). The proportion of inbred animals in reference population reached 98% ($F_X>0$). More than 28% and 4% of the horses had inbreeding coefficients higher than 6.25% and 12.5%. The average values of relatedness coefficient were 0.13 in the reference population. The average relatedness coefficient was found higher than the double of the average inbreeding coefficient, from which an increase in inbreeding coefficient in the next generations may be derived. The average inbreeding value implies a loss of genetic variability that may negatively influence fitness characteristics and increase occurrence of phenotypic defects. The influence of inbreeding depression on performance traits was neither confirmed

by Curik et al. (2003) nor by Wolc and Balinska (2010). However, inbreeding depression was observed in racing performance (Klemetsdal, 1998) and in morphological traits (Gomez et al., 2009). Rate of inbreeding (ΔF) is one of the main parameters of genetic diversity monitoring. Based on the positive values of ΔF in the reference population, an increase in F_X values can be expected in further generations of the Czech Hucul population. The Food and Agriculture Organization of UN (FAO, 1998) stated that the average value of ΔF should not exceed 1%. The estimated average value of ΔF is close to this recommended maximal value. The mean of inbreeding rate for the reference population is close to the value 0.01. The effective population size derived from an increment in inbreeding coefficient reached the value of 54.15. The values of N_e, derived from ΔF, were close to the recommended minimum of N_e (50) for the conservation of genetic diversity (FAO, 1998). The estimated values of N_e were lower than N_e estimated in the other horse breeds including Lipizzaner ($N_e = 102$, Zechner et al., 2002), Austrian Noriker ($N_e = 157$, Druml et al., 2009) and higher than in Lusitano breed ($N_e = 28$, Vicente et al., 2012).

CONCLUSION

A complex pedigree analysis of the Czech Hucul breed has been performed. The results of the analysis of pedigree completeness show a high pedigree completeness level. The moderate values of inbreeding coefficient were estimated due to the high pedigree completeness that increased the accuracy of its computation. However, increased inbreeding level in the analysed time period was observed. This increased inbreeding level is characteristic for small, closed populations as Czech Hucul horse. However, this study indicates that genetic diversity in the Czech Hucul breed is still relatively high and conservation programs should be continued.

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GENOMIC VARIABILITY AMONG CATTLE POPULATIONS BASED ON RUNS OF HOMOZYGOSITY

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Original scientific paper

SUMMARY

In this work, the distribution of different lengths ROH (runs of homozygosity) in six cattle breeds was described. A total of 122 animals from six cattle breeds (Holstein, Simmental, Austrian Pinzgau, Ayrshire, MRI-Meuse Rhine Issel and Slovak Pinzgau) were analysed. The ROH approach was used to distinguish Slovak Pinzgau population from other investigated breeds as well as to differentiate between ancient and recent inbreeding. The average number of ROH per animal ranged from 17.06 in Holstein to 159.22 in Ayrshire. The highest number of short ROH (ancient inbreeding) was found in Simmental, followed by Ayrshire. The Ayrshire and MRI had a higher proportion of longer ROH distributed across the whole genome, revealing recent inbreeding. ROH were identified and used to estimate molecular inbreeding coefficients (F_{ROH}). The highest level of inbreeding from the investigated breeds was found out in Ayrshire with the same tendency for all length categories compared to Slovak Pinzgau with higher ancient inbreeding. Ancient inbreeding was only observed in Holstein population. A similar trend is becoming apparent even for Slovak Pinzgau, showing the second smallest recent inbreeding. Therefore, it is necessary to preserve the given population in the original phenotype and prevent further increase of inbreeding especially in endangered breeds.

Key-words: autozygosity, diversity, genotyping array

INTRODUCTION

High throughput genotyping allows a new and more accurate view on rates and effects of inbreeding in livestock (Ferenčaković et al., 2013a, Purfield et al., 2012). Long stretches of homozygous genome that most likely arise when the individual is the offspring of related individuals represent ROH (runs of homozygosity). When related individuals mate, the offspring carry long sections of the genome that are homozygous and identical by descent (IBD). Long ROH are most likely derived from a recent ancestor; shorter ones, from a more distant one. Long ROH thus correspond to recent inbreeding, whereas shorter ROH indicate more distant ancestral effects (ancient inbreeding) such as breed founder effects (Purfield et al. 2012). Calculating how much an individual's genome occurs as ROH of particular lengths provides information about levels of inbreeding relative to reference populations specific numbers of generations ago (Curik et al., 2014). Another advantage of inbreeding estimation based on genomic information is related to the fact that it is possible to differentiate local vs. genome-wide effect of inbreeding. For instance, Purfield et al. (2012) identified several genomic regions with particularly large ROH inbreeding in cattle, those regions potentially containing genes associated with traits of interest (immunity, carcass, dystocia).

Slovak Pinzgau is a traditional dual purpose breed, introduced approximately 150 years ago (Kasarda et al., 2014). Thanks to its unique traits as longevity, fertility, health, grazing ability it had been bred in mountainous areas of northern Slovakia (Pavlík et al., 2014). Due to significant decline of the population in last decades this breed is considered endangered (Kadlečík et al., 2008). From the beginning, only purebred animals were used for breeding. Approximately 60 years ago, the breed was started grading-up with other breeds in order to increase milk production while unique dual-purpose character was preserved. Jersey breed was

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used rather experimentally whereas Ayrshire, Meuse Rhine Issel (MRI), Red Holstein have been successfully proved and Red Holstein has been used up to this day. Nowadays, sires used originated mostly from Slovakia and some from Austria, due to a small number of sires, but pedigrees are generally connected. Austrian Pinzgau has been bred with Red Holstein and Simmental until the second half of the last century (Pšenica, 1990).

Since Pinzgau belongs to autochthonous breeds of Slovakia, clear distinction from other populations is necessary. There are many known methods based on genomic data as principal component analysis, F_{ST} values, genetic distances, structure analysis, admixture analysis. The aim of this study was to evaluate general ROH analysis for Slovak Pinzgau cattle and five related breeds.

MATERIAL AND METHODS

The population of sires of Pinzgau cattle from Slovakia were analysed. Genomic DNA for each of 19 semen samples was genotyped at a commercial lab using the Illumina BovineSNP50 v2 BeadChip. The genotyping array contained 54,609 SNPs. To understand the genetic relationship within and among breeds, genotypes from 103 other animals belonging to 5 domesticated bovine breeds (Holstein-54, Simmental-23, Austrian Pinzgau-5, Ayrshire-18 and MRI-3) were obtained from Decker et al. (2014a) and Gautier et al. (2010a). Despite the fact that Decker et al. (2014b) and Gautier et al. (2010b) removed closely related individuals from the dataset if pedigree data were available, current numbers of Slovak Pinzgau cattle did not allow us to perform the same exclusion. Due to use of more data sets new genetic map was created. In order to minimize risk of genotype errors and excluding poorly performing SNPs individual quality control was performed. Excluded were animals with more than 5% and SNP markers with more than 10% of missing genotypes by PLINK (Purcell et al., 2007). Minor allele frequency was not used as an exclusion criterion in this analysis, so that the detection of homozygous segments was not compromised. Further analyses were performed including information from 42 852 autosomal SNPs common to all breeds.

In this study, the minimum length of ROH was set at 1 Mb on the basis of the theoretical relationship between distribution of IBD fragments and the number of generations since a common ancestor. ROH were placed into five groups following approach of Ferenčaković et al. (2013a), identified as ROH > 1Mb , ROH > 2Mb , ROH > 4Mb , ROH > 8Mb and ROH > 16 Mb. For each individual in each of the six breeds, and for each ROH length category the total number of ROH detected, the average length of ROH (in Mb) and the sum of all ROH segments by animals (in Mb) were calculated. The inbreeding coefficient based on ROH (F_{ROH}) was defined as the length of all ROH in the genome divided by specified length of the autosomal genome covered by SNP according to McQuillan et al. (2008). The level of F_{ROH} in different length categories was determined to differentiate between ancient and recent inbreeding. The difference among individual breeds based on total length and total number of ROH was confirmed using F-test.

RESULTS AND DISCUSSION

Total length and number of determined ROH > 1 Mb per each of 122 individual is shown in Figure 1. Total number and length of ROH differed significantly ($p<0.0001$) among populations which historically contributed to the Slovak Pinzgau origin. The highest mean number of ROH was found in Ayrshire (159.22), followed by Simmental (143.78).

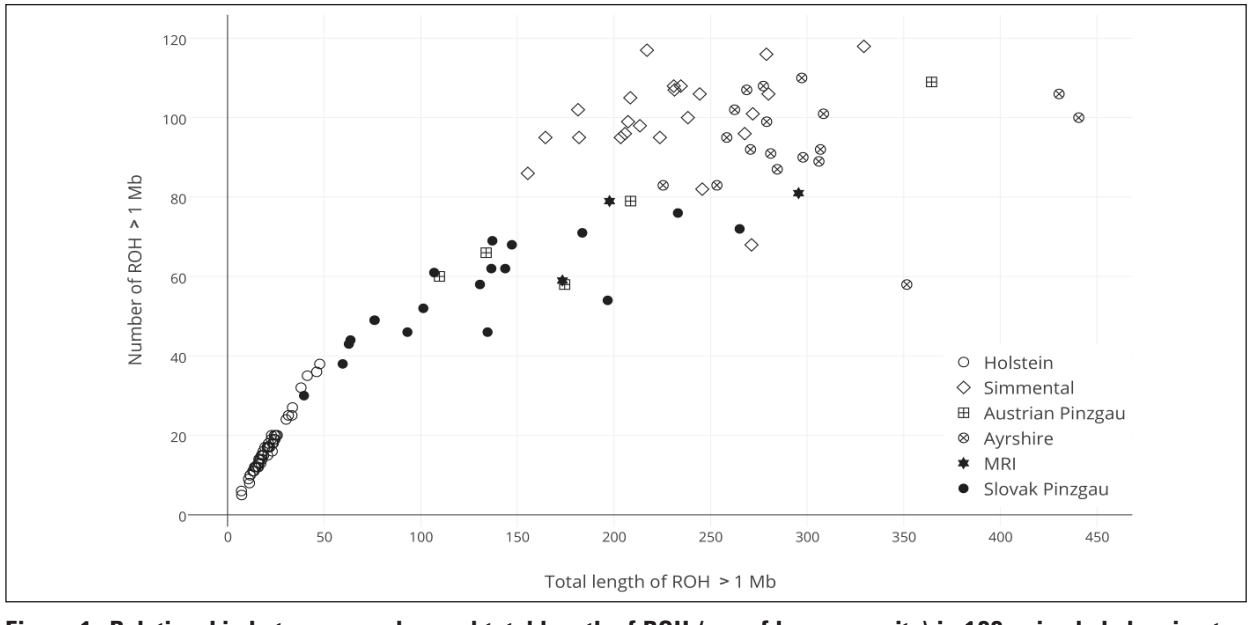

Figure 1. Relationship between number and total length of ROH (run of homozygosity) in 122 animals belonging to 6 breeds examined

The inbreeding coefficient derived from ROH in different length categories was used to differentiate more evident between ancient and recent inbreeding. The average number of the shortest ROH (related to ancient kinship) per animal ranged from 16.89±6.92 in Holstein to 99.96±11.34 in Simmental, similarly to the results of Austrian Simmental obtained by Ferenčaković et al. (2011). The Ayrshire and MRI had a higher proportion of longer ROH distributed across the whole genome, revealing recent inbreeding. Present livestock populations consist of a large number of cows decreasing number of sires and intensive selection for dairy production leading to a reduction of effective population size and increase of inbreeding. Planned mating strategy has been used in order to avoid the problem. Otherwise, only ancient inbreeding was observed in Holstein population (Figure 2).

The similar trend has been becoming apparent even for Slovak Pinzgau considered as dual-purpose cattle as well as for Simmental in study of Marras et al. (2015), whilst Purfield et al. (2012) noticed the highest recent inbreeding in Holstein. Differences in the F_{ROH} depend on selected animals and chosen length categories for ROH computation. The same tendency for all length categories was examined in Ayrshire with the highest values of inbreeding coefficient from all the investigated breeds. Mainly ancient inbreeding in Slovak Pinzgau was found out. The genomic inbreeding coefficients ranged from 0.0212±0.0216 to 0.1206±0.0226 in Ayrshire while from 0.0055±0.0074 to 0.0505±0.0245 in Pinzgau for $F_{ROH>16\ Mb}$ and for $F_{ROH>1\ Mb}$, respectively. The overall mean values of F_{ROH} across all length categories were 0.017±0.0037 in Holstein, 0.0411±0.0341 in Simmental, 0.0409±0.0368 in Austrian Pinzgau, 0.0683±0.0425 in Ayrshire, 0.0496±0.0333 in MRI, and 0.0238±0.0244 in Slovak Pinzgau. Thus, F_{ROH} differed significantly among breeds. The F_{ROH} values examined by Ferenčaković et al. (2013b) for all length categories in Austrian Pinzgau bulls were in between those obtained in the present study for Slovak and Austrian Pinzgau.

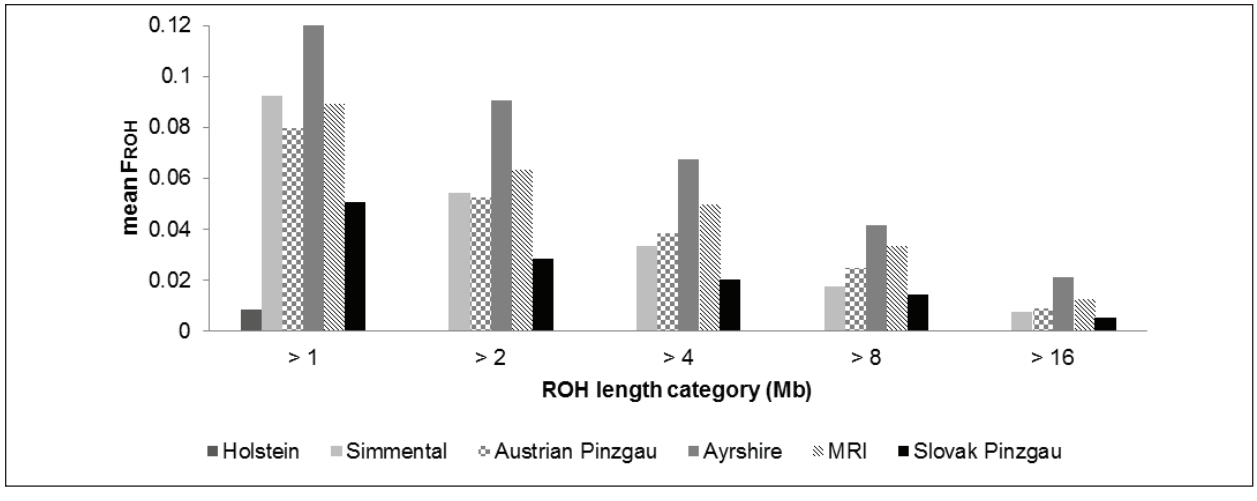

Figure 2. Inbreeding coefficient computed for different length categories ($F_{ROH\ >1,\ >2,\ >4,\ >8}$ and $_{>16\ Mb}$) in six breeds investigated

CONCLUSION

Total number of ROH and total length of ROH have shown genomic differences of the populations historically contributing to Slovak Pinzgau. The study provides results about the inbreeding independent of pedigree information. Historical formation of the breeds as well as present mating strategies within the breeds should be taken in the account for detection ancient or recent inbreeding. The absence of recent inbreeding in Holstein could be caused by selection of individuals for the performed analyses. Higher ancient inbreeding was obtained in Slovak Pinzgau, taking into account only this population. In comparison with other breeds showing the second smallest recent inbreeding it is necessary to preserve the given population in original phenotype. Regular monitoring of genetic diversity including inbreeding trends is necessary as this information is needed in population management.

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GENOMIC BACKGROUND OF ENTROPION IN FLECKVIEH CATTLE

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Original scientific paper

SUMMARY

Runs of homozygosity (ROH) and genome wide association study (GWAS) were used to identify genomic regions of entropion in Austrian Fleckvieh cattle. Entropion is an eye disorder where the eye lids turn inward, causing inflammations, cornea damage or even blindness if left untreated. A total of 196 bulls genotyped by BovineSNP50 BeadChip were analysed, nine of which had the disorder confirmed by a veterinarian, ten were unconfirmed and 177 were healthy control animals. Runs of homozygosity analysis highlighted regions on seven chromosomes where the proportion of animals in ROH significantly ($p<0.001$) differed between cases and controls. None of the 19 genes identified in these regions showed any connection to entropion or eye development. The GWAS study using only cases and controls had a mild peak directly on top of the ITGA9 gene. This gene was previously identified in mice affecting cornea and eye lid development, therefore we consider it being a candidate to influence entropion also in cattle.

Key-words: cattle, SNP, GWAS, runs of homozygosity, eye disorder

INTRODUCTION

Entropion is a non-lethal medical condition characterized by the lower or upper eye lid turning inwards. Subsequently the eyelashes irritate the eyeball and the cornea, causing inflammation and damage to the eye, or even loss of vision when untreated. It appears almost in all species, such as cats (Williams and Kim, 2009), dogs (Willis et al., 1999), pigs (Allbaugh, 2009), sheep (Basrur and Yadav, 1990), goats (Donnelly et al., 2014) and horses (Labelle et al., 2011).

The genetic background of entropion is complex, suggesting involvement of multiple genes, but no consensus on the mode of inheritance. The research focus in farm animals appears to be on sheep, due to high occurrence of entropion and relatively high heritability of 0.08-0.21 (Sakul and Kellom, 1997). Six regions influencing entropion in sheep were identified in a GWAS study by Mousel et al. (2014). To our knowledge there is no similar information available in cattle.

While the frequency of the disorder is likely similar to that of other cattle breeds, breeders of the Austrian Fleckvieh raised concerns about its occurrence. The aim of our study was thus to respond to this request and to search for genomic region(s) affecting the occurrence of entropion in Austrian Fleckvieh cattle.

MATERIAL AND METHODS

The data consisted of 196 genotyped Fleckvieh bulls, managed in two near-by locations by a breeders association, with Illumina BovineSNP50 BeadChip. Nine of those animals had entropion diagnosed by a veterinarian. Additional 10 bulls had changes on the eyelids, indicating possible abnormal condition, but without clear identification of entropion. Therefore we denoted these bulls as "uncertain". The remaining 177 bulls showed no sign of entropion or any other eye disorder and were assigned to the control group. Two evaluation scenarios were considered, given the uncertainty of diagnosis for some bulls: scenario 1 using affected, uncertain and control groups analysed together; scenario 2 using only affected and control bulls, i.e. the uncertain bulls deleted.

No prior checks were performed to compare the relatedness of the affected versus non-affected animals, assuming that inclusion of population structure in the GWAS analyses accounts for relatedness.

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Two methodologies were used to determine the genetic background of entropion: 1. comparison of runs of homozygosity (ROH) patterns between the affected and control groups (scenario 1 only); 2. a genome wide association study (GWAS) considering both scenarios. Two separate quality control approaches for ROH and GWAS followed, similarly to Mészáros et al. (2015).

For the ROH analyses we included only SNPs with GenCall ≥0.7 and GenTrain ≥0.4, following Ferenčaković et al. (2013). No other quality checks were done. The minimal length of ROH was set to 1Mb with minimum of 30 SNPs, without missing or heterozygous SNPs in the run. The analysis was done separately for cases and controls. Regions of interest were identified as those significantly differing ($p<10^{-3}$) in their ROH status between cases and controls. The uncertain cases were not considered for ROH analyses.

The quality control criteria for the genome wide association study included limitation of the minor allele frequency (min. 1%), deviation from Hardy-Weinberg ($p<10^{-6}$) and individual call rate (min. 90%). The number of SNPs remaining after quality control was 36,624 in scenario 1 and 36,682 in scenario 2. The average distance between SNPs was 72.2 kb after quality control in both cases. The population structure was considered using eigenvectors computed with the GemTools R package (Klei et al., 2011). Single SNP regression was used to find significant regions within the genome using R (R Core Team, 2012), using the Bonferroni correction as the significance threshold.

In the follow up analysis the regions of interest were studied in more details. Genes with possible connections to eye development in these regions were identified using the data base of National Center for Biotechnology Information (NCBI).

RESULTS AND DISCUSSION

The ROH analyses were conducted in the first step, to see if the causal regions for the entropion were in homozygous segments. If a certain genomic region would be in a ROH in all cases but only in some of the controls, it could be identified as a very strong candidate region. A similar approach was applied in Drögemüller et al. (2011).

In our case however, there was no region suggesting that entropion in cattle would be caused by recessive homozygotes, which confirmed results of Sakul and Kellom (1997). Several genomic regions showed very different patterns of ROH in cases and controls. The ROH results from chromosome 2 are shown as an example in Figure 1. The proportion of the animals in ROH is shown at the bottom, with apparent differences in some regions. The p values were calculated for each SNP to denote significance of the difference (Figure 1, top). An arbitrary value of $p<10^{-3}$ was used as a threshold to identify regions for further investigation.

Regions on chromosomes 2, 6, 11, 12, 13, 19, and 21 showed significantly different patterns in ROH, harbouring 19 genes in total. None of these 19 genes had a previously identified connection to eye development or disorders however. There was a complete lack of genes in multiple regions.

The genome wide association study was done considering two scenarios. In both cases the population structure was considered via eigenvectors, included as fixed effects into the model.

In the first step the uncertain cases were included into the evaluation, considering them as truly affected cases. After the analysis had been conducted there was only a single significant SNP over the Bonferroni line in the 42 Mb region of chromosome 6 (not shown). There were several genes in the region, but without apparent links to entropion.

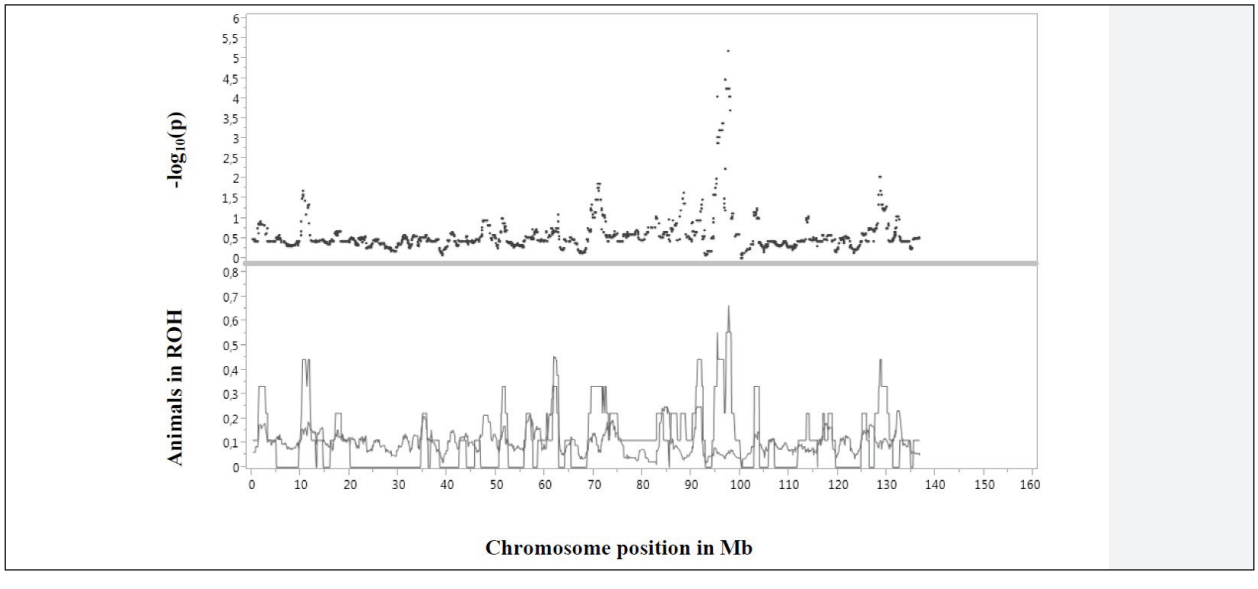

Figure 1. Comparison of segments in run of homozygosity in affected and control animals (chromosome 2)

In the second scenario only the confirmed cases were used in the GWAS run (Figure 2). Several regions were suggestive (i.e. close to the Bonferroni line), thus closely checked for genes. The most important finding was from chromosome 22 at 11.09 Mb, directly on the top of the integrin alpha 9 gene (ITGA9; 10.95-11.31Mb). The gene was previously identified affecting the cornea (Stepp et al., 1995) and eye lid development (Stepp, 1999; Banks, 2000) in mice. The ITGA9 gene has also strong connections to the lymphatic system (Bazigou et al., 2009), and it has been shown to be up regulated during corneal lymphangiogenesis and lymphatic valve formation (Truong et al., 2011).

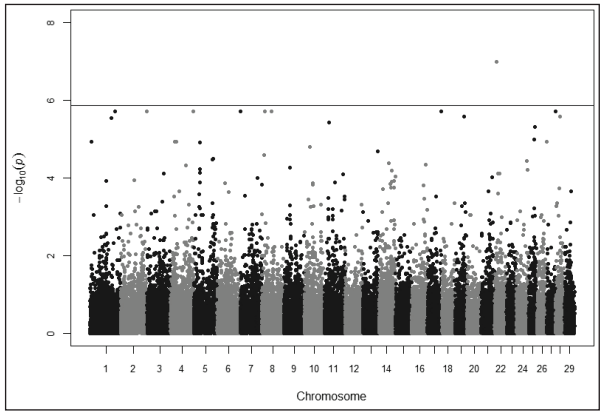

Figure 2. Genome wide association study comparing affected and control individuals. Uncertain cases were not considered here

From our analysis it was apparent that caution should be exercised when including animals with uncertain disease occurrence into genome wide association studies. Our analyses identified different regions of interest, when uncertain cases were considered, as opposed to the analysis with only the certain cases and controls. Possibly some of the uncertain cases had the symptoms of entropion, without being truly affected and therefore introducing a bias.

The most interesting region on chromosome 22 was identified by a single SNP. After critical review of the results we have to acknowledge that the result was not extremely convincing, even though there was an underlying gene with strong connection to the development of eyelids. Typically in a single SNP analysis a strong GWAS signal would involve "tower like" structures in the Manhattan plot, with multiple highly significant SNPs very near to each other. This was not our case. The reason could be that the low number cases did not allow to precisely identify the possibly complex genomic background of entropion. Therefore a follow up analysis with more cases and higher density genotype data would be of interest.

CONCLUSION

Two methods were used to identify possible causes of entropion in Austrian Fleckvieh cattle. The ROH analyses identified regions of interest on seven chromosomes, harbouring 19 genes. None of these genes was associated with eye development or disorders however, by the literature research. The genome wide association study considering affected and control individuals identified the gene ITGA9 on chromosome 22 as a possible candidate influencing entropion in cattle.

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GENEALOGICAL DECOMPOSITION OF THE EFFECTIVE POPULATION SIZE: A CASE STUDY ON CROATIAN AUTOCHTHONOUS CATTLE BREEDS

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Preliminary communication

SUMMARY

Effective population size (N_e) is one of the most important tools used to assess genetic diversity for conservation purposes. Using pedigree data of three Croatian autochthonous cattle breeds (Buša, Istrian and Slavonian Syrmian Podolian) the effective maternal (Ne_F), paternal (Ne_M) and combined maternal-paternal (N_{eFM}) population size was estimated. Additionally, we estimated the effective population size based on the census population sex ratio (N_{es}), the effective population size from the individual increase in inbreeding (N_{eFi}) and the effective population size from individual increase in coancestry (N_{eCi}). We compared these sizes with the values obtained for 20 additional cattle populations, as well as with the newly calculated N_{eFM}. The effective population sizes calculated for three autochthonous breeds were consistently the lowest in amongst all the considered cattle breeds. Utilisation of extremely small numbers of breeding males is the main reason for the observed reduction in the effective population size. The decomposition of effective population size into maternal and paternal components is shown to be an informative parameter in detecting the reduction of the effective population size as a consequence of unequal sex contribution. Still, the impact of the pedigree depth and completeness on the N_{eF}, N_{eM} and N_{eFM} estimation remain to be analysed. A large deviation between N_{es} and all other methods of N_e estimation was observed and it is our recommendation that breeders and stakeholders should consider using alternative methods of N_e estimation when planning breeding programmes as well as in the determination of the endangered status of animal populations.

Key-words: genealogical analysis, effective population size, cattle, sex ratio

INTRODUCTION

Assessment of genetic diversity is necessary for autochthonous genetic stock conservation (Alvarez et al., 2012). Genealogical records, or pedigrees, are both historically and presently important sources for the estimation of quantitative genetic and diversity parameters. Leroy et al. (2014) states that the effective population size (N_e), as developed by Wright (Wright, 1931), stands out amongst the many tools used to assess genetic diversity for conservation purposes. N_e is defined as the size of an ideal (Wright-Fisher) population (N) where individuals are monoecious, selfing is possible, and the amount of genetic drift will be the same as in the actual population being considered (Allendorf, 2013). The simplest method of calculating N_e is based on the sex ratio within the population. Since pedigree information is unavailable for many animal populations, this is the method routinely used by the Food and Agriculture Organization (FAO) and the European Association for Animal Production (EAAP) when estimating the effective population size of animal populations. However, N_e can be estimated from different sources of information, including demographic information, pedigrees or molecular data. The choice of the method of estimation becomes very important in order to obtain a result which can be used to effectively manage animal populations.

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Several alternative genetic diversity indicators have been proposed and of those the most widely used ones are: the genetic drift through temporal changes in allele frequencies (variance of effective population size), the increase in homozygosity (inbreeding effective population size), or the rate at which the unique alleles are lost (eigenvalue effective population size) (Leroy et al., 2014). None of these methods separate the effective population sizes of males and females, rather they combine them. On the other hand, the ratio of the effective population sizes of both sexes is one of the critical points for the estimation of the overall effective population size, so our approach was to estimate these parameters separately.

Recently, mitochondrial DNA (mtDNA) was used to compute the maternal effective population size, along with the discrete generations equivalent and inbreeding parameters (Alvarez et al., 2012). In this pilot study we aimed to extend the genealogical approach by Alvarez et al. (2012) and estimate the effective maternal (N_{eF}) and paternal (N_{eM}), as well as the combined maternal-paternal (N_{eFM}) effective population size in three Croatian autochthonous cattle breeds (Buša, Istrian and Slavonian Syrmian Podolian) using their respective pedigrees. We also estimated the effective population size based on the sex ratio (N_{es}), the effective population size from the individual increase in inbreeding (N_{eFi}) and the effective population size from the individual increase in coancestry (N_{eCi}), and compared them with values obtained for 20 additional cattle populations (Leroy et al., 2014) ,as well as to the newly calculated N_{eFM}.

MATERIAL AND METHODS

Pedigree files for three autochthonous cattle populations: Buša (CRB), Istrian (IST) and Slavonian Syrmian Podolian cattle (SSP) in Croatia have been analysed. The largest pedigree was the IST cattle pedigree (4752), followed by the BH cattle (1707) and the SSP cattle (1129) pedigrees.

The reference population was defined as individuals born in the period from 2004 to 2014 and all effective population sizes were calculated only for these populations. The pedigrees were checked for errors using the programs CFC (Sargolzaei, M., 2006) and Endog (Gutiérrez and Goyache, 2005).

The effective population size based on census population sex ratio (N_{es})

$$N_{es} = \frac{4MF}{M + F},$$

was calculated for all three pedigrees.

The following genealogical parameters were then computed in Endog:

a) effective population size from individual increase in inbreeding (N_{eFi}) according to Gutiérrez et al. (2009):

$$\Delta F_i = 1 - \sqrt[EqGi-1]{(1 - F_i)},$$

b) effective population size from individual increase in coancestry (N_{eCi}) according to Cervantes et al. (2010):

$$\Delta C_{ij} = 1 - \sqrt[(EqGi+EqGj)]{(1 - C_{ij})}$$

Maternal (N_{eF}) and paternal (N_{eM}) effective population sizes were calculated using the version of the in-house program MaGelLAn (work in progress) by the method employed by Alvarez et al. (2012):

$$N_{eF} = \frac{1}{\Delta PI},$$

where PI is defined as the probability where individuals share the same dam or haplotypic line by chance (Bowling et al., 2000). The paternal (N_{eM}) effective population size was calculated using the same method by simply reversing the sexes within the pedigrees.

The results obtained in this way via MaGelLAn were then used to calculate the combined maternal-paternal effective population size N_{eFM} based on the same formula as for N_{es}, substituting N_{eM} instead of M and N_{eF} instead of F.

RESULTS AND DISCUSSION

Three Croatian autochthonous cattle breed populations have been analysed. A total of 3350 IST, 1357 CRB and 866 SSP cattle were included in the reference population (*Pref*). The mean inbreeding percentage (*F*) was largest in CRB (6,59), followed by IST (5,1) and SSP (3,81) cattle populations. When compared to *F* values from 20 cattle populations by Leroy et al. (2014) our populations had at the same time the largest inbreeding and the smallest complete generation equivalents (2, 3 and 3,1) of all cattle populations analysed. The estimated effective population sizes of the three Croatian autochthonous cattle populations, calculated by different approaches, are shown in Table 1.

Table 1. Estimated effective population sizes Croatian autochthonous cattle breeds

Parameters	CRB	IST	SSP
Reference population (Pref)	1357	3350	866
Individual increase in inbreeding effective population size (N_{eFi})	13	22	29
Individual increase in coancestry effective population size (N_{eCi})	13	19	12
Maternal effective population size (N_{eF})	147	78	13
Paternal effective population size (N_{eM})	18	2	3
Founder sex ratio effective population size (N_{eFM})	64	8	8
Census population sex ratio effective population size (N_{es})	1244	3316	861

Buša (CRB), Istrian (IST) and Slavonian Syrmian Podolian cattle (SSP)

The effective population size estimates N_{eFi} and N_{eCi} showed values in the range 12-29 for all three cattle populations. The combined maternal-paternal effective

population size N_{eFM} resulted in a larger range from 8 for IST and SSP to 64 for CRB cattle. By comparing the N_{eFi} and N_{eCi} estimates with those for the 20 cattle populations from Leroy et al. (2014) our cattle populations showed consistently smaller effective population sizes than other populations, up to twice as small as the smallest N_{eFi} and N_{eCi} values in the comparison dataset, 52 and 58, respectively. By estimating N_{eFM} through decomposition to N_{eF} and N_{eM} we noticed large differences between N_{eF} and N_{eM}. The N_{es} values, on the other hand, strongly deviate from all other N_e estimates, being in the range from 861 to 3316, due to the relatively balanced census population sex ratio in the three populations. This overestimation trend is also clearly seen when looking at the N_{es} values of the 20 cattle populations from Leroy et al. (2014). Here, it is evident that the census population sex ratio is overestimated. By comparing the census population sex ratios (N_M/N_F) with effective population sex ratios (N_{eM}/N_{eF}) a two-three fold drop in the values was observed: 0,6→0,3 for CRB, 0,8→0,25 for IST and 1,1→0,4 for SSP. Thus, we consider that the low number of breeding males is the critical factor that caused small effective population size estimates in the analysed breeds.

The low effective population size for IST population is in concordance with Curik et al. (2014) where the current effective population size (N_{eLD}=12) was estimated by high-throughput molecular data following the linkage disequlibrium approach described in Flury et al. (2010). At the same time, according to the Croatian Agricultural Agency report (2013) the breed status is highly endangered with N_e estimated to 152 (721 cows and 40 bulls) when calculated from the census population sex ratio.

Our recommendation is that breeders and stakeholders should not rely only on N_{es} when planning breeding programmes or estimating the endangered status of animal populations, as other methods provide more concordant N_e estimates.

CONCLUSION

The effective population sizes calculated in three autochthonous Croatian cattle breeds were consistently lower in comparison to other cattle breeds from Leroy et al. (2014), even those with smaller reference populations than Croatian breeds, such as Ferrandaisex (*Pref*=587). Utilisation of an extremely small number of breeding males in CRB, IST and SSP breeding is the main reason for the observed reduction in the effective population size. Decomposition of the effective population size to the maternal and paternal components has been shown to be an informative parameter in detecting the reduction of the effective population size due to the unequal sex contribution. Still, the impact of the pedigree depth and completeness on N_{eF}, N_{eM} and N_{eFM} estimation remains to be analysed.

A large deviation between N_{es} and all other methods of N_e estimation was observed and it is our recommendation that breeders and stakeholders should be considered using alternative methods of N_e estimation when planning breeding programmes, as well as in the determination of the endangered status of animal populations.

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LACTATE DEHYDROGENASE GENE GENOTYPIZATION FOR SPECIES IDENTIFICATION IN A FISH FARM ON THE RIVER NERETVA

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Original scientific paper

SUMMARY

There are severale Salmonid species, found in the river Neretva basin, among which S. trutta and S. obtusirostris. Also, natural hybrids such as S. obtusirostris x S. trutta have been observed. In one fish farm on the river Neretva, S. trutta and S. obtusirostris were decided to breed separately. Parental fishes were separated phenotypicaly on the basis of the morphological signs. PCR-RFLP analysis of the exon 3 to exon 4 part of the lactate dehydrogenase (LDH) C1* gene with restriction endonuclease RsaI was employed to identify the presence of other species representatives or intercrosses in two groups of juvenille fishes. Using this method, we were able to identify two S. trutta representatives in the S. obtusirostris group.

Key-words: Lactate dehydrogenase gene, PCR-RFLP, S. trutta, S. obtusirostris

INTRODUCTION

There are severale Salmonid species, found in the river Neretva basin, among which *S. trutta* (brown trout), *Salmo marmoratus* (marble trout), *Salmo obtusirostris* (softmouth trout) *Salmo farioides* and *Salmo dentex*.

Salmo obtusirostris also known as the Adriatic trout, Adriatic salmon, and softmouth trout, is a species of salmonid fish endemic to the rivers of Western Balkans. It is found naturally in four drainages of the Adriatic Sea, in Croatia, Bosnia and Herzegovina and Montenegro: the Neretva-Vrljika system, the Jadro, the Morača-Zeta system and the Krka river drainages. Morphological different characteristics for different softmouth trout populations from different river-systems resulted in the description of three putative subspecies: *Salmo obtusirostris oxyrhynchus* from the River Neretva, Bosnia and Herzegovina, *Salmo obtusirostris salonitana* from the river Jadro, Croatia, and *Salmo obtusirostris krkensis* from the River Krka, Croatia.

In the river Neretva basin, natural hybrids such as *S. obtusirostris x S. trutta* have been observed and reported (Vuković, 1982). The hybridization between autochthonous species was confirmed also in an experiment performed in the fish farm located in the river Buna, a tributary of the river Neretva (Kosorić and Vuković, 1969). Introduction of non-native brown trout has also been practised in the river Neretva and stocking activities have never been well documented.

Lactate dehydrogenase (LDH) C1* gene containing parts of exons 3 and 4 with intermediate intron has been found, firstly in brown trout (Mc Meel et al, Ferguson, 2001), and after that also in *S. obtusirostris* (Snoj et al., 2002) proven as an informative genetic marker.

A mutation in the intron 3 of the (LDH) C1* gene (G/C substitution) was connected with the disruption of the *Rsa*I restriction enzyme recognition site, being specific for *S. obtusirostris* (Razpet, 2004; Razpet, 2007).

The same mutation was found in *Salmo obtusirostris salonitana and in Salmo obtusirostris oxyrhynchus (Odak, 2004).*

In one fish farm on the river Neretva, *S. trutta* and *S. obtusirostris* were decided to breed separately.

Parental fishes were separated phenotypically on the basis of the morphological signs. The aim of this work was, based on the LDH genotype, to find intercrosses or representatives of the other species in two groups of juvenille fishes. In the first group, there should

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be only representatives of *S. trutta*, while in the second one only representatives of *S. obtusirostris.*

MATERIAL AND METHODS

In the fish farm, juvenille fishes were raised in two groups. In the first group, there were juvenille fishes of *S. trutta*, and in the second juvenille fishes of *S. obtusirostris*. Fin clips were taken from 21 individuals from both groups (together 42 samples) and stored in 96% ethanol. In the lab, genomic DNA was isolated from the preserved fin clips using Bio Basic EZ-10 Spin Column Animal DNA Mini-Preps Kit (Bio Basic Canada Inc.), following the supplier's instructions.

PCR of the lactate dehydrogenase (LDH) C1* gene containing parts of exons 3 and 4 with intermediate intron has been performed as described by Mc Meel et al. (2001). Twenty μl PCR contained 100 ng of genomic DNA, 1,5 mM $MgCl_2$, 0,2 mM of each dNTP, 1 μM of each primer (Ldhxon4R and Ldhxon3F), and 1U of *Taq* DNA polymerase (Fisher scientific). Amplification was performed in the thermal cycler (*Eppendorf*) as described before (Mc Meel et al., 2001).

PCR products were cleaved by *Rsa*I restriction endonuclease, with a recognition site gt/ac. The amplified part of the (LDH) C1* gene in *S. trutta* contained two *Rsa*I recognition sites, leading to three bands after restriction in the lenght of 74, 135 and 166 bp (genotype NP). On the other hand, PCR product of *S. obtusirostris* posessed only one restriction site because of the disruption of the second recognition site due to a G/C transformation (MP genotype), leading to only two bands in the length of 74 and 300 bp after restriction, respectively (Figure 1). Products of restriction reactions were checked on the 2% agarose gel.

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CLUSTAL 2.1 multiple sequence alignment


S.obtusirostris      TGTTACCACGACGATACGAGAGTTCGCCGTCACAGAGTAGTCTGACCGTGGGAGAACAAT 60
S.trutta             TGTTACCACGACGATACGAGAGTTCGCCGTCACAGAGTAGTCTGACCGTGGGAGAACAAT 60
                     ************************************************************

S.obtusirostris      CAATCAATGAGAGTACTGTGTATCATTTGTGTCTAGTATTTCTTCAGTAATTTGTCATAT 120
S.trutta             CAATCAATGAGAGTACTGTGTATCATTTGT--CTAGTATTTCTTCAGTAATTTGTCATAT 118
                     ******************************  ****************************

S.obtusirostris      CATTAATAGATCTAATGGCAGGACTATTACATGTCAAAGTAGGATTTCAGAAATTGCTTT 180
S.trutta             CATTAATAGATCTAATGGCAGGACTATTACATGTCAAAGTGGGATTTCAGAAATTGCTTT 178
                     ************************************** *******************

S.obtusirostris      GAGAAACTTCATTCATACATTTCCCTTTCACCCTTTTCCCCCCCATCTCCCTTTCATACA 240
S.trutta             GAGAAACTTCATTCATACATTTCCCTTTCACCC------CCCCCATCTCCCTTTCATACA 232
                     *********************************      *********************

S.obtusirostris      CTTCCCCTCTCAGAGAGACTACTTCATTTAACACACAGACATTTGACATGCAGATGGTGT 300
S.trutta             CTTCCCCTCTCAGAGAGAGTACTTCATTTAACACACAGACATTTGACATGCAGATGGTGT 292
                     ****************** ****************************************

S.obtusirostris      CCATCTTGGTTTTCTGGTTTCCTGGTGCCCAATTGCTACACACTCACCTTTGCTGGCGAC 360
S.trutta             CCATCTTGGTTTTCTGGTTTCCTGGTGCCCAATTGCTACACACTCACCTTTGCTGGCGAC 352
                     ************************************************************

S.obtusirostris      TATCTTGGGCGTTTTGAGGAA 381
S.trutta             TATCTTGGGCGTTTTGAGGAA 373
                     *********************
```

Figure 1*. Clustal 2.1* alignment of the PCR-amplified part of the (LDH) C1* gene in *S. trutta* and *S. obtusirostris* with underlined *Rsa*I restriction enzyme recognition sites

RESULTS AND DISCUSSION

In 21 fishes analyzed from the first group (*S. trutta*), PCR-RFLP analysis of the exon 3 to exon 4 part of the lactate dehydrogenase (LDH) C1* gene with restriction endonuclease *Rsa*I revealed presence of only NP genotype, specific for *S. trutta*, with two *Rsa*I restriction sites and tree bands on the agarose gel after restriction (Figure 2).

In the second group of juvenille fish, there should be softmouth trutt individuals, with characteristic MP genotype of the lactate dehydrogenase (LDH) C1* gene. However, after PCR amplification and subsecvent restriction of the PCR product with *Rsa*I restriction endonuclease, 19 samples had MP genotype and two ones had NP genotype (Figure 2), being not speciffic for *S. obtusirostris*.

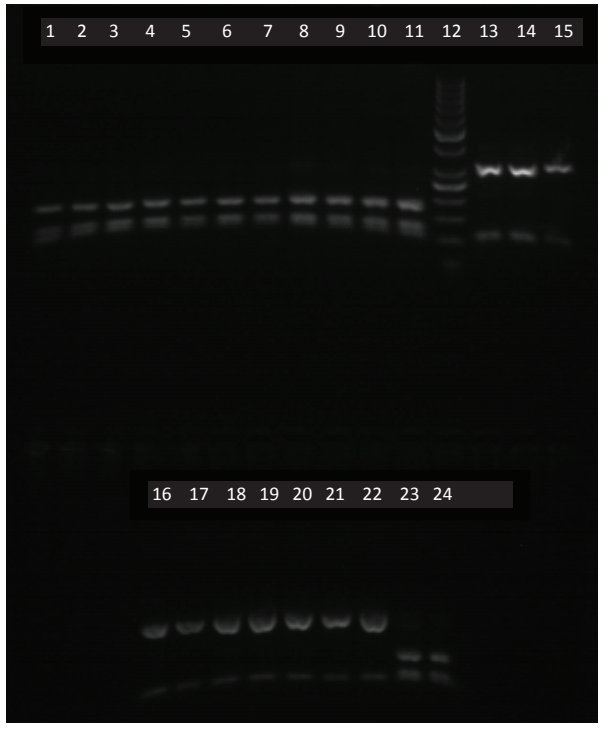

Figure 2. PCR-RFLP results on agarose gel. Samples 1-11 are from the *S.trutta* group and they are all of NP genotype with tree bands after restriction. Samples 13-24 are from the *S.obtusirostris* group. While samples 13-22 posess MP genotype, specific for *S. obtusirostris*, samples 23 and 24 have NP genotype. Sample 12 is 50bp marker

In the both analized groups no intercrosses of MP and NP genotypes were found. The assumption is that the presence of the NP genotype in the group of juvenille softmouth trutt is either consequence of the presence of pure *S. trutta* fish in the *S. obtusirostris* parental group, or juvenille fish were accidentally mixed during handling. In the fish fry morphological signs, characteristic for adult fishes of both species, were not well defined and distinguishing between representatives of both species is difficult. Based on the results, the suggestion was to first devide adult fishes into two groups, based on morphological signs. Further, parental lines of both species should be marked and sampled for genetic analysis for detection of intercrosses or representatives of other species in parental groups. Combination of morphological signs and genetic methods was allready proven to be very powerfull method in distinguishing between *S. marmoratus* and hybrids with alien *Salmo* species in the Soča river basin (Delling et al., 2000).

Disadvantage of the McNeel's method is that it could be used only to elucidate *S. obtusirostris* representatives from other *Salmo* species, found in the river Neretva. While the MP genotype of the (LDH) C1* gene is a characteristic only for *S. obtusirostris*, the NP genotype is not present only in *S. trutta*, but also in *S. marmoratus* and other *Salmo* species, found in the river Neretva basin (Razpet, 2006). Other genetic methods like microsatellite genotyping, mitochondrial DNA analysis and SNP genotyping were described in different phylogenetic studies of *Salmo* species in the river Neretva (Snoj et al., 2002; Razpet et al., 2007; Pustovrh et al., 2014). The described methods could also be used for species identification in fish farms. The problem is that these methods are much more expensive than PCR-RFLP genotyping and therefore they are financially not suitable for the fish farm owners.

CONCLUSIONS

PCR-RFLP analysis of the exon 3 to exon 4 part of the lactate dehydrogenase (LDH) C1* gene was employed to identify the presence of other species representatives or intercrosses in the two groups of salmonides (*S. trutta* and *S. obtusirostris* group), raised in a fish farm on the river Neretva. Using this method, we were able to identify two *S. trutta* representatives in the *S. obtusirostris* group. There is also a great interest of fish farmeres to raise other salmonides from the river Neretva with a help of genetic methods. Due to financial limitation, there is a need for developing the low-cost genetic methods distinguishing between other commercially interesting salmonides from the river Neretva.

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EFFECT OF BREED AND SAMPLING PLACE ON THE MINERAL CONTENT OF CATTLE HAIR

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Original scientific paper

SUMMARY

Mineral intake is important for high production level. Estimation of exact mineral intake is difficult in grazing and/or group housed animals like cattle. Accessing of long term mineral status seems to be possible using hair mineral analyses. However, several factors can affect the results. Therefore, the aim of this study was to test the effect of sampling location and breed on the mineral content of beef cows' hair fed the same feeding regime. Ten Hungarian Simmental and ten Charolais cow were selected from the same farm. Coloured hair samples free of visible contamination were obtained from the withers, side and quarter of the cows. Hungarian Simmental samples were used to test the effect of sampling location. Since it did not show significant effect, Charolais samples were analysed as pooled. Samples were mineralized using nitric acid and hydrogen peroxide using ultrasonic cleaning unit. Calcium, magnesium, sodium, copper, selenium and zinc content were determined by ICP-OES (Perkin-Elmer, Optima 3300 DV). Statistical analyses were carried out by SAS (SAS Institute Inc., Cary, NC) GLM procedure. Significant breed differences were detected in the case of calcium, magnesium and copper. The measured values were above or around the normal ranges, suggesting that the mineral status of the herd was adequate. Sampling location of short hairs had no influence on the mineral profile.

Key-words: cattle, mineral content, hair analyses

INTRODUCTION

Minerals play various roles in the living organisms. Due to the increase of production potential and level of farm animals, the proper mineral supplementation has high importance. The actual amount of metabolically available minerals depends on many factors: mineral intake, chemical form of mineral supplementation, age, mineral interactions, phytase enzyme supplementation, etc. Therefore, it has a practical importance to monitor the actual mineral status. Analysing the plasma or urine mineral content to check the mineral status is an obvious option. However, the plasma mineral content is quite variable due to the stage of intestinal absorption (absorption and plasma level is increasing as the partly digested feed reaches the place of absorption, but decreases when available substrate is decreased) and/or mineral mobilization from stores. Therefore, plasma or urine mineral content represent limited information about the overall mineral supply (Gabryszuk et al., 2010). For that reasons, researchers were looking for other biological samples, which can give information about the mineral status of longer period. It is proven that in the course of hair development minerals are accumulated from blood into the cortex of hair (Combs, 1987). Developed hair became isolated from the continuously changing metabolic processes. Therefore, hair mineral content can represent the average mineral supplementation of a longer period. The first applications of hair mineral analyses were to detect and prove mineral poisoning of famous humans, like Bonaparte Napoleon (Kintz et al., 2007). However, in such a case only the detection of presence is required, while in case of farm animals the connection of intake and hair mineral content should be established. The research results are controversial regarding to the precision and interpretation of hair mineral analyses (Namkoong et al., 2013; Darsch and Roider, 2002; Combs, 1987). For the practi-

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cal application we need to establish reference values and gain information about the factors influencing the hair mineral content (Mikulewicz et al., 2013; Combs, 1987). It is known that hair mineral content of animals originating from different farms and genotypes can be different (Gabryszuk et al., 2010; Patkowska-Sokola et al., 2009). The obvious reason is the various feeding regimes applied on the different farms. Information is still lacking on the hair mineral content of different breeds of cattle having the same feeding regime. Body location of sampling and the contamination of hair samples can also alter the measured mineral content (Fisher et al., 1985). Therefore, the aim of our study was to investigate the effect of breed (Hungarian Simmental and Charolais) and hair sampling place on the hair mineral content of beef cattle.

MATERIAL AND METHODS

Ten Hungarian Simmental (beef type) and ten Charolais cows were randomly selected from the herd of the Derecske Petőfi Agricultural Ltd. (Derecske, Hungary). The sampling was carried out in March. The animals received the same feeding regime consisting of meadow hay and concentrate. Coloured hair samples free of visible contamination were obtained from the withers, side and quarter of the Hungarian Simmental cows using bended scissors. As results did not show any sampling site effect, Charolais cows were sampled by the same method, but hair samples were mixed and analysed like that. Results of the mixed samples served to test breed differences. From the samples calcium (Ca), magnesium (Mg), sodium (Na), copper (Cu), selenium (Se) and zinc (Zn) content were analysed. Organic contamination was removed by washing with ethyl-alcohol (96%, Sigma-Aldrich). Dried samples were mineralized by 2 ml nitric acid (distilled, Sigma-Aldrich) in ultrasonic cleaning unit at 60°C for 30 min. After cooling 2 ml of 30% hydrogen peroxide (Sigma-Aldrich) were added and samples were mineralized for 90 min at 100°C. After mineralization solutions were filled up to 10 ml with distilled water and filtered throughout MN 619 G □ (155 mm diameter) filter paper. Measurement of solutions was carried out with ICP-OES (Perkin-Elmer, Optima 3300 DV). Statistical analyses were conducted by SAS (SAS Institute Inc., Cary, NC) GLM procedure at P=0.05 level.

RESULTS AND DISCUSSION

Calcium content of Hungarian Simmental cows was higher than Charolais, but both fell within the reference range (Table 2). Calcium is the most abundant mineral in the body. Despite that, its majority can be found in bones. It has several important functions in soft tissues as well. Ca is transported in ionized form in the plasma, and its level regulated in narrow limits (Georgievskii, 1982). This can be the reason that researchers found out only week correlation between calcium intake and hair content, making hair analyses limited value for assessing Ca supply (Combs, 1987). The evaluation is further complicated by the interaction with other elements. Anke (1966) reported that dietary Ca had an antagonistic effect on hair P and Zn level. Kornegay et al. (1981) reported that hair of pigs fed a reduced P diet contained higher amount of Zn and Mn. It is known that Ca and P compete for transport mechanisms during intestinal absorption, and P in a form of phytic acid binds other minerals. These mechanisms can be the reasons of such observations. However, it is still not explained why we detected breed differences in spite of the similar nutrition. Since experimental animals were adult, differences in metabolic activity due to different growth rate cannot be suspected as a reason. Furthermore, it is very interesting that Gabryszuk et al. (2010) found only 25-30% Ca level in cattle hair kept on organic farms, showing deficient Ca supply. These cows were grazing and, depending on pasture yield and availability of other feeds, the feeding ration was supplemented only with hay, straw, silage and cereals. The large differences in hair Ca content suggest that in spite of well controlled blood Ca level, hair Ca level can respond to certain factors, needed to be identified.

Magnesium level of Hungarian Simmental cow's hair was significantly higher than found in Charolais (Table 1). However, both values are much higher than the suggested normal level. Despite that, Holstein Friesian cows from organic farms expressed levels quite below the reference values (Gabryszuk et al., 2010). Increasing the level of Mg in the diet induced higher Mg content in cattle hair (Anke, 1966). Fisher et al. (1985) demonstrated that Mg content in hair samples were depended on the Mg content of pasture where cattle grazed. They also stated that higher Mg content of hair does not necessary mean sufficient supply because hair can be contaminated by manure rich in magnesium (especially in case of tail switch hair). However, in our case this likely have not been the responsible factors, as samples originated from uncontaminated short haired body parts and were washed before analysis. Therefore, we can conclude that the Mg supply of the experimental animals were more than adequate. Blood plasma analyses could confirm that conclusion (Fisher et al., 1985). Fisher et al. (1985) demonstrated that black hair can contain markedly higher amount of magnesium, compared to lightly coloured hair samples. The breed differences we found can be attributed to the different pigment content of Charolais and Hungarian Simmental coloured hair.

No breed effect was found in the case of sodium. Unfortunately we could not find any research suggesting normal Na levels of cattle hair. However, the values we found were about 13 times higher than in cows kept on organic farms (Gabryszuk et al., 2010). In our case the cows had free access to salt blocks. Gabryszuk et al. (2010) do not mention salt supplementation of their experimental cows. Based on that we can suspect that

hair Na level reflects the salt supply of cattle. However, the determination of normal values needs further investigations.

Unlike to Ca and Mg, Charolais cows had higher copper level in their hair samples (Table 1). Early research results demonstrated that hair copper level relates liver copper reserves when the level is below 20 μg/g (Kellaway et al., 1978). The values were found are around the lower end of the normal range. However hair samples of organic cattle showed deficient supply again (Gabryszuk et al., 2010). Suttle and McMurray (1983) developed an assessment system for cattle and sheep based on three criteria. According to that system, if cattle hair contains less than 4 mg/g copper it shows prolonged deficiency with probable production drop. When this low hair copper value is coupled with plasma tither higher than 0.59 it shows infection or stress.

Both tested breed had statistically similar selenium content in their hair. The measured values are far above the normal range, suggesting an oversupply of Se in the tested herd. Olson (1969) concluded that Se concentrations of 5 to 10 ppm in cattle hair may indicate selenium toxicity. Acute selenium toxicity can be caused by consuming large amount of high seleniferous accumulating plants during grazing or by accidental overdosing supplementation. Signs of toxicities include laboured breathing, abnormal movement and posture, prostration and diarrhoea followed by death in few hours. The acute selenosis is not a frequent problem, since grazing animals avoid accumulator plants if possible (NRC, 2005). In spite of the high hair Se level in this study, no signs of acute or chronic selenosis had been observed on the animals. One reason can be that overdosing inorganic Se supplements not always result in toxicities, but maybe reflected in hair. It has to be stressed out that selenium deficiency is more frequent in farm animals, in spite of the fact that the importance of selenium was discovered throughout its toxic effect (NRC, 2005).

Zn level in the examined population did not show breed differences (Table 1). The values somewhat below the normal range, suggest zinc deficient feeding. Nevertheless, animals in organic farms developed much lower levels (Gabryszuk et al., 2010). Zn is widely distributed in the body; highest levels were detected in bone, liver, skin, and hair (Georgievskii, 1982). Early studies summarized by Combs (1987) demonstrated that dietary Zn intake correlated with hair levels. In cattle and goat, hair reflects more sensitively to the differences in Zn intake than any other tissue (Miller, 1970). Contrary, it has been also concluded that the severity and duration of Zn deficiency cannot be determined (Combs, 1987). Studies with animal hairs used hair samples from unshaved body parts. As hair/wool length approaches its final length, the growth and mineral accumulation slows down. This can be a reason of variable results. Therefore, the adequacy of mineral nutrition should be evaluated based on hair samples collected from the previously shaved skin surfaces.

Table 1. The effect of breed on the mineral content of cattle hair

Breed	Minerals (mg/kg)						Source
	Ca	Mg	Na	Cu	Se	Zn	
Charolais	1722	650.8	4916	7.58	7.02	80.7	this trial
Hungarian Simmental	2406	912.2	4165	5.66	9.20	84.4	this trial
RMSE**	376	180	1300	1.21	3.06	16.6	
P	0.0007	0.0045	0.2131	0.0023	0.1283	0.6233	
Holstein Friesian (organic farms)	587	63	368	2.26	0.91	37.6	Gabryszuk et al., 2010
Normal values	-	-	-	7	-	129	Haenlein and Anke, 2011
Normal values	1000-2500	130-455	-	6.7-32	0.5-1.32	100-150	Puls, 1994
Normal values	-	25-30; 100-125*	-	8.7	-	-	Fisher et al., 1985

*For non-coloured and black hairs; **root mean square error

Hair growth of most species occurs in phases. The length and season of active hair growth periods depends on species, season and body location (Combs, 1987). In the case of cattle body hair has shorter growth and rest periods than tail switch. Furthermore, hair contamination can markedly alter the concentration of some mineral in hair. In that sense, body hair is more suitable than tail switch for analyses. The results of this study demonstrate that the sampling site on the short haired body parts has no influence on mineral composition (Table 2).

Table 2. The effect of sampling place on the mineral content of Hungarian Simmental cattle hair

Sample origin	Minerals (mg/g)					
	Ca	Mg	Na	Cu	Se	Zn
withers	2402	920.5	4166	5.69	9.20	84.6
side	2406	907.7	4154	5.72	9.19	82.5
quarter	2410	908.7	4175	5.58	9.22	86.3
RMSE*	472	217	814	0.54	3.65	22.0
P	0.9992	0.9893	0.9983	0.8333	0.9999	0.9291

*root mean square error

CONCLUSION

Breed differences exist in Ca, Mg and Cu of hair mineral content even in case of similar nutrition ($P<0.05$). This may reflects metabolic differences. Sampling site of short haired body parts has no influence on hair mineral content. Results of hair analyses showed that the herd had satisfactory mineral status.

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PREDICTION OF GLOBAL AND LOCAL SIMMENTAL AND RED HOLSTEIN FRISIAN ADMIXTURE LEVELS IN SWISS FLECKVIEH CATTLE

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Original scientific paper

SUMMARY

In this study we estimated levels of local ancestry for individuals of the Swiss Fleckvieh dairy cattle population. It is a composite breed descending from two pure breeds, Simmental (SIM) and Red Holstein Friesian (RHF). Illumina BovineSNP50 Beadchip genotyping data for a total of 500 pure and admixed animals were used for the analysis. The global ancestries estimated by Hidden Markov model were 0.68 and 0.32 for RHF and SIM respectively. Local ancestry levels investigated along chromosomes 2, 3 and 13 indicated that there were some regions across the chromosomes exhibiting substantial fluctuations in admixture. On chromosome 2, in the range of 28 to 31, 41 to 46 and 54 to 56 Mb RHF ancestry is substantially higher than average (0.77-0.78). These regions on chromosome 2 are wide, indicating recent admixture. Along the segments on chromosome 2, many QTLs related to dairy, conformation, reproduction, health and carcass traits were found. We observed sharper excess in favour of SIM on chromosome 3, whereas different regions with excess of RHF and SIM were found out on Chromosome 13. At the first part of chromosome 13, an excess of RHF was observed. Moreover, in regions between 40 and 57 Mb excess of SIM, referred to recent admixture was detected. In respect of RHF chromosome segments in admixed animals, dairy, reproduction and health QTLs were found. In positions where more Simmental segments were detected, QTLs related to meat and carcass traits as well as udder health traits were found. In conclusion, the authors believe that estimation of local admixture levels in crossbred populations can add information to the composite breeds history of selection.

Key-words: cattle, admixture, SNP, global ancestry, local ancestry, QTL

INTRODUCTION

Crossbreeding is a mating system that is widely used in dairy cattle to improve milk production as well as health, reproduction and survival traits. Since differences between breeds are larger than the differences within breeds, extra benefits can be achieved from heterosis due to crossbreeding (Swalve, 2007).

The amount of heterosis depends on the difference in allele frequencies between pure ancestral populations and it will be maximized when one allele is fixed in one pure population and the alternative allele is fixed in the other population (Caraviello, 2004).

With recombination, taking place at each generation, the genome of admixed animals is a mosaic of segments originating from different ancestral populations. In a recently admixed population, the fraction of ancestry (termed global ancestry) from each pure population varies substantially across individuals. However, the proportion of ancestries along the chromosomes (termed local ancestry) of an individual varies as well, and the wideness of the mosaic segments can also lead us to infer the age of the admixture (Sankararaman et al., 2008; Padhukasahasram, 2014; Zhang and Stram, 2014).

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Pedigree information is conventionally used to estimate global ancestry and assumes equal contributions of all ancestors of a generation (Sölkner et al., 2010). Admixture analysis based on the single nucleotide polymorphism (SNP) chip data is expected to replace pedigrees with high accuracy (0.97) or pedigree analysis in case of non-pedigreed populations (Frkonja et al., 2012).

The estimation of genetic ancestry in human populations is widely used to control population stratification in association studies (Kang et al., 2009; Zhang and Stram, 2014) and admixture mapping (Seldin, 2007; Chen et al., 2014). Admixture estimation based on molecular data has also been performed in livestock. Bray et al. (2014) investigated the historic population processes using microsatellite markers in a Lincoln Red breed. Some studies have also used SNP chip data to detect the levels of the admixture in sheep and cattle populations (Sölkner et al., 2010; Frkonja, et al., 2012, García-Ruiz et al., 2015).

In this study, we estimated the local admixture with LAMP program for Swiss Fleckvieh admixed animals, using SNP chip data to consider the trends of the admixture at local levels across three autosomal chromosomes (2, 3 and 13). Our aim was to monitor which chromosome segments on these three chromosome show deviations from global admixture, higher/lower proportions of the ancestral Simmental (SIM) and Red Holstein Friesian (RHF) populations. Such deviating regions were then inspected for genes and QTL to infer causes of selective pressure in the recently admixed population.

MATERIAL AND METHODS

Swiss Fleckvieh is a composite breed of Simmental and Red Holstein Friesian that has been established over the last forty years with the emphasis on high milk production derived from the Holstein Friesian as well as on additional traits like beef value, fitness traits and longevity of Simmental breed. The genotype data from the Illumina Bovine SNP50 beadChip were available for 100 pure RHF, 100 pure SIM and 300 Swiss Fleckvieh bulls. The quality control of the data was performed with PLINK 1.07 (Purcell et al., 2007). Dataset was controlled to exclude SNPs with call rate of $<95\%$ that were monomorphic (based on minor allele frequency) and with p-value$<1.0\times10^{-6}$ deviating from Hardy Weinberg Equilibrium (HWE). The animal samples with more than 5% missing genotypes and SNPs with more than 5% missing data were removed from the dataset. SNPs located on sex chromosomes were also not used in the analysis. There were 39,525 SNPs and 485 animals left after pruning and filtering.

We performed unsupervised global ancestry estimation with the full SNP set applying Hidden Markov Models (HMM) using ADMIXTURE (Alexander et al., 2009) with the number of ancestral populations fixed at 2. Global estimates were used as a reference metric to scale the local ancestry estimates.

Estimation of local ancestry can also be performed based on HMM for every single SNP on each chromosome separately. In this study we used LAMP software calculating local ancestry based on HMM. We ran LAMP in the LAMPANC mode and used the allele frequencies of the Red Holstein Friesian and Simmental as information of ancestral populations. The following configuration parameters were used: admixture proportions (alpha) = 0.68, 0.32 based on the results from ADMIXTURE, number of generations since admixture (g) = 7, recombination rate (r) = 1e-8, fraction of overlap between adjacent windows (offset) = 0.2. LAMP relies on a predefined set of ancestry informative markers that are in low linkage disequilibrium ($r^2<0.1$ for each pair of selected SNPs). We did not include LD in this research.

Furthermore, LAMP estimates the locus-specific ancestry for each individual with respect to pure breeds. Under the assumption of a dihybrid population model, marker specific ancestries were estimated across 3 autosomal chromosomes (2, 3 and 13) for each animal separately. The genome wide mean estimations were used as reference line for local ancestry detection. For 300 admixed animals, we computed the average locus-specific ancestry level. We then calculated r ancestry by subtracting the genome wide ancestry from the average locus specific ancestry for each of the two ancestry components (Tang et al., 2007).

RESULTS AND DISCUSSION

At first individual admixture proportions of Swiss Fleckvieh animals based on both pedigree and 39,525 SNPs information were estimated. Figure 1 presents individual admixture levels of admixed animals based on pedigree and SNP information. Animals were ordered from the highest to lowest RHF proportions based on pedigree. Global admixture based on pedigree and using 39,525 SNPs indicated the high correlation between estimations (0.97) as, also, inferred by Frkonja et al. (2012) for the same dataset. The average admixture level based on pedigree was 0.69 RHF (with 0.20 standard deviation) whereas based on SNP data it was 0.68 RHF (with 0.19 standard deviation).

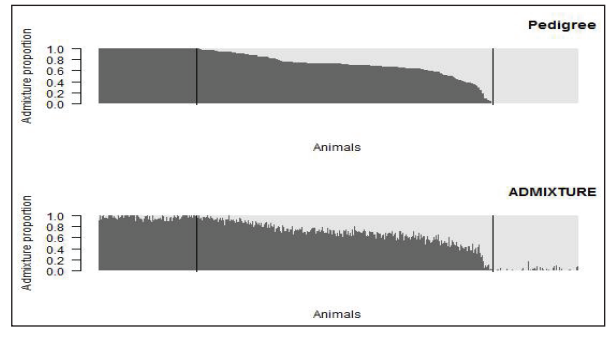

Figure 1. Genetic contributions derived from pedigree and from SNP information (39,525) using ADMIXTURE (RHF = 1, SIM = 0)

Figure 2 displays average ancestry across chromosomes 2, 3 and 13 derived from LAMP program for admixed animals, keeping the same order as in Figure 1, i.e. sorting animals from high to low RHF levels based on pedigree information. The average ancestry regards to RHF across chromosomes 2, 3 and 13 was estimated 0.73, 0.70 and 0.65 respectively.

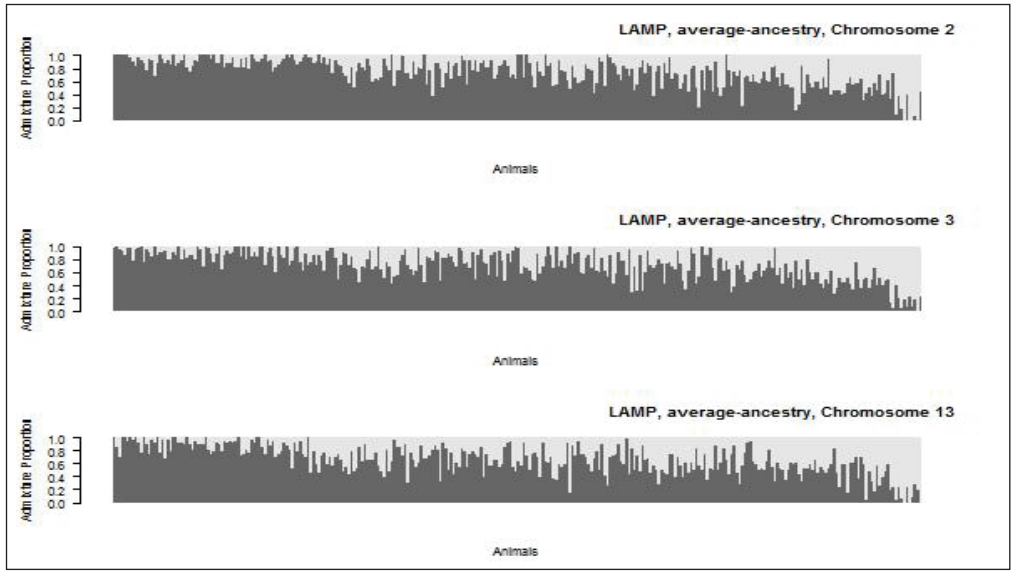

Figure 2. Average ancestry estimates across chromosomes 2, 3 and 13 (RHF=dark grey, SIM= light grey)

Figure 3 shows the average excess or deficiency of local ancestry from genome wide ancestry at each SNP location on chromosomes 2, 3 and 13 for all 300 admixed animals. The graphs indicate that some SNPs have different amount of proportion of each breed across these chromosomes. Deviations on most locations are in accordance with genome wide ancestry. However, some regions exhibit extreme fluctuations.

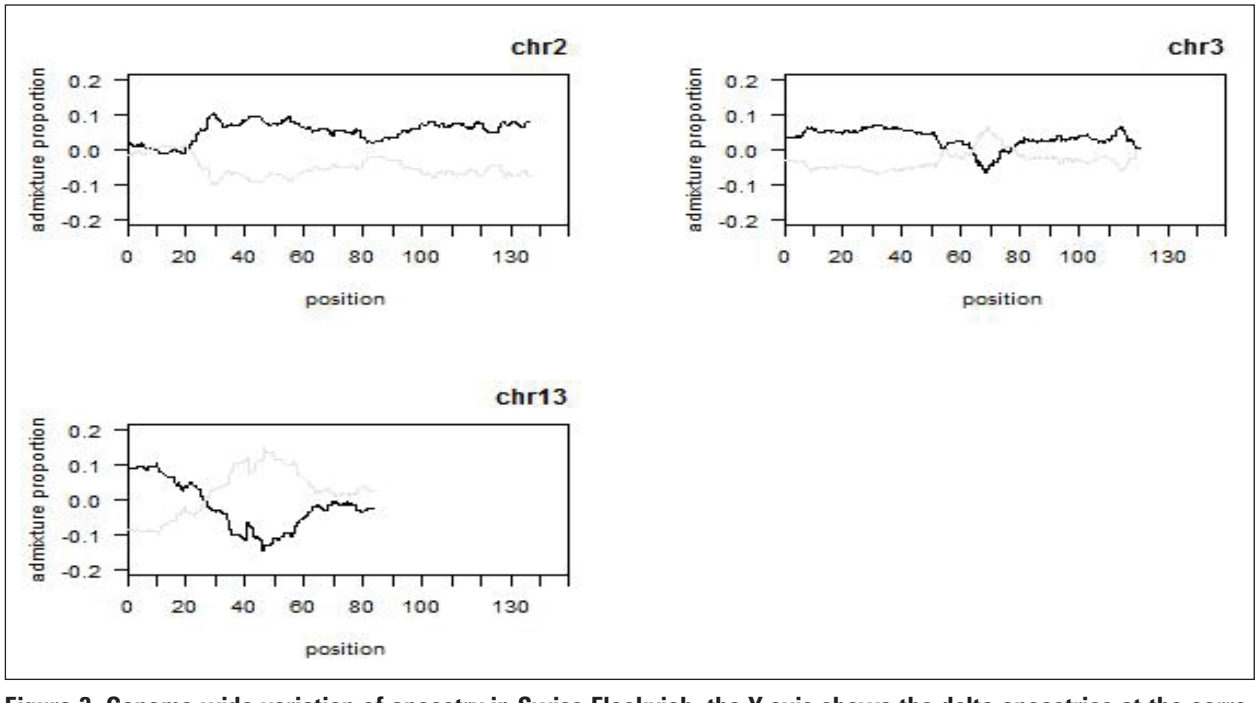

Figure 3. Genome wide variation of ancestry in Swiss Fleckvieh, the Y-axis shows the delta ancestries at the corresponding SNP for 300 admixed animals (RHF=dark grey, SIM= light grey lines)

Based on the results in Figure 3, we observed some excess and deficiency of local ancestry from global ancestry estimation, not probable only by chance, and indicated that the genomes of the admixed animals in these parts have higher proportions of one breed and long range of LD. The genomes of admixed animals contain wide segments of each ancestral breed (graphs not shown), indicating recent admixture. Therefore, extreme deviations from genome wide admixture may have been caused by recent selection.

On chromosome 2 some excesses of RHF have been indicated in 28 to 31 Mb (0.77-0.78), 41 to 46 Mb (0.77) and 54 to 56 (0.77) Mb positions, proportions that deviate by 0.10 or more from estimates of global admixture (0.68). The fact that the chromosome segments deviating from expectation are wide indicates recent admixture and not enough time for selection to sharpen the signal.

We used *Cattle*QTLdb website to examine whether such regions harbour QTL. The results are summarized in Table1. On chromosome 2 we found QTLs associated to dairy, conformation, reproduction, health and carcass traits. On chromosome 3, we have searched for QTLs in the region of 67 to 72 Mb. In this region only one QTL associated with fertility was detected.

The SNP pattern on chromosome 13 indicates excess of RHF ancestry around Mb 0-15 and SIM ancestry around Mb 40-60. The most important reason for the excess of RHF segments in the first part of the chromosome may be selection for calving ease, as this used to be one of the most important problems in the Simmental breed. A QTL for calving ease was detected in this region in previous studies (Table 1). On the other hand, selection for udder health (somatic cell score, mastitis), female fertility and meat/carcass traits certainly preferred SIM segments in the middle of chromosome 13, see Table 1 for information about respective QTL.

Table 1. The segment of chromosome 2, 3 and 13 QTLs are located in these area (Cattle QTL database).

Chr	Position (bp)	Breed	Trait / Type
2	27034490-29073969		Age at puberty/Reproduction
2	30080851-32170510	Holstein-Friesian	Lean meat yield, subcutaneous fat/ Meat and carcass
2	30080851-32170510	Holstein-Friesian	Post-partum interval to commencement of luteal activity/ Reproduction and fertility
2	30080851-32170510	Holstein-Friesian	Viral diarrhea susceptibility/Health
2	44067070-44085642	Holstein-Friesian	Milk yield, milk fat and protein yield/Milk
2	45093319-45313175	Holstein-Friesian	Milk fat and protein/Milk
2	45093319-45313175	Holstein-Friesian	Fertilization rate/Reproduction
2	46834289-46834365	Holstein-Friesian	Milk yield, milk fat and protein yield/Milk
2	46834289-46834365	Holstein-Friesian	Udder height, udder cleft/Udder morphology
2	46834289-46834365	Holstein-Friesian	Bovine tuberculosis susceptibility/Health
3	71791844-71793206	Brahman	Interval to 1st estrus after calving, age at puberty/Reproduction
13	9258332-10558420	Holstein-Friesian	Milk kappa-casein and alpha-casein percentage/Milk
13	9258332-10558420	Holstein-Friesian	Gestation length/Gestation and fertility
13	9258332-10558420		Subcutaneous fat/Meat and carcass
13	10665897-11438802	Holstein-Friesian	Calving ease (direct and maternal)/Parturation
13	10665897-11438802	Holstein-Friesian	Gestation length/Gestation
13	10665897-11438802	Holstein-Friesian	Somatic cell count and mastitis/Health
13	10665897-11438802	Holstein-Friesian	Milk yield, milk fat and protein percentage/Milk
13	46178647-52998234	Norwegian red	Clinical mastitis/Health
13	46834289-46834365	Ayrshire,Norwegian	non retun rate/ interval to first estrus after calving/Reproduction
13	51062875-56847265		Somatic cell count ,mastitis/Health
13	53561417-56839719		Body weight, conformation traits
13	57016938-57993671	Gelbvieh	Yield grade/Meat and carcass

CONCLUSION

This study considered three sample chromosomes of the bovine genome to explore the variability of levels of admixture along the genome of Swiss Fleckvieh cattle, a composite of Simmental and Red Holstein Friesian. Substantial deviations (>0.1) from global admixture were observed for several regions, implying the possibility of strong recent selection in the crossbred popu-

lation. The signals found are wide, which is consistent with the small number of generations (∼10) since the start of crossbreeding in this population and not enough generations having passed for narrowing the signatures of selection.

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EFFECT OF DIETARY NPP LEVEL AND PHYTASE SUPPLEMENTATION ON THE LAYING PERFORMANCE OVER ONE YEAR PERIOD

Tischler, A., Halas,V., Tossenberger, J.

Original scientific paper

SUMMARY

Our trial aimed to study the effect of different dietary non-phytin phosphorus (NPP) levels with and without phytase enzyme supplementation on laying performance and eggshell quality of Tetra SL-LL in the last 25 weeks of the long-term (17 months) egg production. A total of 69 Tetra SL-LL layers were allocated into 3 dietary treatments. Two diets with different levels of NPP (2.45 or 2.15 g/kg, HP and LP, respectively) were formulated, and 0 or 300 FTU/kg phytase enzyme was added to low NPP feed (LP and LP+E, respectively). Dietary Ca was uniformly adjusted to 38.2 g/kg of feed in each treatment. In the course of the trial intensity of egg production (%), egg weight (g/egg), number of the broken eggs and feed intake (g/d/bird) were recorded. Every 2 weeks 20 eggs per treatment were broken to determine the shell strength and thickness. Our results show that low NPP diet had detrimental effect on the intensity of egg production (P<0.05). However, dietary treatments had no effect on weight of eggs. They significantly affected eggshell thickness (P<0.05), but not egg shell strength (P>0.05) and phytase added to the LP diet resulted in the lowest number of broken eggs (P<0.05). In conclusion, NPP content of the layer diet can be reduced from 2.45 to 2.15 g/kg in the last 25 weeks of the elongated laying term (12-17 month of laying), if supplemented with 300 FTU/kg phytase enzyme without compromising the egg production, and in the same time it can improve eggshell quality and reduce the number of broken eggs.

Key-words: laying hen, phosphorus, phytase, long-term laying period, egg production, eggshell quality

INTRODUCTION

In practice, the leading breeding companies keep layers in production up to 90-100 week of age (babolna-tetra.com, hyline.com, isapoultry.com). It is well be documented that egg shell quality problems arise towards the end of the laying period causing considerable economic loss. Therefore, the precise Ca and P supply is crucial in that period (Tischler et al, 2013). Addition of phytase to high phytate containing diets improves P digestibility due to release of bounded P in cereal grains and oilseed meals. Several studies examined different doses of phytase in laying hens to evaluate the needs for improving performance parameters.

Van der Klis et al (1997) found that deprivated P containing diets supplemented by 250 FTU/kg enzyme supported similar egg production as fed adequate P diet, and at the same time, 500 FTU/kg phytase added to low P diet did not result in further improvement on laying performance. Some other publication also showed that providing supplementary 300 FTU/kg to a low NPP diet can be efficient for improving laying intensity and egg shell quality (Lim et al, 2003; Augspurger et al, 2007) even for older (70-76 wk of age) intensive laying hens (Gordon and Roland, 1997, Boling et al, 2000).

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Therefore, the aim of our study was to confirm the effect of dietary NPP level and phytase supplementation on egg production and egg shell quality in the last interval of the elongated (from 12 to 17 month) laying period.

MATERIAL AND METHODS

In the trial, three diets were fed to 69 Tetra SL-LL hens from 44 to 68 week of age. Treatments consisted of a control feed with 2.45 g/kg non-phytin phosphorus (HP), a 20% reduced 2.15 g/kg NPP (LP) and a 2.15 g/kg NPP plus 300 unit/kg phytase enzyme (LP+E) diets with constant dietary energy (11.6 MJ AMEn/kg), protein (160 g/kg), Ca (38.2 g/kg) and amino acid content (8.2 g Lys, 7.3 g Met+Cys per kg feed). Composition and nutrient content of HP and LP diets are showed in Table 1. Layers performance was examined in the last 6 months of the elongated (17 months) laying period. Egg production and egg weight were daily recorded by cages, whereas feed intake was measured weekly. Performance of the hens were characterized by intensity (%), egg weight (g/egg), broken eggs (egg/d/cages) as well as feed intake (FI, g/d/bird) and feed conversion ratio (FCR, kg FI/kg egg mass). Every two weeks, 20 eggs per treatment were collected and broken to determine eggshell strength and thickness. Zwick Roell ZO05 type of instrument was used for cracking eggshell. Thickness was measured by Mitutoyo electronic micrometer with 0.001 mm accuracy averaged from three measurements per egg. Data were analysed by two-way ANOVA (SAS, 2004), as follows: $\mathbf{Yijk = \mu + Pi + Tj + Pi*Tj + e_k}$, where: Yijk = dependent variable, μ = general mean, Pi = effect of dietary treatment (i=3; HP, LP, LP+E), Tj = effect of laying time (as for feed intake, feed conversion ratio, egg weight and egg shell strength j=6; M12-M17, as for egg production, broken eggs and egg shell thickness j=25; wk44-wk68), Pi*Tj = effect of interactions, e_k = undefined error. Statistical significance was based on a 5% probability level.

RESULTS AND DISCUSSION

There was no interaction between dietary treatments and laying period at any of the examined parameters.

Table 1. Composition and nutrient content of feed in dietary treatments HP and LP

Ingredients (g/kg)	HP	LP+E	LP	Nutrients (g/kg)	HP	LP+E	LP
Corn, grain	653.7	654.6	658.3	DM[2]	897.0	896.9	896.8
Soybean meal (CP: 47,3 %)	224.0	224.0	224.0	CP[3]	160.0	160.1	160.3
Fat, vegetable	2.0	2.0	2.0	AMEn	11.6	11.6	11.6
MCP[1]	7.3	5.8	0.0	Lysine	8.2	8.2	8.2
Limestone	95.0	95.6	97.8	M+C[4]	7.3	7.3	7.3
Salt	4.0	4.0	4.0	Threonine	6.4	6.4	6.4
L-lysine-HCl	0.8	0.8	0.7	Ca	38.2	38.2	38.2
DL- methionine	2.4	2.4	2.4	tP[5]	5.03	4.71	3.46
L-threonine	0.8	0.8	0.8	Non Phytate-P	2.45	2.15	2.15
Premix 1%	10.0	10.0	10.0	Na	1.6	1.6	1.6

[1]Mono calcium phosphate, [2]Dry matter, [3]Crude protein, [4]Methionin+Cystein, [5]Total phosphorus

Effect of dietary treatments and laying term on the intensity of egg production is showed in Figure 1. Treatment LP unlike HP and LP+E treatments was significantly lower ($P<0.0001$). Therefore our data suggest that 2.15 g/kg dietary NPP is insufficient to support well egg production. It is well documented that inadequate P supply reduces the laying intensity (Liu et al., 2007). Our data clearly show that there is no difference between the production intensity of hens fed diet with recommended 2.45 g/kg NPP and 2.15 g/kg NPP + 300 FTU. Egg production positioned on 92% at the beginning of the last 25 weeks of the elongated laying period, followed by progressive lowering. In the final weeks it was around 55%, which intensity still might be acceptable for keeping hens in production.

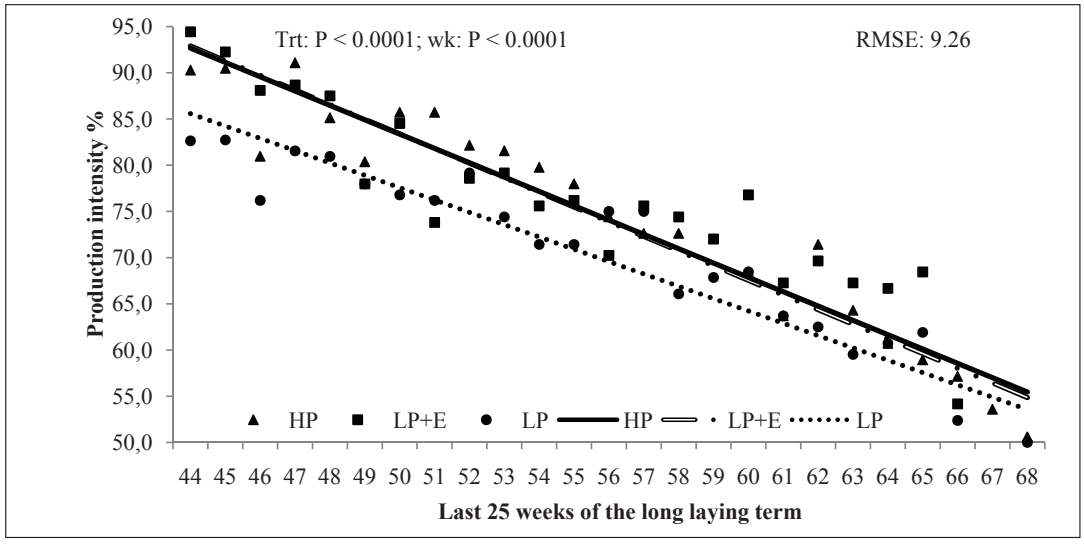

Figure 1. Effect of dietary NPP levels and phytase supplementation on laying intensity %

Mean production intensity % in different treatments were: HP $\dot{x}=74.0^{A}$, LP+E $\dot{x}=73.8^{AB}$, LP $\dot{x}=69.5\%^{B}$, respectively

The effect of treatments on the feed intake, feed conversion, egg weight, and egg shell parameters are shown in Table 2. The experimental treatments showed significant differences between LP and LP+E diets (P=0.026) on feed intake, birds consumed the least from 2.15 g/kg NPP + 300 FTU/kg diet. In the first month of the experimental period layers took 117.0 gram meals daily and consumption was progressively decreased by 99.0 g/day. Jalal and Scheideler (2001) found out that P deprivated birds compensated their P supply by higher feed intake. FCR was significantly affected by trial time: at the beginning of the trial it was lower by 0.47 than in the last month. Dietary NPP levels or phytase supplementation did not affect egg weight, which is in accordance with results of Keshavarz (2003). The average egg weight was ±63 g all along the experimental term being the desirable egg size (M) in the European countries. Due to the high variability, shell strength was affected either by different NPP level or by time factor (P>0.05). Breaking power was approximately 3.5 kg in all three treatments. Considering egg shell quality, shell thickness was affected by both dietary treatments (P<0.001) and time (P<0.011). Leeson et al (1992) reported that 0.35% available phosphorus significantly reduced egg shell quality (P<0.01). Rao et al (2003) found that between 196-336 day of age layers need 2.8 g NPP kg^{-1} diet for optimum egg production and egg shell quality. Egg shell was constantly thinner at LP+E treatment compared to HP treatment during the experimental period, but still kept within acceptable range (Arpasova, 2010).

Table 2. Effect of dietary NPP level and phytase supplementation on daily feed intake, feed conversion, mean egg weight, shell strength, and shell thickness

		Feed intake g/day/bird	Feed conversion ratio kg/kg	Egg weight g/egg	Shell strength kg	Shell thickness mm
Treatments	HP	107.8^{AB}	2.36	62.4	3.53	0.344^{A}
	LP+E	104.9^{B}	2.30	62.4	3.59	0.333^{B}
Months	LP	112.8^{A}	2.29	62.9	3.37	0.339^{AB}
	12	117.0^{A}	2.17^{B}	62.0	3.65	0.341^{A}
	13	110.4^{ABC}	2.12^{B}	62.4	3.50	0.339^{AB}
	14	110.9^{AB}	2.25^{AB}	62.2	3.40	0.340^{AB}
	15	108.0^{ABC}	2.24^{AB}	62.7	3.55	0.339^{AB}
	16	104.4^{BC}	2.47^{AB}	63.5	3.45	0.340^{AB}
	17	99.0^{C}	2.64^{A}	62.6	3.50	0.336^{B}
RMSE		13.75	0.53	2.67	1.06	0.03
P ≤	Trt	0.026	NS	NS	NS	0.001
	Month	0.001	0.008	NS	NS	0.010

1HP: 2.45 g/kg dietary NPP, LP+E: 2.15 g/kg dietary NPP + 300 FTU/kg phytase, LP: 2.15 g/kg dietary NPP, 2Last 6 months of the long-term, 3Root mean square error, 4Statistical significance

Figure 2 presents that dietary treatments as well as the laying period significantly affected the cracked eggs (P<0.0001; P=0.02, respectively). Approaching the end of the long laying term and considering HP and LP diets the number of broken eggs increases. It is notable that the loss of production due to egg break increased approximately 3 times in HP, two times in LP group and remained at the initial value in LP+E treatment. The number of the broken eggs pro rata to the total produced eggs was 8.03% in HP, 5.55% in LP and no more than 4.12% in LP+E treatment (data are not shown). Summing up the results on egg shell strength, thickness and the number of broken eggs we may hypothesize that egg shell gets more flexible at LP+E diet compared to other treatments. This might be due to a smoother P supply from enzyme released phytate and likely better crystallography parameters.

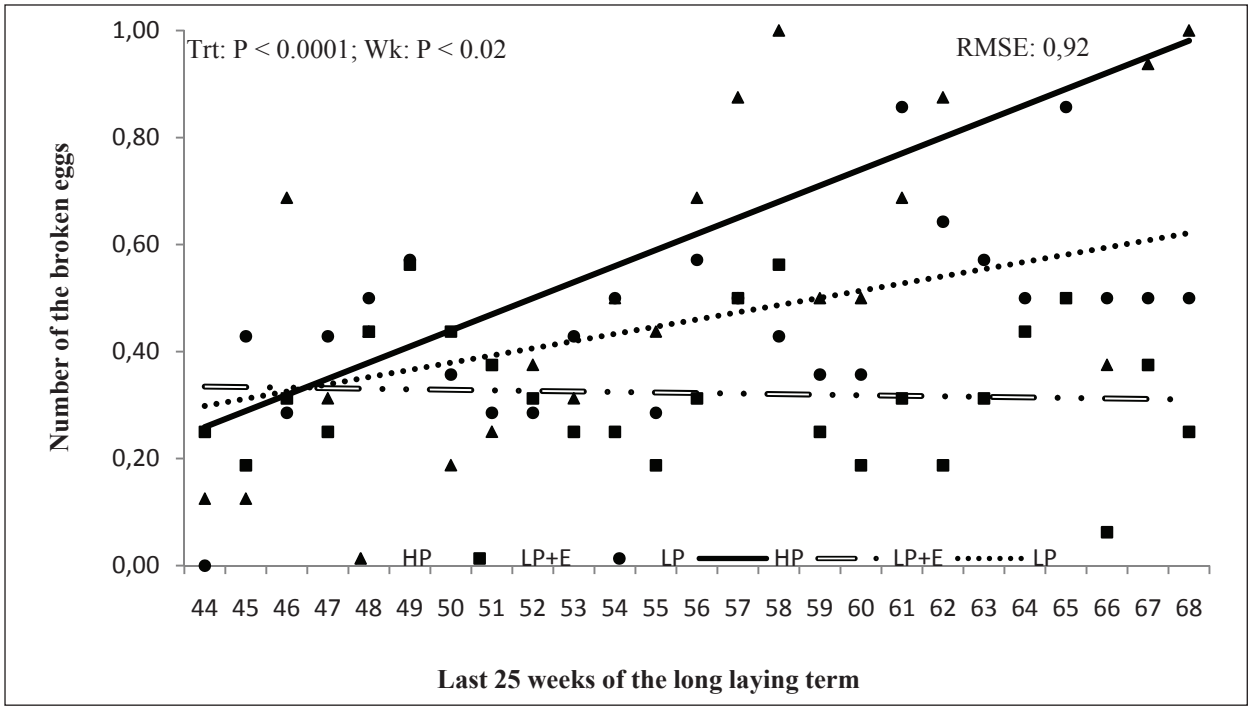

Figure 2. Effect of dietary NPP levels and phytase supplementation on the number of broken eggs

Average broken eggs number of different treatments was: HP $\dot{x}=0.62^{A}$, LP+E $\dot{x}=0.32^{B}$, LP $\dot{x}=0.46^{B}$ eggs, respectively

CONCLUSION

Based on our data, it can be concluded that in the last 25 weeks of the elongated laying term, dietary NPP content can be lowered by 20% if supplemented with 300 FTU phytase per kg feed, without compromising the production intensity and feed consumption of laying hens. Low P diet with phytase supplementation ensures acceptable egg shell strength, even thinner shell but less broken eggs due to likely more flexible shell.

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A HIGHER PROPORTION OF PUFA IN DIET INCREASES THE PUFA CONTENT IN RABBIT MEAT, BUT REDUCES THE OXIDATIVE STABILITY OF MEAT

Trebušak, T., Levart, A., Salobir, J., Pirman, T.

Original scientific paper

SUMMARY

The aim of our study was to determine the changes in the fatty acid composition of rabbit meat, if palm fat (99% of saturated fatty acids (SFA)), as a source of fat in rabbit diet, was replaced with linseed oil (71% of polyunsaturated fatty acids (PUFA), containing 52% of α-linolenic acid (n-3 PUFA)). The Ganoderma lucidum or olive leaves were added in the diet as potential antioxidant in order to protect the PUFA against oxidation. 48 SIKA rabbits were randomly divided by mass and gender in four groups: CONT- 6% palm fat, CONT+ 6% linseed oil, REISHI 6% linseed oil and 1% Ganoderma lucidum, OLIVE 6% linseed oil and 1% olive leaves. After 22 days of the experimental procedure, the samples of back muscle were taken and divided in 7 portions. One was for fatty acid determination, other six for malondialdehyde (MDA) determination after different storage condition; fresh, 6 days at 4°C or 3 months at -20°C, raw or cooked (60 minutes, 85°C). Addition of linseed oil resulted in a significant higher proportion of PUFA (n-3 PUFA) and monounsaturated fatty acid (MUFA) and lower proportion of SFA in the back muscle, but the oxidative stability of meat was reduced, since the level of MDA was significantly higher. After cooking, the level of MDA increased in all the groups, but more in the groups with linseed oil in the diet, the addition of Ganoderma lucidum or olive leaves slightly decreased the level of MDA, but the difference was not significant.

Key-words: nutrition, rabbit meat, oxidative stability, PUFA, MDA

INTRODUCTION

Nowadays people are more and more aware of the importance of healthy diet, and healthy effect of polyunsaturated fatty acids (PUFA) is implied in this context. Western diets have excessive amounts of omega-6 polyunsaturated fatty acids (n-6 PUFA), compared to the omega 3 polyunsaturated fatty acids (n-3 PUFA), leading to a high n-6/n-3 PUFA ratio, more than 15/1, while the optimal ratio would be 4/1 (Simopoulos, 2002). Rabbit meat already contains high amount of n-3 PUFA compared to the other source of meat (Dalle Zotte, 2004), which is the result of alfalfa presence in the rabbit diet. Nevertheless, the fatty acid composition of rabbit meat can be improved by the addition of n-3 fatty acids in rabbit diet (Dalle Zotte, 2004) and in that way rabbit meat could be considered as a functional food (Dalle Zotte and Szendro, 2011). However, PUFA can be easily oxidized, forming aldehydes like malondialdehyde (MDA) during storage or cooking of meat and consequently has impact on the nutritional value of meat (Gray et al., 1996; Tres et al., 2014). Incorporation of antioxidants in the diet can prevent lipid oxidation. Interest of using non-vitamin antioxidants of plant origin (polyphenols, flavonoids) has increased in the last years.

The objectives of the study were to evaluate the impact of adding PUFA in rabbit diet on the fatty acid composition of back muscle, the susceptibility of raw and cooked meat to oxidation under different storage conditions and the effectiveness of addition of potential antioxidants, *Ganoderma lucidum* or olive leaves on the oxidative stability of the meat.

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MATERIAL AND METHODS

Animals and diets

All procedures were performed by the current legislation on animal experimentation in Slovenia. Forthy-eight SIKA rabbits (80 days old, average initial body mass 2580±299 g) were randomly divided (by mass and gender) in four groups. The CONT- group received diet with 6% of palm fat (99% of saturated fatty acids - SFA), other three groups received diet with 6% of linseed oil (more than 70% of PUFA), CONT+ was unsupplemented, REISH was supplemented with 1% of *Ganoderma lucidum* and OLIVE was supplemented with 1% of olive leaves. All other ingredients of the diets were the same and at the same level, except the alfalfa level that was decreased in REISHI and OLIVE groups by 1%, as it was the supplementation level.

Experimental procedure and sample collection

Throughout of the experiment, 22 days; animals had free access to the water (nipple) and pelleted diet and were individually housed in wired cages. During the experiment, the body mass of the animals were recorded each week and just before slaughtering. Diet intakes were recorded daily, animals received weighted daily meal and the residue from the day before was weighted and discarded. Rabbits were slaughtered at the 102 days of age whereas a sample of back muscle (*M. longisimus dorsi*) from each animal was taken and divided in 7 equal portions in order to determine the MDA concentration in raw and cooked samples, stored in three different ways: fresh (at -70°C until the analyses were performed), 6 days in refrigerator at 4°C and 3 months in freezer at -20°C. Cooking was performed at 85°C one hour, after the storage. In one sample of back muscle, the fatty acid composition was determined for each meat treatment. Before the analyzing, samples were homogenized in laboratory homogenizer by liquid nitrogen.

Determination of fatty acid composition and MDA concentration

The fatty acid composition of diets and back muscle were analyzed using a gas chromatographic method after the in situ transesterification of lipids. Methyl esters of fatty acids were prepared according to the procedure of Park and Goins (1994) whereas analysis of fatty acids methyl esters (FAMEs) was performed by gas chromatography using an Agilent 6890 series gas chromatograph. FAMEs are identified by retention time and results are expressed as a percentage of the total fatty acids content.

The MDA concentration was determined following the method of Vilà et al. (2002) with minor modifications by HPLC using reversed-phase chromatography column. A Waters Alliance 2690 equipped with Waters 474 scanning fluorescence detector was used to determine MDA concentration.

Statistical analysis

The data were analyzed using the General Linear Models procedure of the SAS/STA module (SAS 8e, 2000) considering the diet as the only main effect. Differences among the groups were determined using Tukey's multiple comparison test. Unless stated otherwise, a least significant difference of 0.05 was used to separate treatment means. Results in the tables are presented as least square means (LSM) ± SEM with *P*-values.

RESULTS AND DISCUSSION

Growth performance of rabbits were similar in the all groups and normal by the age (weight gain between 28.8 g/day and 34.8 g/day and feed conversion rate between 5.18 g/g and 6.09 g/g), with tendency of better values in groups with linseed oil addition and no additional effect of olive leaves or *Ganoderma lucidum* supplementation. This is in accordance with the notification of Meartens et al. (1986), that more saturated fatty acids are less digestible, although some other authors (Bianchi et al., 2009; Casado et al., 2013) detected lower growing performance, when linseed or linseed oil was added in the diet.

Since the differences in the composition of the diet were only in the source of fat (palm fat or linseed oil) and in the supplementation (1%) of olive leaves or *Ganoderma lucidum* or no supplementation, the composition (Table 1) of the diets was similar. On the contrary, the fatty acid composition of the diets differs. After the substitution of palm fat with linseed oil the proportion of SFA decreased and the proportion of monounsaturated fatty acids (MUFA) and especially PUFA increased, with no additional supplement effects (Table 2).

Table 1. Proximate composition (g/kg) and fatty acid composition (% of total fatty acids) of the diets

	CONT –	CONT +	REISHI	OLIVE
Chemical composition (g/kg DM)				
Dry matter (g/kg)	933	912	924	922
Crude protein	191	196	196	193
Crude fat	116	95	92	93
Crude fibre	244	250	247	246
Crude ash	74	76	77	76
Main fatty acids (% of total fatty acids)				
C12:0	0.20	0.04	0.04	0.04
C14:0	0.89	0.12	0.12	0.12
C16:0	35.81	8.00	8.06	8.07
C18:0	41.73	4.00	3.98	3.99
∑ C18:1	7.75	23.48	23.70	23.56
C18:2 n-6	9.01	22.01	22.20	21.92
C18:3 n-3	2.78	40.23	39.77	40.21
∑ SFA	80.08	13.48	13.53	13.56
∑ MUFA	8.05	24.14	24.36	24.22
∑ PUFA	11.86	62.38	62.11	62.22
∑ n-3 PUFA	2.85	40.33	39.86	40.31
∑ n-6 PUFA	9.01	22.05	22.25	21.92
n-6/n-3 PUFA	3.16	0.55	0.56	0.54

CONT- 6% palm fat in a diet; CONT+ 6% linseed oil in a diet; REISHI 6% linseed oil in a diet with addition of 1% *Ganoderma lucidum*; OLIVE 6% linseed oil in a diet with addition of 1% olive leaves; DM – dry matter; SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids

Table 2. Fatty acid composition (% of total fatty acids) of back muscle (*M. longisimus dorsi*)

	CONT -	CONT +	REISHI	OLIVE	SEM [1]	P-value
C12:0	0.15	0.09	0.14	0.11	0.02	0.057
C14:0	1.86 [a]	1.44 [b]	1.28 [b]	1.40 [b]	0.10	0.001
C16:0	23.21 [a]	17.31 [b]	17.66 [b]	17.62 [b]	0.34	<0.001
C16:1 n-7	3.48	2.64	2.41	2.55	0.45	0.319
C18:0	10.48 [a]	6.02 [b]	6.84 [b]	6.49 [b]	0.33	<0.001
∑ C18:1	23.38	23.50	22.76	23.12	0.30	0.286
C18:2 n-6	23.91	24.25	24.30	24.17	0.50	0.940
C18:3 n-3	3.15 [a]	14.68 [b]	12.50 [b]	13.48 [b]	0.74	<0.001
C20:4 n-6	4.12	3.43	4.44	3.91	0.39	0.309
C20:5 n-3	0.14 [a]	0.39 [b]	0.45 [b]	0.41 [b]	0.04	<0.001
C22:5 n-3	0.63 [a]	1.11 [b]	1.36 [b]	1.23 [b]	0.11	<0.001
C22:6 n-3	0.11 [a]	0.15 [ab]	0.19 [b]	0.17 [ab]	0.02	0.005
∑ SFA	38.52 [a]	27.50 [b]	28.98 [b]	28.43 [b]	0.53	<0.001
∑ MUFA	27.80	27.06	26.11	26.60	0.74	0.397
∑ PUFA	33.68 [a]	45.44 [b]	44.91 [b]	44.97 [b]	0.85	<0.001
∑ n-3 PUFA	4.07 [a]	16.52 [b]	14.68 [b]	15.50 [b]	0.68	<0.001
∑ n-6 PUFA	29.59	28.90	30.21	29.45	0.65	0.546
n-6/n-3 PUFA	7.28 [a]	1.81 [b]	2.11 [b]	1.96 [b]	0.13	<0.001

CONT- 6% palm fat in a diet; CONT+ 6% linseed oil in a diet; REISHI 6% linseed oil in a diet with addition of 1% *Ganoderma lucidum*; OLIVE 6% linseed oil in a diet with addition of 1% olive leaves; SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; [a, b] values with different superscripts are significantly different ($P < 0.05$); [1] n = 46

In the fatty acid composition of the back muscle, the linseed oil addition resulted in a significant higher proportion of PUFA (substituting SFA), in particular n-3 PUFA, α-linolenic acid and also EPA and DHA (Table 2). The result was narrower n-6/n-3 PUFA ratio in all the three groups with linseed oil in the diet, without any additional effect of *Ganoderma lucidum* or olive leaves, as compared to the group with palm fat in a diet. Some other authors (Gondret et al., 1998; Bernardini et al., 1998; Kouba et al., 2008; Gigand and Combes, 2008; Li et al., 2012; Du et al., 2013; Tres et al., 2014) also observed that fatty acid composition of the rabbit diet

have influence on the fatty acid composition of the meat. Two weeks of feeding of fortified diet with PUFA is enough to have changes in fatty acid composition in meat (Maertens et al., 2008).

However, higher amount of PUFA in meat (back muscle) leads to the lipid oxidation and degradation of n-3 PUFA into oxidative products, like MDA. MDA concentration in a back muscle was significantly higher in a groups supplemented with linseed oil, as compared to the group with palm fat (Figure 1). *Ganoderma lucidum* or olive leaves slightly reduce the level of MDA, but not to the level determined in the palm fat group, and the difference with CONT+ was not significant.

In the Figure 1 it could be seen that cooking increased the level of oxidation in the meat, but the highest oxidation degree was determined after 6 days of storage in the refrigerator in raw meat in the groups with linseed oil supplementation. The addition of *Ganoderma lucidum* was more effective in raw samples (except fresh row) and the olive leaves in cooked samples. Although for both those supplements *in vitro* antioxidative potential was already proven, it seems that in *in vivo* conditions they have no antioxidative potential, being in accordance with the results of Dal Bosco et al. (2014) on Spirulina or Thyme. If meat is cooked, the antioxidative protection was even less effective.

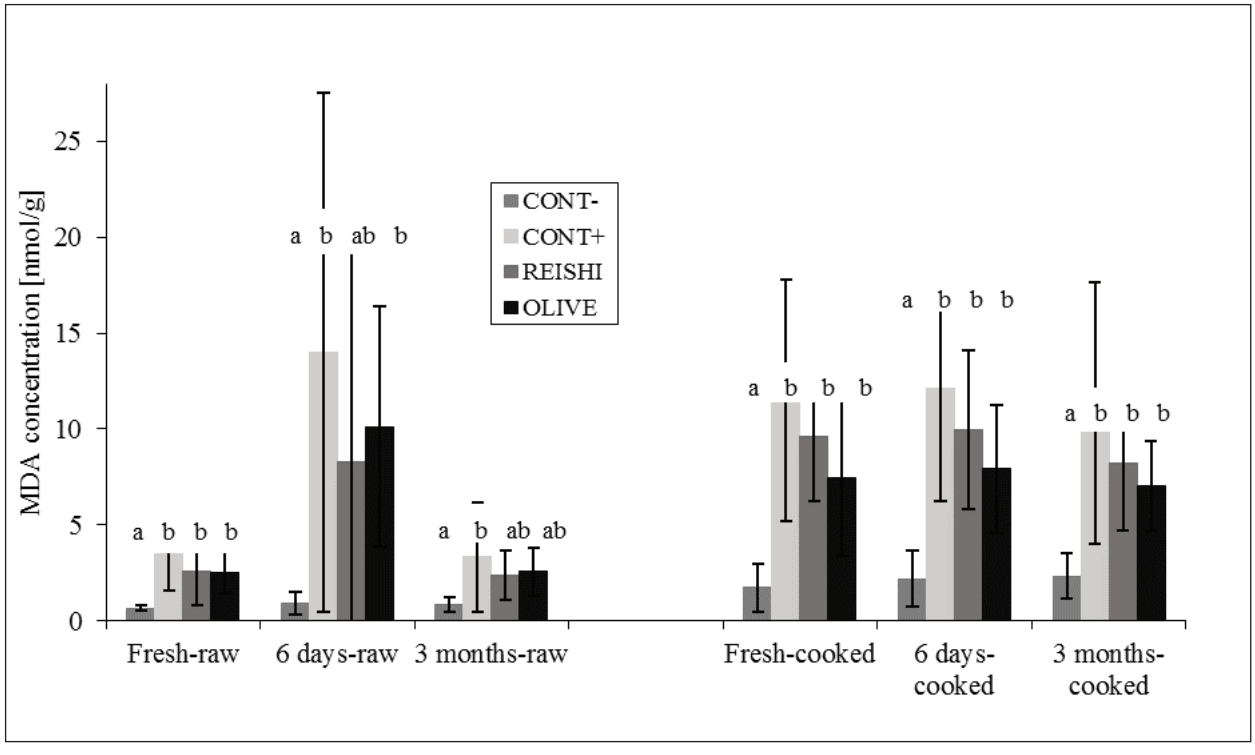

Figure 1. MDA concentration in raw and cooked (85 °C, 60 min) back muscle (nmol/g) after different storage conditions, fresh, refrigerator stored (4 °C, 6 days) or frozen stored (-20 °C, 3 months) (CONT- 6% palm fat in a diet; CONT+ 6% linseed oil in a diet; REISHI 6% linseed oil in a diet with addition of 1% ***Ganoderma lucidum*****; OLIVE 6% linseed oil in a diet with addition of 1% olive leaves)**

CONCLUSION

The meat oxidative stability was worse with PUFA content of meat increases. The addition of potential antioxidants (*Ganoderma lucidum* or olive leaves) does not protect meat from oxidation. Addition of *Ganoderma lucidum* decreased the concentration of MDA in both ways of stored raw samples to the level not statistical different from the palm fat group, but olive leaves only in the meat stored in freezer at -20 °C. Cooking (heat processing) forwards the process of oxidation. The addition of *Ganoderma lucidum* negligible reduces the MDA concentration as well as olive leaves (slightly higher), but none of them did not completely prevent oxidation. Better way of using those (potential) antioxidants could be in the form of extracts, that could be worthy of future investigation.

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EFFECT OF PROTEIN SHORTAGE AND CONJUGATED LINOLEIC ACID SUPPLEMENTATION ON QUALITY TRAITS AND MODELLING OF COAGULATION, CURD FIRMING AND SYNERESIS OF HOLSTEIN-FRESIAN MILK

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Original scientific paper

SUMMARY

Aim of the present study was to evaluate the effect of diets with optimal (CP 15% DM) or suboptimal (CP 12.3% DM) protein content, supplemented (CLA+) or not (CLA-) with rumen-protected conjugated linoleic acid (rpCLA) on some cheese-making properties. Twenty Holstein-Fresian mid lactating dairy cows have been reared following a 4×4 Latin square experimental design of 4 periods, 3 weeks each. Individual milk samples, collected during the third week of each period, were analysed for chemical composition, traditional milk coagulation properties (MCP: RCT, k_{20} and a_{30}) and for recording curd firmness (CF) every 15 s over a 90 min period. Data acquired from each sample were used to model CF over time calculating the following parameters: rennet coagulation time (RCT_{eq}), asymptotic potential CF (CF_P), CF rate constant (k_{CF}), syneresis rate constant (k_{CF}), maximum CF achieved within 90 min (CF_{max}) and time to CF_{max} (t_{max}). Data were analysed using period, diet and group (random) as sources of variation. Cows evidenced a strong individual variability within groups and were classified as early (RCT<20 min) or late (RCT>20 min) coagulating cows. Dietary protein shortage reduced milk protein and lactose content, while rpCLA supplementation depressed milk fat synthesis. Results showned that traditional MCP parameters were worsened by reduction of dietary protein in the case of milk produced by early coagulating cows, while rpCLA supplementation affected negatively all three traits on all cows. The study of CF model parameters evidenced that CP12 diets have improved CF (CF_P and CF_{max}) respect to CP15 when fed to late coagulating cows while worsened CF (CF_P and CF_{max}) and reduced k_{CF} when fed to early coagulating cows. The results of the present study underline the complex relationship between dietary fat and protein and their consequences on milk technological properties highlighting the need for further investigations.

Key-words: bovine milk, milk coagulation properties, curd firming modelling, dietary protein, rumen-protected conjugated linoleic acid

INTRODUCTION

The use of low protein diets is gaining interest because of environmental concerns and the increasing cost of protein sources (Schiavon et al. 2010 and 2013; Gallo et al. 2015). Conjugated linoleic acid isomers (CLA) content in animal products has gained attention primarily for the beneficial effect of these molecules on human health (Pariza et al. 2001) and have shown a favourable interaction with dietary protein reduction in young bulls fattening (Schiavon et al., 2012; Schiavon and Bittante, 2012). A dietary supply of these isomers decreased milk fat content in dairy cows (Glasser et al., 2010) and worsened milk coagulation properties (MCP) in ewe milk (Bittante et al., 2014). In cheese production interest in MCP of milk is increasing (Bittante et al., 2012). These

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information are relevant for cheese processing, particularly for Protected Designation of Origin (PDO) cheeses (Bertoni et al., 2001; Bittante et al. 2011a and 2011b). The combination of dietary protein shortage and the use of fat supplements in dairy cows could exert some effects on MCP, thus more detailed information about this interaction are required.

Traditional measurements of the coagulation attitude are made with the Formagraph (McMahon and Brown, 1982) which usually determine 3 parameters: rennet coagulation time (RCT), time to curd firmness of 20 mm (k_{20}) and curd firmness 30 min after enzyme addition (a_{30}). Different factors affect MCP and especially experimental conditions (Stocco et al., 2015) and cows genetics (Cassandro et al., 2005; Bittante et al., 2012). The existence of late- and non-coagulating milk samples, characteristic of some breeds as the Holstein-Fresian and Scandinavian ones (Tyrisevä et al., 2004), make difficult to measure traditional MCP within the 30 min duration of classical lactodynamographic test and thus the parameters cannot be measured. This problem was overcome by Bittante (2011) and Bittante et al. (2013) who modelled all the observations of curd firmness (CF) collected every 15 s on each individual milk sample extending the period of observation from 30 to 90 min. This experiment aimed to study the effect of a reduced dietary CP, with or without a CLA supplementation, on milk attitude to cheese-making, evaluating milk composition, traditional MCP traits and coagulation, curd firmness and syneresis model parameters of Holstein-Friesian dairy cows.

MATERIAL AND METHODS

The study was conducted at the University of Padova (Legnaro, Italy) and all experimental procedures were reviewed and approved by the University Ethical Committee (CEASA).

Experimental design and diets. Twenty Holstein-Fresian balanced for milk yield, DIM, parity, BW and BCS were randomly assigned to 4 pens (5 cows each), and treated according to a 4×4 Latin square experimental design with 4 periods of 3 weeks each. Cows were fed *ad libitum* total mixed rations based on corn silage, corn grain, meadow hay, sugar beet pulp, alfalfa hay, wheat bran and soybean meal. The control diet (CP15) has been formulated following NRC (2001) recommendations for 30.0 kg/d milk yield (3.5, 3.4 and 4.7 of protein, fat and lactose, respectively). Low CP diet (CP12) was formulated by replacing soybean meal with barley grain, decreasing CP content from 15 to 12.3% DM and increasing starch from 22.7 to 26.3% DM. Other constituents did not vary among diets. A rumen protected mixture of conjugated linoleic acid isomers (rpCLA; SILA, Noale, Italy) was supplemented by top dressing 80 g/d/cow of rpCLA, details about rpCLA composition is given in Schiavon et al. (2011).

Sampling and analysis. Milk samples have been collected during 5 consecutive d of the third experimental week for each of the 4 periods, one sample per cow for each milking event (2 per d), 800 samples were analysed in total. Milk coagulation properties have been analysed using two mechanical lactodynamographs (Formagraph, Foss) according to Cecchinato et al. (2013). Briefly milk samples (10 ml) have been allotted to racks with 10 cuvettes each, heated at 35°C and mixed with 200 μl of rennet solution (Hansen Standard 215, Oacovis Amrein AG, Bern, Switzerland) diluted to 1.2 % (wt/vol). Curd firmness measures (one every 15 sec for 90 min = 360 values each sample) enabled to apply the 4-parameter equation described by Bittante et al. (2013):

$$CF_t = CF_p \times [1 - e^{-k_{CF} \times (t-RCT_{eq})}] \times e^{-k_{SR} \times (t-RCT_{eq})}$$

where CF_t is the curd firmness at time t (mm); CF_P is the asymptotic potential maximum value of curd firmness (mm); k_{CF} is the curd-firming instant rate constant (% min^{-1}); k_{SR} is the curd syneresis instant rate constant (% min-1); RCT_{eq} is the rennet coagulation time (min).

In the initial phase of the test the k_{CF} prevail over k_{SR} and CF_t reaches its maximum value at CF_{max} in time t_{max} at which the two rate constant are equal but opposite in sign.

Statistical analysis. The 20 dairy cows differed largely in terms of MCP within each group, and were classified into two sub-groups: early coagulating (r < 20 min; n = 10) and late coagulating (r > 20 min; n = 9) cows that were analysed separately using the mixed procedure in SAS 9.2 (SAS Inst. Inc., Cary, NC) with the following model:

$$Y_{ijklm} = \mu + P_i + G_j + CP_k + rpCLA_l + CP \times CLA_{kl} + e_{ijklm}$$

where y_{ijklm} is the observed trait; μ is the overall intercept of the model, P_i is the fixed effect of the ith period (i = 1, …, 4), G_j is the random effect of the jth group of cows (j = 1, …, 4), CP_k is the fixed effect of the dietary CP level (k = 1, 2); CLA_l is the fixed effect due to the presence or absence of CLA (l = 1, 2), CP × CLA_{kl} is the interaction between CP and CLA and e_{ijkl} is the random residual. Group was assumed to be independently and normally distributed with a mean of zero and variance σ_j^2.

RESULTS AND DISCUSSION

The traditional MCP traits confirmed a negative effect of rpCLA on milk fat content (Glasser et al., 2010). Early and late coagulating cows differ by about 7 min. for rennet coagulation time, the difference, constant across diets (Table 1), confirms a large variability of MCP among cows of the same breed (Bittante et al., 2015).

Table 1. Effect of diets with a crude protein content of 15 (CP15) or 12.3 (CP12) % DM supplemented or not with 80 g/d of rumen-protected conjugated linoleic acid (CLA) on milk quality and on traditional milk coagulation properties of dairy milk1

	Diet				SE	*P*-values		
	CP15	$CP15_{CLA}$	CP12	$CP12_{CLA}$		CP	CLA	CP × CLA
Milk quality (all cows):								
Fat, %	3.66	3.12	3.69	3.12	0.12	0.90	0.002	0.88
CP, %	3.52	3.54	3.42	3.31	0.07	0.026	0.41	0.29
Lactose, %	4.76	4.73	4.72	4.67	0.02	0.020	0.05	0.59
RCT (min):								
early coagulating cows[2]	14.95	15.44	14.86	16.20	0.97	0.47	0.05	0.38
late coagulating cows[3]	23.67	25.34	22.26	26.00	1.89	0.61	**< 0.001**	0.15
k20 (min):								
early coagulating cows[2]	3.90	4.21	4.53	6.16	0.96	**< 0.001**	**0.005**	0.06
late coagulating cows[3]	9.92	11.31	8.93	10.51	0.87	0.15	**0.016**	0.88
a30 (mm):								
early coagulating cows[2]	45.67	45.54	43.13	37.42	4.25	**< 0.001**	**0.037**	**0.049**
late coagulating cows[3]	16.39	13.92	20.90	14.20	2.69	0.08	**< 0.001**	0.12

[1]RCT = rennet coagulation time; k_{20} = time interval to achieve a curd firmness of 20 mm; a_{30} = curd firmness after 30 min from rennet addition; [2]Cows (n=10) with a RCT before the beginning of the trial <20 min. [3]Cows (n=9) with a RCT before the beginning of the trial > 20 min

The supply of rpCLA negatively affected the traditional MCP traits, as observed on bovine (Bittante et al., 2014; Vacca et al., 2015). In fact, RCT was delayed, the k_{20} was increased and a_{30} reduced respect to the diet without rpCLA. The effects were more evident in late- than in early-coagulating cows. The reduction of dietary CP slightly reduced milk protein, lactose contents and worsened traditional k_{20} and a_{30} traits only in the early coagulating cows. Results obtained from CF_t modelling confirmed those observed for MCP. The rpCLA supply delayed milk gelation and decreased the asymptotical potential maximum curd firmness in late coagulating cows. Even though the two instant rate constants depicting increasing and decreasing phases of lactodynamographic pattern (kCF and kSR) were not modified, the final result indicated a CF_{max} decrease (Table 2). The rpCLA supply had no effect on MCP of early coagulating cows. A different effect of dietary CP reduction on technological properties of milk from early- and late-coagulating cows was observed (Table 2). In the first group of the cows the dietary CP reduction worsened the CF_P and k_{CF} model parameters of milk, leading to lower CF_{max}, thus confirming the traditional MCP results. The trend observed for late-coagulating cows was different as the dietary CP shortage had a favourable effect on CFt modelling: CF_P, k_{CF} and CF_{max}, were increased.

Table 2. Effect of diets with a crude protein content of 15 (CP15) or 12.3 (CP12) % DM supplemented or not with 80 g/d of rumen-protected conjugated linoleic acid (CLA) on modelling of coagulation, curd firming and syneresis of dairy milk[1]

	Diet					*P*-values		
	CP15	$CP15_{CLA}$	CP12	$CP12_{CLA}$	SE	CP	CLA	CP × CLA
RCT_{eq} (min):								
early coagulating[2]	15.69	15.90	15.12	16.19	0.94	0.77	0.17	0.36
late coagulating[3]	23.30	24.68	21.95	24.58	1.55	0.28	**0.003**	0.36
CF_P (mm):								
early coagulating[2]	57.78	57.96	55.33	52.76	2.50	**< 0.001**	0.19	0.14
late coagulating[3]	48.47	45.94	50.48	47.74	1.52	0.05	**0.006**	0.91
k_{CF} (% min^{-1}):								
early coagulating[2]	12.62	13.66	11.97	11.04	0.99	**0.0066**	0.93	0.11
late coagulating[3]	6.45	6.41	7.23	7.17	0.51	**0.031**	0.88	0.98
k_{SR} (% min^{-1}):								
early coagulating[2]	0.14	0.15	0.13	0.11	0.03	0.34	0.72	0.67
late coagulating[3]	0.18	0.16	0.14	0.10	0.04	0.14	0.37	0.62
CF_{max} (mm):								
early coagulating[2]	54.88	55.40	52.85	50.04	2.95	**< 0.001**	0.22	0.08
late coagulating[3]	42.72	39.91	45.39	41.71	1.14	**0.027**	**0.0013**	0.66
t_{max} (min):								
early coagulating[2]	72.79	70.37	74.44	76.87	2.547	0.06	0.99	0.27
late coagulating[3]	79.62	83.10	83.06	82.60	2.524	0.37	0.35	0.23

[1]RCT = rennet coagulation time; CF_p = asymptotic potential curd firmness; k_{CF} = curd firming instant rate constant; k_{SR} = syneresis instant rate constant; CF_{max} = maximum curd firmness; t_{max} = time at achievement of CF_{max}. [2]Cows (n = 10) with a RCT before the beginning of the trial <20 min; [3]Cows (n = 9) with a RCT before the beginning of the trial >20 min

CONCLUSION

This study shows that a reduction of dietary CP decreases protein and lactose milk contents, where rpCLA supply decreases milk fat content. For the first time in bovine the effects of both dietary treatments on milk coagulation traits were studied. The parameters of the curd firming model made clear an opposite effect of the reduction of dietary CP on milk produced by early-coagulating cows (unfavourable) and by the late-coagulating cows (favourable). The rpCLA supply did not exert any modification of MCP of early-coagulating cows, but worsened MCP in late-coagulating cows. Further researches are needed to define the upper and lower limit of dietary protein shortage also related to the variability of cow's individual milk yield and characteristics. More studies are required on rpCLA dosage in order to avoid not desired side effects on milk technological characteristics.

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PREDICTION OF GROSS FEED EFFICIENCY IN ITALIAN HOLSTEIN FRIESIAN BULLS

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Original scientific paper

SUMMARY

The aim of this study was to predict gross feed efficiency of Italian Holstein Friesian bulls selected for production, functional and type traits. A total of 12,238 bulls, from the April 2015 genetic evaluation, were used. Predicted daily gross feed efficiency (pFE) was obtained as ratio between milk yield (MY) and predicted dry matter intake (pDMI). Phenotypic trend for MY, predicted body weight (pBW) and pFE were calculated by the bull birth year. The results suggest that pFE can be successfully selected to increase profitability of dairy cattle using the current milk recording system. Direct measurements on DMI should be considered to confirm results of pFE obtained in the present study.

Key-words: gross feed efficiency, correlation, genetic trend, Holstein Friesian bulls

INTRODUCTION

Improving feed efficiency is a hot topic in dairy cattle breeding. Feed costs are a major proportion of the total costs of the dairy herd and thus reducing feed costs for the same output will improve farm profitability. Another benefit from improving feed efficiency is the reduction of greenhouse gases emissions (Hegarty et al., 2007; Wall et al., 2007; Cassandro et al., 2010, 2013). Several countries have set up projects to record dry matter intake (DMI) data (Veerkamp et al., 2000; de Haas et al., 2012; Pryce et al., 2014), but the recording of large datasets to estimate genetic parameters for feed efficiency is complicated and expensive. One way to obtain estimated breeding values (EBV) for traits difficult to collect at population level is to use genomic selection (Meuwissen et al., 2001), where phenotypes such as DMI are measured in a subset of the population, and genomic predictions are calculated for other animals that have genotypes but not phenotypes (Pryce et al., 2014). Although this approach is appealing, allowing industry-wide selection for improved efficiency, the size of the reference population from which the genomic prediction equations are derived is currently too small within each country to achieve satisfactory levels of accuracy of genomic breeding values (Verbyla et al., 2010). Another way to obtain EBV for feed efficiency is to predict this trait by combining official milk recording data and type traits. The aim of this study was to predict gross feed efficiency of Italian Holstein Friesian bulls selected for production, functional and type traits, and to assess phenotypic correlations of gross feed efficiency with milk yield and composition traits.

MATERIAL AND METHODS

A total of 12,238 bulls, from the official April 2015 genetic evaluation performed by the Italian Holstein Friesian Cattle Breeders Association (ANAFI), were used. Estimated breeding values (EBV) for milk yield (MY, kg/305 d), fat content (FAT, %/305 d), protein content (PRT, %/305 d), stature and body depth rescaled on phenotypic data of cattle born in the period 2007-2009, were provided by ANAFI. Predicted body weight (pBW, kg) was calculated as proposed by Cassandro et al. (1997). Dry matter intake (pDMI, kg/305 d) was derived using information of MY, FAT, and pBW for each bull, as reported by Chase and Sniffen (1985). Daily gross feed efficiency (pFE) was predicted as ratio between MY and pDMI. Phenotypic trend for MY (kg/305 d), pBW and pFE was calculated by birth year of bulls. Pearson cor-

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relations and descriptive statistics were computed using SAS software version 9.2.

RESULTS AND DISCUSSION

Descriptive statistics and Pearson correlations for the studied traits are reported in Table 1. Predicted FE and BW averaged 1.47±0.07 and 669.1±4.7 kg, respectively, as well as means for MY, FAT and PRT were 10,144±701 kg/305 d, 3.72±0.22% and 3.39±0.11%, respectively. Unfavourable correlations were estimated between pFE and milk composition traits, whereas favourable relationship was assessed between pFE and MY. All correlations were statistically significant (P<0.001). Similar results were reported by Connor et al. (2013) and Manzanilla Pech et al. (2014), whereas Vallimont et al. (2011) reported greater estimates of pFE than those obtained in the present work.

Table 1. Descriptive statistics[1] for milk yield (MY), fat content (FAT), protein content (PRT), predicted body weight (pBW) and predicted gross feed efficiency (pFE) of Holstein Friesian bulls (n=12,238). Pearson correlations (r_p) of MY and pFE with other traits are also provided

Trait	Mean	SD	Minimum	Maximum	r_p with MY	r_p with pFE
MY, kg/305 d	10,144	701	7,734	12,711	-	0.94
FAT, %/305 d	3.72	0.22	3.02	4.76	-0.32	-0.61
PRT, %/305 d	3.39	0.11	2.91	3.93	-0.21	-0.37
pBW, kg	669.1	4.7	652.85	685.37	0.47	0.33
pFE	1.47	0.07	1.18	1.70	0.94	-

[1]SD = standard deviation

Trends of MY and pFE by the bulls birth year are depicted in Figure 1. Milk yield increased by 62 kg per year during the last three decades. This result represents the 0.56% of the current phenotypic mean. The pFE followed similar trend with an annual increase of +0.004 kg of MY per kg of DMI. This result represents the 0.26 % of the current phenotypic mean. The lower value for pFE compared with MY is the result of the indirect selection strategy used by ANAFI to improve feed efficiency. Figure 2 shows trends for pBW and pFE. Body weight increased by 0.27 kg/year which represents an annual increase of +0.04% of the current mean value of pBW. These findings suggest that feed efficiency can be improved together with milk traits. However, body weight should not increase further.

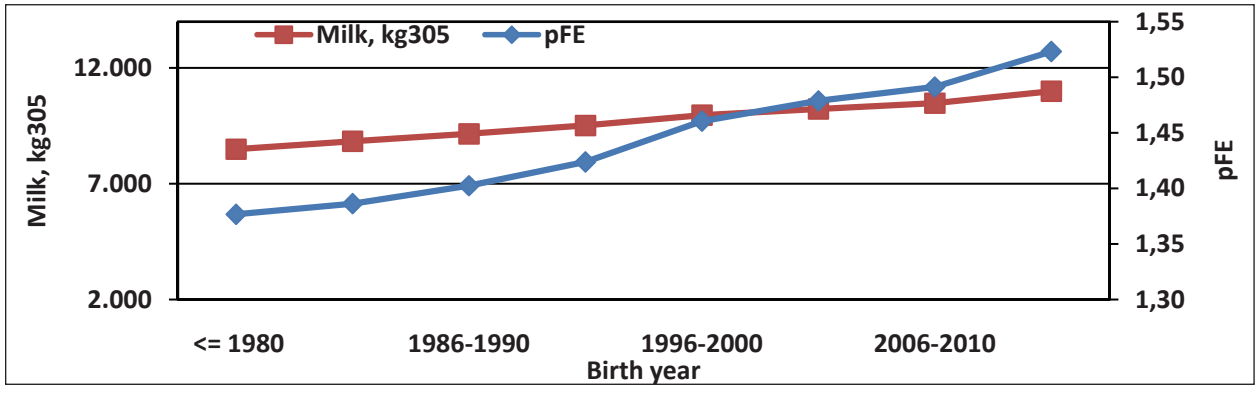

Figure 1. Trend of milk yield (MY, kg/305 d) and predicted feed efficiency (pFE) for Holstein Friesian bulls evaluated in Italy (ANAFI, April 2015)

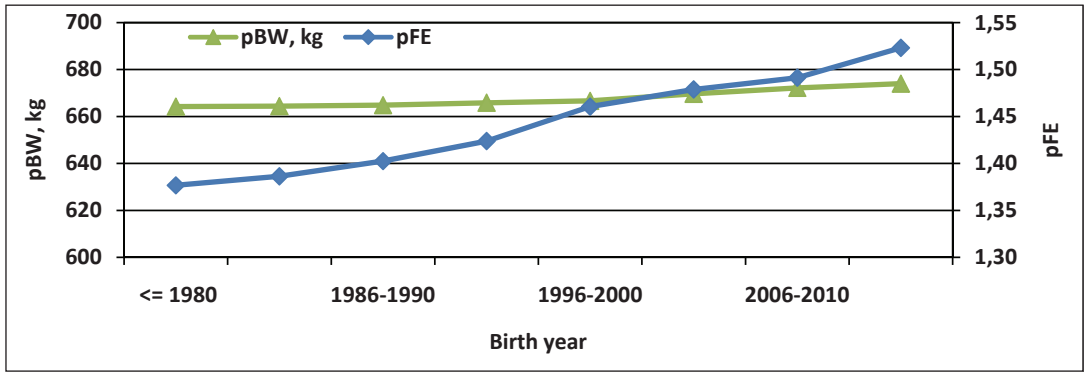

Figure 2. Trend of predicted body weight (pBW, kg) and predicted feed efficiency (pFE) for Holstein Friesian bulls evaluated in Italy (ANAFI, April 2015)

CONCLUSION

The results of this explorative study suggest that pFE can be successfully selected to enhance profitability of dairy cattle using current milk recording system. Recent advances in the dry matter intake at individual level using a roughage intake control system or similar tools seem to be very helpful to set up specific selection strategies for feed efficiency. A larger dataset with direct measurements on DMI should be considered to confirm results of the present study.

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THE CONTRIBUTION OF SOCIAL GROUP EFFECT TO VARIATION IN BOARS GROWTH

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Original scientific paper

SUMMARY

The aim of the study was to estimate the contribution of group to phenotypic variance of daily gain on different intervals. The focus was on data structure and differences of variance components estimated with and without effect of the social group. Growth of 806 boars from 443 litters was obtained during the field test in nucleus herd of Pietrain breed. A group was defined as pen mates. Most frequently, the group was formed from 6 and 7 boars. Data sets were prepared with SAS Software, variance components were estimated using VCE-6. The results showed the significant contribution of the group to phenotypic variance of daily gain. Inclusion of the effect of social group reflected in lower heritability and the smaller contribution of common litter environment. Further analysis revealed different contributions of components to phenotypic variance of growth rate on different intervals. The proportion of variation caused by common litter environment was larger on the interval from ±32.0 kg to ±48.8 kg of body weight (22%), compared to interval from ±39.6 kg to ±104.1 kg, explaining 1% of phenotypic variation, that could be the consequence of less defined pretest environment. The social group explained 6% of phenotypic variance for daily gain on interval from ±39.6 kg to ±104.1 kg of body weight, however, the contribution was larger on the interval from ±32.0 kg to ±48.8 kg (23%). The results confirmed the group as an environmental component, causing more variation in daily gain shortly after group formation (±32.0 kg), when a hierarchy is established, and later after its set, the contribution decreases.

Key-words: pigs, genetic evaluation, daily gain, social interaction, group effect

INTRODUCTION

Pigs show several social behaviours such as cooperation, altruism, and aggression. A tendency to establish a social hierarchy often leads to aggressiveness in a group. In limited resources, pigs also compete for food and other limited resources. Traits underlying these behaviours are influenced by interactions between pen mates.

Social environment of an individual is reflected also in its production level and welfare. However, phenotype of an individual is not only affected by its own genes, but also by the genes of pen mates present in individual's social environment (Griffing, 1967; Bijma et al., 2007).

Social environmental effect has biological origin and contributes the heritable variation, in animal breeding known as associative effect or social effect (Griffing, 1967; Muir, 2005; Bijma et al., 2007). Due to its potential for improving performance and animal welfare, models for implementation of social effect in a genetic evaluation were developed. Estimability of social genetic effect depends on effects included in the model, especially on implementation of group effect (Van Vleck and Cassady, 2005; Chen et al., 2008). Bergsma et al. (2008) showed that group effect included as random effect take into account nonheritable social effects to avoid overestimated social genetic variance. Bergsma et al. (2008) also reports covariances among pen mates expressed as genetic variance when pen effects are omitted from the model, due to the relatedness among pen mates. Thus, heritability decreased after inclusion of group effect.

The aim of study was to estimate the contribution of the group to phenotypic variance of daily gain on different intervals. The focus was on data structure and differences in estimates of variance components after including effect of the group.

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MATERIAL AND METHODS

Data of boar's performance was obtained from field test in nucleus herd of Pietrain breed. Performance test and genetic evaluation were conducted in accordance with national breeding program SloHibrid (Kovač and Malovrh, 2012). Boars that finished field test in April 2006 to August 2014, were included in the analysis. Total of 806 boars from 443 litters were considered. The group was defined as the group of pen mates. For the purpose of performance test, 8 pens are available. The surface area of the 6 pens is 7.92 m^2 and 10.06 m^2 of the other two. The group size varied between 2 and 12 individuals, 82% of groups sized of 5 to 8 boars. Preliminary analysis showed no effect of pen size or stocking density on growth and backfat thickness. The majority of groups consisted of boars originated from 3 to 4 litters.

Daily gain (DG) on several intervals up to 100 kg was analyzed (Table 1). Weighing in different stages revealed a large variability of animal's age and weight. Average DG from birth to the beginning of test ranged from 227 to 498 g/day. At the beginning of the test, the oldest boars were more than twice older than the youngest. The differences in the DG from the 1st weighing to the end of test ranged up to 690 g/day. The average relatedness within pen was 0.30, ranged from 0.151 to 0.524. The groups differed also in surface area per animal. On the average, twice as minimal required surface was provided per boars.

Table 1. Descriptive statistics

Variable		N	$\bar{x}$	σ	min	max
At the beginning of test	Weight (kg)	806	32.0	3.9	21.5	45.0
	Age (days)	806	83.5	7.1	48	109
At 1st weighing	Weight (kg)	806	39.6	4.7	26.0	56.0
	Age (days)	806	97.6	7.0	77.0	123.0
At 2nd weighing	Weight (kg)	806	48.8	5.7	35.0	69.0
	Age (days)	806	111.6	7.1	91	138
End of the test	Weight	806	104.1	9.6	80.0	136.0
	Age (days)	806	182.2	10.7	147.0	222.0
	Backfat (mm)	793	8.0	1.3	4.7	12.3
Daily gain (g/day)	Birth to the beginning of test	806	361.5	42.3	227	498
	Beginning to 2nd weighing	806	602.0	129.2	74	1222
	From 1st weighing to end	806	765.7	110.3	439	1129
	From birth to end	806	571.8	59.6	385.0	768.4
Average group relatedness		133	0.304	0.078	0.151	0.524
Surface area (m^2/animal)		806	1.41	/	0.7	4.0

N –number of observations; σ – standard deviation; $\bar{x}$ - mean; min – minimum; max – maximum

Two datasets were prepared. **Dataset 1** consisted of performance data at the end of the field test (weight and backfat thickness at the last two weighing). DG from birth to the end of test and backfat (BF) was analyzed. Fixed part of the statistical model included season as month-year interaction, model for BF consisted of effect of weight at BF measured as covariate. Common litter, direct additive genetic, permanent environment were treated as random effects. Permanent environment effect was used due to repeated measures of an animal.

(Co)variance components were estimated with two-trait repeatability model (eq. 1). Models were used (written in matrix notation):

$$\mathbf{y} = \mathbf{X\beta} + \mathbf{Z}_c\mathbf{c} + \mathbf{Z}_a\mathbf{a} + \mathbf{Z}_p\mathbf{p} + \mathbf{e} \qquad (1)$$

where **y** is the vector of observations. Unknown parameters for fixed effects are presented in vector β with incidence matrix **X.** Vectors **c, a** and **p** with incidence matrix $\mathbf{Z_c}$, $\mathbf{Z_a}$ and $\mathbf{Z_p}$ present common litter effect, direct additive genetic effect, permanent environment effect, respectively, **e** is vector of residual. To estimate the contribution of group to phenotypic variance, the group was added as random effect (equation 2).

$$\mathbf{y} = \mathbf{X\beta} + \mathbf{Z}_c\mathbf{c} + \mathbf{Z}_a\mathbf{a} + \mathbf{Z}_g\mathbf{g} + \mathbf{Z}_p\mathbf{p} + \mathbf{e} \qquad (2)$$

where **g** is a vector of random group effect and $\mathbf{Z_g}$ incidence matrix. It was assumed that random effects and residual follow normal distribution:

$$\mathbf{c} \sim N\left(\mathbf{0}, \mathbf{I}_c\sigma_c^2\right),\ \mathbf{a} \sim N\left(\mathbf{0}, \mathbf{A}\sigma_A^2\right),\ \mathbf{g} \sim N\left(\mathbf{0}, \mathbf{I}_g\sigma_g^2\right),$$
$$\mathbf{p} \sim N\left(\mathbf{0}, \mathbf{I}_p\sigma_p^2\right),\quad e \sim N\left(\mathbf{0}, \mathbf{I}_e\sigma_e^2\right) \qquad (3)$$

where $\mathbf{I_c}$, $\mathbf{I_g}$, $\mathbf{I_p}$, $\mathbf{I_e}$ are identity matrices of the appropriate dimensions, and **A** is a relationship matrix.

Dataset 2 was used to obtain the contribution of the social group to phenotypic variance of DG on different intervals. Following traits were analyzed: daily gain (DG_1) from the beginning of the test (±32.0 kg) to 2nd weighing (±48.8 kg), daily gain (DG_2) from 1st weighing (±39.6) to the test end (±104.1 kg) and BF thickness of the last weighing. Permanent environment effect was omitted from the model 1 and 2 (equation 1, 2) as one measure for an animal was observed. Three-trait model was used.

Fixed part of model was developed with SAS 9.3 (SAS Inst. Inc., 2011). Covariance components were estimated by the residual likelihood method (REML) using statistical package VCE-6 (Groeneveld et al., 2010).

RESULTS AND DISCUSSION

Heritability estimates for DG (dataset 1) from the first model 1 was 61% (Table 1), being higher than the heritability, usually estimated in commercial breeding farms (Clutter and Brascamp, 1998). The common litter environment contributed 14% and permanent environment accounted for 5% of phenotypic variance. Heritability for BF was 55%, which is in line with the literature (Clutter and Brascamp, 1998), larger contribution of unexplained variance could be the result of feeding *ad libitum*. After including group effect in the model for DG (15% variance), heritability dropped from 61% to 55% for DG, and common litter variance from 14% to 8%, indicating a partial additive genetic effect due to relatedness between pen mates (Bergsma et al., 2008). The group did not cause the variation in BF.

Table 2. Ratios for variance components (bold) and corresponding correlations between traits (italic) with standard errors estimated with both models in dataset 1

Variance component	Model 1		Model 2	
	Daily gain	BF	Daily gain	BF
Common litter environment	**0.14**±0.04	*-0.14±0.34* **0.04**±0.04	**0.08**±0.03	*0.25±0.61* **0.02**±0.03
Direct additive genetic effect	**0.61**±0.08	*-0.01±0.15* **0.55**±0.10	**0.55**±0.09	*-0.04±0.15* **0.53**±0.09
Group effect	/	/ /	**0.15**±0.02	*-0.79±0.30* **0.05**±0.03
Permanent environment	**0.05**±0.06	*-0.29±0.61* **0.14**±0.08	**0.05**±0.05	*-0.15±0.57* **0.13**±0.07
Residual	**0.20**±0.02	*-0.33±0.06* **0.28**±0.02	**0.18**±0.01	*-0.33±0.06* **0.27**±0.02

Daily gain - from the birth to the test end (±104.1 kg); BF- backfat thickness

Results of dataset 2 revealed varied contributions of variance components for DG on different intervals of body weight (dataset 2; Table 3). Heritability for DG was low (8%) on the interval from ±32.0 kg to ±48.8 kg of body weight and high (82%) on interval from ±39.6 kg to ±104.1 kg, indicating the majority of variation originated from genetic differences among tested animals. Low heritability in the first interval and correlation of breeding values for DG in these two intervals 0.57 revealed the selection in early stages that could not be performed. The proportion of variation caused by common litter environment was 36% in the interval from ±32.0kg to ±48.8kg of body weight, and accounted 4% in the interval ±39.6 kg to ±104.1 kg. Substantial proportion in the first interval could be explained by varied lactation length and less defined pretest environment.

The results from model 2 revealed the significant variance for the group effect (Table 3). The group effect contributed 6% of phenotypic variation of DG on the interval from ±39.6 kg to ±104.1 kg whereas the contribution was larger in the interval from ±32.0 kg to ±48.8 kg (23%). Bergsma *et al*. (2008) reported group contributed 27% of phenotypic variance in DG from ±27.0 kg to finishing. Our results revealed group causing more variation in DG shortly after group formation (±32.0 kg), when a hierarchy is established, and later after its set, the contribution decreases.

Table 3. Ratios for variance components (bold) and corresponding correlations between traits (italic) with standard errors estimated with both models in dataset 2

Variance component	Model 1			Model 2		
	DG_1	DG_2	BF	DG_1	DG_2	BF
Common litter environment	**0.36**±0.05	*0.18±0.31*	*0.40±0.34*	**0.22**±0.05	*0.58±0.43*	*0.44±0.32*
		0.04±0.03	*-0.20±0.78*		**0.01**±0.02	*-0.47±0.57*
			0.05±0.05			**0.05**±0.04
Direct additive genetic effect	**0.08**±0.05	*0.57±0.35*	*0.03±0.34*	**0.05**±0.03	*0.67±0.34*	*-0.16±0.39*
		0.82±0.08	*0.03±0.14*		**0.76**±0.06	*0.06±0.14*
			0.63±0.10			**0.61**±0.07
Group effect	/	/	/	**0.23**±0.05	*-0.19±0.27*	*0.66±0.87*
		/	/		**0.06**±0.03	*0.61±0.94*
		/	/			**0.00**±0.00
Residual	**0.56**±0.05	*-0.07±0.20*	*-0.10±0.13*	**0.49**±0.05	*-0.06±0.16*	*-0.06±0.11*
		0.15±0.08	*0.12±0.27*		**0.17**±0.08	*0.05±0.21*
			0.32±0.10			**0.33**±0.07

DG_1 – daily gain from the beginning of the test (±32.0 kg) to 2nd weighing (±48.8 kg); DG_2 -from 1st weighing (±39.6 kg) to the test end (±104.1 kg); BF- backfat thickness

CONCLUSION

Contributions of components to phenotypic variance of DG and BF were estimated. Heritability for DG was low on the first interval (±32.0 kg to ±48.8 kg) and high on the interval ±39.6 kg to ±104 kg. It indicates selection in early stages that not be performed. The proportion of variance caused by common litter environment was larger on the interval from ±32.0 kg to ±48.8 kg of body weight (22%), compared to interval from ±39.6 kg to ±104.1 kg, explaining 1% of phenotypic variation. The group explained 6% of phenotypic variance of daily gain on interval from ±39.6 kg to ±104 kg of body weight. However, the contribution was larger on the interval from ±32.0 kg to ±48.8 kg (23%). The group effect caused more variation in DG shortly after group formation (±32.0 kg), when a hierarchy is established, and later after its set, the contribution decreases.

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MILK SUPPLY OF RABBIT KITS

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Scientific review

SUMMARY

In general, rabbit does nurse the kits once-a-day for 3-4 minutes. During this time the kits are able to consume their daily feed requirement, which is about equal to 1/6 of their body weight. The milk intake, body weight gain and survival of the kits depend on the milk production and their mother nursing of willingness. However, the does are not able to cover the nutrient requirements of the suckling kits, especially on the 3rd week of lactation. The goal of this study is to examine the nutrient supplementation of nursing kits and to highlight the deficiencies of the nursing systems. The study summarizes the effect of using two nursing does per litter.

Key-words: milk production, suckling kits, nursing

INTRODUCTION

Several scientists have examined the methods of increasing the body weight and the weight gain of the rabbits during the fattening period. On the other hand, the possibilities of taking advantage of the growth potential of the rabbit kits before weaning have been studied in few papers. In this study we summarized the main scientific results in this field.

MILK SUPPLEMENTATION OF SUCKLING KITS

Rabbit does nurse their kits once a day (24h) with circadian periodicity (Zarrow et al., 1965; Drewett et al., 1982; Jilge and Stahle, 1993; Morgado et al., 2008). The kits have to consume their daily feed intake in a short nursing time, being about 3-4 minutes. It means that they are able to consume milk equal to 1/6 of their body weight (Lebas, 1975). According to other scientists it can even reach the 35% of their weight (Morgado et al., 2008). That is why they can double their birth weight till the age of 6 days (Davies et al., 1964).

The circadian nursing periodicity can be observed in the behaviour of the kits as well. The rabbit kits spend the day in the nest covered by hair (Hudson and Distel, 1982). The kits provide their body temperature huddling together in the nest, thus they can minimize their energy expenditure. Just before the nursing time (2-2.5 hours) the kits become active, they move to the top of the nest and their body temperature increases. With this process they prepare themselves for the arrival of the doe to suck as soon as possible (Caba and González-Mariscal, 2009). According to Jilge et al. (2000) the average body temperature (24 hours) of the suckling kits increases by 0.5% just before the mother arrival, however during the nursing process it even increases by 0.3-0.6%.

Even so, other scientists observed two or three nursings a day. According to Hoy and Selzer (2002) in a "free-range" system, the number of daily nursing events reached 2-3 events per day at the second week of the lactation. They also observed the main nursing period of the domesticated rabbit which was between 7 and 9 p.m. This finding is in close connection with the observation of Seitz et al. (1998). In their experiment the rabbit does visited the nests 0.8-2.2 times a day, and the average duration between two nursing events was 16.5 hours. Matics et al. (2004) published similar results.

Till the 9th day of the lactation 25% of the does nursed their kits more than once in 24 hours, later, between the 10th and 16th day of lactation the frequency of more nursing events per day decreased a little (21%). The frequency of daily nursing events increased as well, when the previously controlled (once-a-day) nursing does had free access to the nest (to the kits). Other experiments showed that the frequency of the nursing events can be increased by changing their time (let the does into the nest earlier than usual) (González-Mariscal, 2006).

Until the age of 15-18 days, the kits can consume only milk. Compared to some other domesticated animal species (as shown in Table 1), the fat and energy

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content of the rabbit milk are quite high (Maertens et al., 2006). During the first part of lactation the milk is enough to satisfy the high energy needs of the kits. However, in the lactation third week, the does are not able to satisfy the nutrient requirement of the kits (Gyarmati et al., 1999; Parigi-Bini et al., 1992; Xiccato et al., 1995; Xiccato et al., 1999). The growth and the survival of the kits depend on the milk production and on the does behaviour. Due to the lack of the milk, the hunger will lead the kits to start consuming solid feed. The less milk, the sooner and faster they eat solid feed.

By utilizing the high growth potential of the kits, they could reach the slaughter weight at younger age. The effects of the better nutritional status of the suckling kits on the production are an important and unanswered question.

Table 1. Milk yield and milk composition of some domesticated animals (Maertens et al., 2006)

	Hybrid rabbit	Pig	Dairy cow
Live weight *(kg)*	4.2	230	650
Peak of milk yield *(kg/day)*	0.3	8.9	47.5
Fat content of milk *(g/100 g)*	12.9	6.5	3.5-4.0
Protein content of milk *(g/100 g)*	12.3	5.1	3.0-4.0
Energy content of milk *(MJ/kg)*	8.4	4.5	2.7-3.2

USING TWO DOES FOR ONE LITTER

Herczeg (1981) tried to force one doe to nurse its kits twice a day, but the kits did not grow faster. A few years later Spencer and Hull (1984) published a new and efficient technique: nursing one litter with two does. In this experiment rabbits were used as animals model to examine the effect of overfeeding human babies. A few years later McNitt and Moody (1988) carried out an experiment with meat-type rabbits. Despite of the experiment success, this method had not become a practical method on rabbit farms.

In the experiment of Gyarmati et al. (2000) - till the age of 21 days - the kits were nursed by two does and they consumed 89% more milk than the control group, which led to a 70% higher body weight at the age of 21 days. The kits were able to keep the higher body weight through the fattening period, so they reached the slaughter weight (2.5 kg) 9 days earlier than the control group. The results of Szendrő et al. (2002) confirmed this outcome. In their experiment the difference between the two groups was +6-7% in favour of the experimental group.

By the refinement of this method, Szendrő et al. (2001) published a farmer-friendly method, where the kits in the experimental group - nursed by two does - reached the slaughter weight (2.5 kg) 5-6 days earlier. Reproduction rhythm of 42-day with two groups of does was used, and they were inseminated 21 days after each kindling. Two does were housed in special, larger (95x54 cm) wire-mesh cages, halved into two independent parts for the two does, with two closable doors into the large nest box. The doe that kindled nursed its kits in the morning, while the other (foster) doe (weaned their kits at 21 days) had free access to the nest box from 3 p.m. to the next morning.

According to the results of Gyovai at al. (2004) there were major differences between the two groups (nursed by one or two does) at the end of the first week. They investigated the production and reproduction performance through their life. Significant differences were in body weight and condition at first insemination. Total number of rabbit born was 9% higher in group nursed by two does. The best results were achieved when the does in their young age were nursed by two does and then fed restricted till the first insemination (60.3 kits born total/year) compared to the traditional method (nursed by one doe and fed *ad libitum;* 53.1 kits born total/year). Overfeeding the suckling kits results in better condition in adult age, which is the base of the long-life production (Xiccato, 1999).

In summary, the milk consumption of the kits can be higher by using two nursing does. The kits will have an increased appetite, lasting after the weaning, so the rabbits will consume more feed during the fattening period. Nursing by two does have beneficial effect on growing rabbits and rabbit does. The weakness of this method consists in the needs of more work and more practice, accuracy, increased space (unique cages) and early kits weaning.

CONCLUSION

According to the results, a satisfactory covering of the nutrient requirements of the suckling kits is a relevant and important task.

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THE PHYSICAL FORM OF CORN INFLUENCES THE RUMEN BACTERIAL BIODIVERSITY– PRELIMINARY RESULTS

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Preliminary communication

SUMMARY

The aim of this study was to investigate the rumen bacteria in terms of genetic biodiversity and variation due to different physical form of corn in cow diet. A total of twenty dry cows were fed for 3 months with the same diet, only differed for corn physical form, ten received corn grains, while the other ones received corn flour. To investigate the biodiversity of the bacterial 16S rRNA gene clone library analysis has been conducted and then the sequencing has been carried out using Ion Torrent PGM™ System. Bacterial population was tested using R statistical software. The Kruskal-Wallis one-way analysis of variance (Kruskal-Wallis, 1952) confirmed that the bacterial populations were different when the animals were fed grain compared with flour corn. Both the OUT's abundance (Operational Taxonomic Unit) and the biodiversity indexes presented a significant difference among the two sample groups, underlining the large changes that take place even with small diet modifications in ruminal environment. There is still the need to deepen how exactly the diet changes the rumen phylogenetic structure and the consequences on bacteria's activity.

Key-words: rumen bacterial biodiversity, diet composition, physical form, OTUs.

INTRODUCTION

Dietary component and variation cause shifts in rumen bacterial ecology playing a role in animal health and productivity. Because of this complexity further investigations are required. For several years the rumen has been studied for its role in nutrient digestion and to manipulate its microbial ecosystem to increase animal performance and efficiency. Microbial population is not stable and changes to ruminal environmental characteristics and diet (Biavati and Mattarelli, 1991; Tajima et al., 2000). Feeding large proportions of starch to ruminants increase rumen microbial activity and the animal productivity but on the other hand, can negatively affect the rumen environment and its functionality, the fibre digestibility, and the animal health (Theurer, 1985).

The use of grain instead of cereal flour in ruminant nutrition can affect the site of starch utilization leading to a shift from the rumen to the intestine with positive effect on efficiency of energy utilization and on rumen environment. For this reason there is a great interest to investigate the impact of the physical form of cereals on the rumen microbial population diversity. New technologies of DNA sequencing ("ultra-high throughput" Ion Torrent Personal Genome Machine, PGM) allow the simultaneous analysis of huge amounts of sequences at very low cost, improving accuracy in quantification, enabling the identification even of minor species.

The PGM™ System has been used by Patel et al (2014) to describe rumen microbiome of Indian cattle (Kankrej breed) under different dietary treatments, where cattle were gradually adapted to a high-forage diets. The study revealed significant differences between all the diet treatments. The aim of this study was to investigate

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the rumen bacteria in terms of genetic biodiversity and its variation feeding finishing dry cows with corn grain and flour.

MATERIAL AND METHODS

A total of twenty dry cows of three different breeds (Holstein, Brown Swiss, and Simmental) reared in L. Toniolo experimental farm (Legnaro, PD, Italy) were fed for 3 months a finishing diet composed of corn silage 44.6%, corn 34.2%, sunflower 8.3%, straw 5.8%, sugar beet pulp 3.7%, others additives 3.4% of DM (chemical composition: starch 38.8%, NDF (Neutral Detergent Fiber) 37.4%, crude protein 10.9%). The cows were divided in 2 homogeneous groups fed the same diet that differed only for the corn physical form, ten received corn grains, while the other ones received corn flour. Rumen fluid samples were collected from each one using rumen probe and stored at -80°C. Subsequently, the DNA has been obtained using a method based on guanidine hydrochloride buffer and common DNA extraction columns (Yaffe et al., 2012) and then purified with silica and DNAse (Rohland and Hofreiter, 2007). After the isolation the bacterial 16S rRNA gene clone library analyse has been conducted and then the sequencing of V1-V2 region has been carried out using Ion Torrent PGM™ System. After sequencing, data were combined and sample identification numbers assigned to multiplexed reads using the MOTHUR software environment (De La Fuente et al. 2014). Data were denoised, low quality sequences, pyrosequencing errors and chimeras were removed, then sequences were clustered into OTU's at 97% identity using the pipeline available from http://www.brmicrobiome.org/#!16s-profiling-ion-torrent/cpdg (Pylro et al., 2014). OTU's containing fewer than 5 reads were excluded due to the likelihood of them being a sequencing artifact. Samples were normalised by randomly resampling to the lowest number of sequences per sample using Daisychopper (De La Fuente et al., 2014). The OTUs' study was made using R software. A principal component analysis (PCA), and subsequently, a k-mean cluster analysis, were performed to test the whole dataset without any prior information. The cluster analysis has shown that the cow breed did not affect the separation of samples in different groups so this factor has been removed from analysis. After this preliminary step the Kruskal-Wallis one-way analysis of variance (Kruskal-Wallis, 1952) has been applied to verify the difference between the two diets treatments. The number of sequences of each normalised sample was 14,289 sequences/sample and the number of sequences per each OTU was log transformed. Three indexes had been used to study the bacterial biodiversity. The Simpson's Index was computed as $D = \sum(n / N)2$, the Shannon's diversity index as and Richness as mean of the number of OTUs of each sample.

RESULTS AND DISCUSSION

The 16S rRNA gene clone library analyses and the sequencing of V1-V2 region allowed obtaining 4.108 operational taxonomic units (OTUs) as sum of all samples analysed. As shown in Figure 13622 and 3089 different OUTs have been globally identified for grain and flour groups, respectively. The log transformation of the sequence number evidenced a different distribution of OTUs abundances among diet treatments. In almost all the OTUs, the grain group had higher abundance of sequence, compared to flour group, with the exception of only 7 OTUs where flour groups showed a much higher abundance.

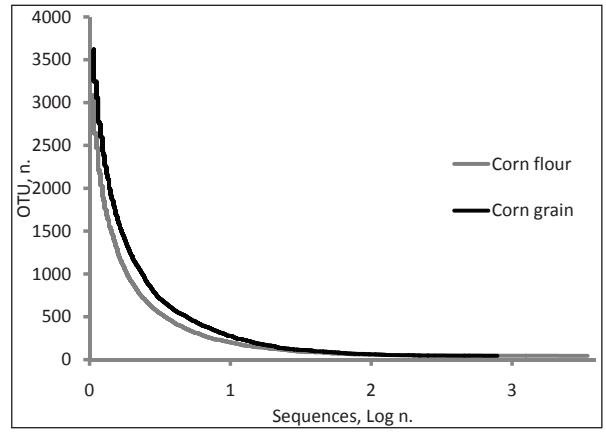

Figure 1. OTUs and Sequences graphical distribution for both groups (Flour and Grain corn).

The k-means cluster analysis graphically and clearly explained the differences among the groups and allowed two clusters identification. As shown in Figure 2, the two ellipses divided the twenty animals in two clusters that clearly identify the diet treatments even if there is an intersection area where some samples were not assigned and there is also an attribution error in corn flour ellipse. The Kruskal test, used to statistically analyse the biodiversity indexes, identified an outlier within the corn grain group, that behaviours differently from the other and was excluded from the statistical analysis. As reported in Table 1, the diversity indexes were significantly different for flour and grain groups.

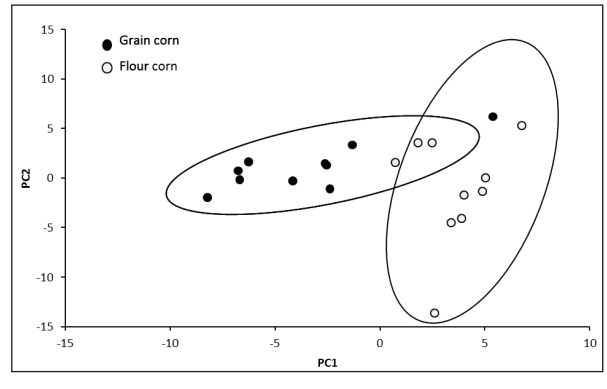

Figure 2. OTUs Cluster Analysis regarding principal component 1 (PC1) and principal component 2 (PC2).

The Shannon index increases as both the richness and the evenness of the community increase, and evidenced that animal fed corn grain instead of corn flour were characterized for a more biodiverse rumen microbial population. The richness index suggests that the grain group has a much higher diversity in term of OTUs number compared to the flour (1692 vs. 1261 OUTs, respectively). Finally, also the Simpson index that measure the microbial dominance, suggests also a slightly higher evenness of microbial population of grain group compared to the flour group.

Table 1. X^2, mean of corn flour group (flour) and corn grain group (grain) and P-value of Simpson, Shannon and Richness diversity indexes

		Corn treatment		
Biodiversity Index	X^2	Flour	Grain	*P-value*
Shannon	5.85	4.29	4.9	0.015
Richness	7.4	1261	1692	0.006
Simpson	3.72	0.91	0.89	0.053

To our knowledge, there are no studies that investigate the specific effect of diet physical form on rumen bacterial dynamics. However, some authors, who have worked on different levels of forages and concentrates, reported important variations in the rumen bacterial biodiversity. In particular, Fernando et al. (2010) found out a reduction of biodiversity increasing the proportion of concentrates in the diet. This result suggests that diet manipulation has an important role on rumen bacterial selection. In the present study these sensible effects of diet treatments on rumen diversity can be related to the different fermentative properties of corn fed to the animal as whole grains or after milling. The reduction of grain particle size is commonly associated to an increase of rumen fermentation rate and to a reduction of starch passage rate (Theurer, 1985). The rumen degradation of starch stimulate the microbial activity and the production of high proportions of VFA. However, at the sometime, the increase of starch fermentation in the rumen is commonly related to a reduction of cellulolytic bacteria activity and fibre digestion (Russell, 2002). Indeed, when the rate of VFA production overcome the buffering and absorption capacity of rumen, their accumulation lead to fluctuation of rumen pH and may have a selective effect on microbial population (Tajima et al., 2001). The reduction of bacteria biodiversity can impair the fermentative activity of the rumen microbial consortium (Wang and McAllister, 2002). Indeed, rumen bacteria adhere and colonize feed particles in the rumen, however, not all bacteria are equipped with a complete array of digestive enzymes. Co-culture of different microbial species demonstrated the importance of cross-feeding among bacterial species in attaining greatest bacterial growth rates and complete digestion of feed (Huntington, 1997).

CONCLUSION

The present study confirmed the significant difference between rumen bacterial populations in cows fed corn with different physical form within the same diet. Thus underlines the extreme dynamism of the bacteria and the susceptibility to even small changes in diet composition. There is a different rumen environmental equilibrium for the two theses that proved the variation in terms of bacterial diversity. The technology applied in this research does not allow to investigate the rumen bacterial activity. Anyway, this work represents a preliminary study that requires further investigations to understand the relation between the physical form of diet and the phylogenetic structure of the rumen population.

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HEAT STRESS AND MILK PRODUCTION IN THE FIRST PARITY HOLSTEINS – THRESHOLD DETERMINATION IN EASTERN CROATIA

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Original scientific paper

SUMMARY

In the light of increasingly rapid climate change worldwide, one of the primary goals is to reduce financial losses of dairy farmers and to enable the sustainable farming. In order to realize those goals, the necessity of implementation of breeding values for heat resistance in breeding strategies, have become more and more pronounced. Estimation of breeding values requires determination of temperature-humidity index (THI) threshold value. Therefore, the objective of this research was to determine the THI threshold value for the first parity Holsteins in environmental conditions in Eastern Croatia. With that purpose individual test-day records of the first parity Holsteins with records of ambient temperature and relative humidity in the barns were analysed. Data were collected in regular milk recording from January 2006 to December 2012. The THI threshold values for daily milk yield were determined by least square analyses of variance for each given THI value (from 65 to 76) using the PROC MIXED (SAS). The THI <68 did not cause significant change in daily milk production of the first parity Holsteins. Significant decrease of daily milk yield was observed at THI ≥ 68 with estimated drop from 0.240 to 0.716 kg milk/day (THI from 68 to 76). The THI=68, as the lowest value at which significant decrease in daily milk yield was determined, was taken as the threshold value for the first parity Holsteins in Eastern Croatia.

Key-words: first parity Holsteins, heat stress, temperature-humidity index, threshold, Eastern Croatia

INTRODUCTION

In the last few decades, we have witnessed more expressed and increasingly rapid climate change worldwide meaning, that in regions that currently are not characterized as extreme climate conditions, in future dairy cattle will be exposed to the unfavourable climatic conditions (IPCC, 2007). In accordance with this forecast, Reiczigel et al. (2009), in Hungary, determined increase of heat stress days/year (temperature-humidity index – THI>68) from 5 to 17 in the period of 30 years. Considering dairy cattle breeding in indoor housing, optimal microclimate conditions in the barns are necessary in order to realize the productive potential of individual cows. The interrelation between ambient temperature and relative humidity is relevant for animal welfare, reproduction traits and dairy farm profitability. Any extreme combinations are potentially harmful. On one hand, environmental conditions with low temperature and high humidity induce the cows to increase heat production and feed consumption in order to compensate body energy losses. Moreover, when the animal is overheated, high humidity may lead to infections of respiratory tract or udder. On the other hand, high temperature and low relative humidity may dehydrate mucous membranes thus increasing vulnerability to viruses and bacteria (Romaniuk and Overby, 2005). The combination of high temperature and high relative humidity has the most detrimental effect through inducing heat stress in cows. Under heat stress conditions,

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lactating cows tend to reduce level of dry matter intake (DMI) and milk production (West et al., 1999). Moreover, beside milk production reduction, heat stress is associated with changes in milk composition, somatic cell counts (SCC) and mastitis frequencies (Bouraoui et al., 2002.; Collier et al., 2012; Correa-Calderon et al., 2004; Ravagnolo et al, 2000.; St-Pierre et al., 2003; West, 2003). Additionally, deteriorate effect on reproductive performances was also observed (Bohmanova et al., 2007; Ravagnolo et al., 2000). Numerous studies showed that the high producing cows are much more susceptible to heat stress than low producing ones (Bohmanova, 2006; Collier et al. 2006). Kadzere et al. (2002) suggested that, due to intensive genetic selection for milk production, the thermoregulation physiology of a cow has been changed. The high producing cows have larger frames and larger gastrointestinal tracts allowing digestion of more feed and results in more metabolic heat, which consequently reduces cow's ability to maintain normal temperature at unfavourable temperature conditions. Finally, high producing cows experience heat stress earlier than low producing cows since the thermoneutral zone of high producing cows is at lower temperatures. The most common measure of heat stress in dairy cows is the temperature-humidity index (THI) that present combination of ambient temperature and relative humidity and is a useful and easy way to assess the risk of heat stress (Kibler, 1964). Du Preez et al. (1990a,b) determined that milk production and feed intake is affected by heat stress if THI values are higher than 72. Bouraoui et al. (2002) put the THI threshold on 69, while Bernabucci et al. (2010) as well as Collier et al. (2012) on 68. Vitali et al. (2009) suggested that the risk of cow's death started to increase when THI reached 80. The significant decrease of daily milk traits (yield and contents) was also determined in Croatian environmental conditions with the highest decline during summer period in Eastern and Mediterranean Croatia (Gantner et al., 2011). In many dairy-producing areas of the world heat stress condition represents a significant financial burden, for example in the USA between $897 and $1,500 million per year (St-Pierre et al., 2003). There are many methods to decrease the impact of heat stress including the shading, cooling, nutrition (Kadzere et al., 2002; West, 2003) and selection for resistance on heat stress (Bohmanova, 2006). Ravagnolo et al. (2000) determined antagonistic relationship between cow's production and heat tolerance implying deteriorate effect of selection on productivity and cow's resistance to heat stress. The high yielding Holstein cows in Israel is good example how selection on production could be successful in terms of heat stress (Aharoni et al., 1999). Implementation of breeding values for heat resistance in breeding strategies would certainly reduce financial losses of dairy farmers and enable sustainable farming. Estimation of breeding values requires determination of THI threshold value. Therefore, the objective of this research was to determine the THI threshold value for first parity Holsteins in environmental conditions in Eastern Croatia.

MATERIAL AND METHODS

For statistical analysis the individual test-day records of the first parity Holsteins collected in regular milk recording performed by alternative milk recording method from January 2006 to December 2012 were used. Monthly, at each recording, milk yields were measured during the evening or morning milkings. Additionally, ambient temperature and relative humidity in the barns were recorded at each recording. Logical control of milk data was performed according to ICAR standards (2003). Daily temperature-humidity index (THI) was calculated using the equation by Kibler (1964):

$$THI = 1.8 * Ta - (1 - RH)(Ta - 14.3) + 32$$

where Ta is average temperature in Celsius degrees and RH is relative humidity as a fraction of the unit. Records with lactation stage in (<6 days and >305 days), age at first calving in (<21 and >36 months), missing or parity >1, and missing or nonsense Ta and RH value were deleted from dataset. Data, provided by the Croatian Agricultural Agency, after logical control, consisted of 171,665 test-day records from 23,604 first parity cows reared on 1,805 farms in Croatia. Variability of ambient temperature (Ta) and relative humidity (RH) per recording year in Eastern Croatia is presented in Table 1.

Table 1. Descriptive statistics of ambient temperature (Ta) and relative humidity (RH) measured during the milk recording regarding the recording year in Eastern Croatia

Recording year	Ambient temperature (ºC)					Relative humidity (%)				
	Mean	SD	CV	Min	Max	Mean	SD	CV	Min	Max
2006	13.9	7.68	55.3	-9.0	37.0	69.5	10.08	14.5	35.0	99.0
2007	14.5	7.63	52.4	-3.0	39.0	69.6	10.58	15.2	30.0	98.0
2008	14.5	7.56	52.1	-6.0	38.0	69.0	10.82	15.7	30.0	97.0
2009	13.7	8.08	59.2	-9.0	40.0	68.6	11.72	17.1	32.0	98.0
2010	12.7	7.95	62.8	-9.0	36.0	70.9	12.45	17.6	35.0	99.0
2011	12.9	8.38	64.9	-9.0	39.0	70.7	11.93	16.9	30.0	98.0
2012	13.8	8.90	64.6	-9.0	40.0	69.2	12.59	18.2	30.0	99.0

The THI threshold values for daily milk yield were determined by least square analyses of variance for each given THI value (from 65 to 76) using the PROC MIXED procedure in SAS (SAS Institute Inc., 2000). The following mixed model was used:

$$y_{ijklmn} = \mu + b_1(d_i/305) + b_2(d_i/305)^2 + b_3 \ln(305/d_i) +$$
$$+ b_4 \ln^2(305/d_i) + S_j + A_k + T_l + e_{ijklmn}$$

where y_{ijklm}=estimated daily milk yield; μ=intercept; b_1, b_2, b_3, b =regression coefficients; d_i=days in milk (i= 6 to 305 day, lactation curve by Ali and Schaeffer, 1987); S_j=fixed effect of calving season class j (j=1/2006 to 12/2012); A_k=fixed effect of age at calving class k (k=21 to 36 month), T_i=fixed effect of THI class (l=0 (*normal condition – values under the given threshold*) or 1 (*heat stress condition – values equal and above the given threshold*)), and e_{ijklm}=residual.

The significance of the differences between the THI classes was tested by Scheffe's method of the multiple comparisons. The lowest threshold value at which significant differences in milk yield was determined has been taken as the THI threshold value.

RESULTS AND DISCUSSION

Least square means from analysis of variances regarding the fixed effect of THI class (0, 1) on daily milk yield are shown in Table 2. Environmental conditions in the barns that characterise THI values in 65, 66 and 67 did not cause significant difference in daily production of the first parity Holsteins. High statistically significant ($p<0.001$) decrease of daily milk yield was observed at THI value above 67, from 68 to 76. When THI value exceeded 67, the estimated drop in milk yield was from 0.240 to 0.716 kg/day. The highest decrease was determined in environmental condition characterised by THI=74. The lowest value, at which significant differences in milk yield was determined has been taken as the threshold value. Therefore, in the environmental conditions of Eastern Croatia, THI threshold value for the first parity Holsteins 68 was set to 68. Significant drop in daily production for dairy cattle at the same THI value was also determined by Bernabucci et al. (2010) and Collier et al. (2012). Bouraoui et al. (2002), in a Mediterranean climate, observed milk production decrease in condition characterised by THI ≥ 69.

Table 2. Least square means of cow's milk yield (kg/day) regarding the given THI threshold value

ThHo	Ls0	Ls1	Estimated difference
THI65	20.09 ± 0.100	20.10 ± 0.105	-0.005 ± 0.044n.s.
THI66	20.09 ± 0.093	20.11 ± 0.099	-0.028 ± 0.047n.s.
THI67	20.10 ± 0.093	20.06 ± 0.100	0.043 ± 0.049n.s.
THI68	**20.14 ± 0.093**	**19.90 ± 0.101**	**0.240 ± 0.050*** **
THI69	20.17 ± 0.094	19.71 ± 0.102	0.461 ± 0.051***
THI70	20.18 ± 0.093	19.60 ± 0.102	0.588 ± 0.052***
THI71	20.17 ± 0.093	19.59 ± 0.104	0.583 ± 0.054***
THI72	20.17 ± 0.091	19.47 ± 0.104	0.701 ± 0.057***
THI73	20.16 ± 0.093	19.45 ± 0.107	0.715 ± 0.059***
THI74	20.15 ± 0.092	19.44 ± 0.108	0.716 ± 0.063***
THI75	20.12 ± 0.093	19.74 ± 0.110	0.377 ± 0.064***
THI76	20.12 ± 0.093	19.71 ± 0.112	0.407 ± 0.067***

ThHo–given threshold value; 0–class under, and 1–class above the given threshold value;***-$p<0.001$; n.s.-non significant

Du Preez et al. (1990a,b) determined that dairy cows in Southern African conditions were affected by heat stress when THI values were higher than 72. The significant decrease of daily milk yield, when THI≥72, was also determined in Eastern and Mediterranean Croatia (Gantner et al., 2011). Bohmanova et al. (2007), in USA, determined different threshold values regarding different regions (72 in Georgia, and 74 in Arizona). The difference between determined threshold values could be due to better adapted cows, farm management or special housing characteristics.

CONCLUSION

Based on analysed data it could be concluded that temperature-humidity index (THI) <68 did not cause significant change in the first parity Holstein's daily production. Significant decrease of daily milk yield was observed at THI ≥68 with estimated drop from 0.240 till 0.716 kg milk/day (THI from 68 to 76). The THI=68, as the lowest value at which significant decrease in daily milk yield was determined, has been taken as the THI threshold value for the first parity Holsteins in Eastern Croatia.

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STUDY OF MILK PROTEIN COMPOSITION AND COAGULATION PROPERTIES OF BURLINA LOCAL CATTLE BREED

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Original scientific paper

SUMMARY

Burlina is a dual-purpose cattle breed, mainly reared in mountain areas of Veneto Region (Italy). The aim of this study was to investigate milk protein composition and milk coagulation properties (MCP) of Burlina breed. A total of 80 individual milk samples were collected and milk casein fractions were detected and quantified with reversed-phase high-performance liquid chromatographic analysis whereas MCP were determined with Formagraph. Sources of casein fractions variation and MCP were investigated using a linear model including herd, parity, and days in milk as fixed effects. Casein fractions showed increasing concentrations across days in milk, but not specific trends across parities. Milk coagulation properties exhibited better values in early than late lactation stages. Also, they deteriorated across parities.

Key-words: local breed, casein fraction, milk coagulation property

INTRODUCTION

Burlina is a native dual-purpose cattle breed reared in north-east of Italy, mainly in mountain areas of Veneto Region (Battagin et al., 2010). This local population has been widely appreciated in the past by farmers, but during the last century the number of animals has decreased drastically. Official statistics report that the population size decreased from 15,000 animals in 1930 to few hundreds in 1990, mainly because of the progressive substitution with the more productive Holstein-Friesian cows (Del Bo et al., 2001). Since the 1980s, the Burlina has been inserted in a conservation program, promoted by public authorities and organisations. Aim of this program is to enhance the genetic variability and to encourage the conservation of a native animal genetic resource to preserve pastures in marginal and fragile environments (Del Bo et al., 2001; Dalvit et al., 2008).

The present study aims to characterize milk from Burlina cattle breed, giving particular emphasis to milk casein fractions and milk coagulation properties (MCP). There is scientific evidence that casein fractions and MCP are useful information for cheese processing, yield and quality, especially in countries where dairy industry is based on traditional cheeses (Cassandro, 2003).

MATERIAL AND METHODS

Data

Individual milk samples (n=80) of Burlina cows from parity 1 to 12 and from 6 to 386 days in milk (DIM) were collected in 4 herds between March and April 2015. Immediately after sampling, milks were added with preservative, transferred at 4°C to the laboratory of the Breeders Association of Veneto Region (Padova, Italy) and analyzed for milk chemical composition using a MilkoScan FT6000 (Foss Electric A/S, Hillerød, Denmark) as well as somatic cell count (SCC) using a Fossomatic (Foss Electric A/S, Hillerød, Denmark). Following the recommendations of the International Committee for Animal Recording (ICAR, 2014), values of fat and protein contents, outside a range of 1.5 to 9% and 1 to 7%, respectively, were identified as outliers. Values of SCC were transformed to somatic cell score (SCS) through the formula $SCS=3+\log_2(SCC/100{,}000)$. Milk coagulation properties (MCP), namely rennet coagulation time (RCT) and curd firmness (a_{30}), were determined by Formagraph (Foss Electric A/S, Hillerød, Denmark). An aliquot of each sample was transferred to

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the Department of Agronomy, Food, Natural resources, Animals and Environment of the University of Padova (Legnaro, Italy). Analysis of casein fractions was carried out using a high-performance liquid chromatography station, Agilent 1260 Series (Agilent Technologies, Santa Clara, CA, USA), equipped with a reversed-phase analytical column C8 (Aeris WIDEPORE XBC8, Phenomenex, 3,6 **μm**, 300., 250 x 2,1 I.D.). The analysis was conducted following the method proposed by Maurmayr et al. (2013).

Statistical analysis

The normal distribution of studied traits was checked using Shapiro-Wilk's test. Pearson correlations between traits were estimated through the CORR procedure of SAS (SAS Institute Inc., Cary, NC, USA). Sources of variation of casein fractions and MCP were investigated using the GLM procedure of SAS. The model included the fixed effects of herd, parity (4 classes: parity 1, parity 2, parity 3, and parities 4 to 8), and DIM (4 classes: 6 to 60 d, 61 to 120 d, 121 to 180 d, and ≥181 d). A multiple comparison of means was performed for the fixed effects using Bonferroni's test ($P<0.05$).

RESULTS AND DISCUSSION

Descriptive statistics and significance of fixed effects

Table 1 shows descriptive statistics of quality traits, casein fractions and technological characteristics of individual milk samples. The most abundant casein fraction was α-casein (**α-C**n), which averaged 13.98±2.85 mg/mL, followed by **β**-casein (**β-Cn**) and κ-casein (**κ-Cn**), averaging 10.21±2.01 and 4.71±1.42 mg/mL, respectively. Contents of casein fractions found in the present work were similar to those reported by De Marchi et al. (2009) in a study aiming to characterise milk composition of the Simmental breed. Concerning MCP, RCT and a_{30} was averaged 20.08±4.77 min and 21.58±11.95 mm, respectively.

Significance of fixed effects included in the analysis of casein fractions and milk technological traits is reported in Table 2. The coefficient of determination ranged from 0.15 (RCT) to 0.37 (**κ**-Cn), suggesting that there is an important portion of the phenotypic variance not explained by the factors included in the statistical model for the studied traits. The κ-Cn and β-Cn fractions were significantly ($P<0.05$) affected by herd and DIM whereas α-Cn was influenced only by parity ($P<0.05$). Herd was an important factor in explaining the variation of pH and SCS, along with DIM for pH and parity for SCS ($P<0.05$).

Table 1. Descriptive statistics[(1)] of milk quality, casein fractions and milk technological traits

Trait[(2)]	N	Mean	SD	Minimum	Maximum
Fat, %	74	3.66	1.27	1.50	7.30
Protein, %	80	3.38	0.44	2.07	5.10
Casein, %	80	2.63	0.36	1.60	4.04
κ-Cn, mg/mL	55	4.71	1.42	0.44	7.61
α-Cn, mg/mL	80	13.98	2.85	5.94	20.66
β-Cn, mg/mL	80	10.21	2.01	6.00	15.37
RCT, min	61	20.08	4.77	9.45	29.00
a_{30}, mm	61	21.58	11.95	1.66	50.82
pH	80	6.63	0.10	6.16	6.88
SCS	78	3.16	1.75	0.16	7.82

[(1)] SD = standard deviation; [(2)] κ-Cn = κ-casein; α-Cn = α-casein; β-Cn = β-casein; RCT = rennet coagulation time; a_{30} = curd firmness; SCS = somatic cell score

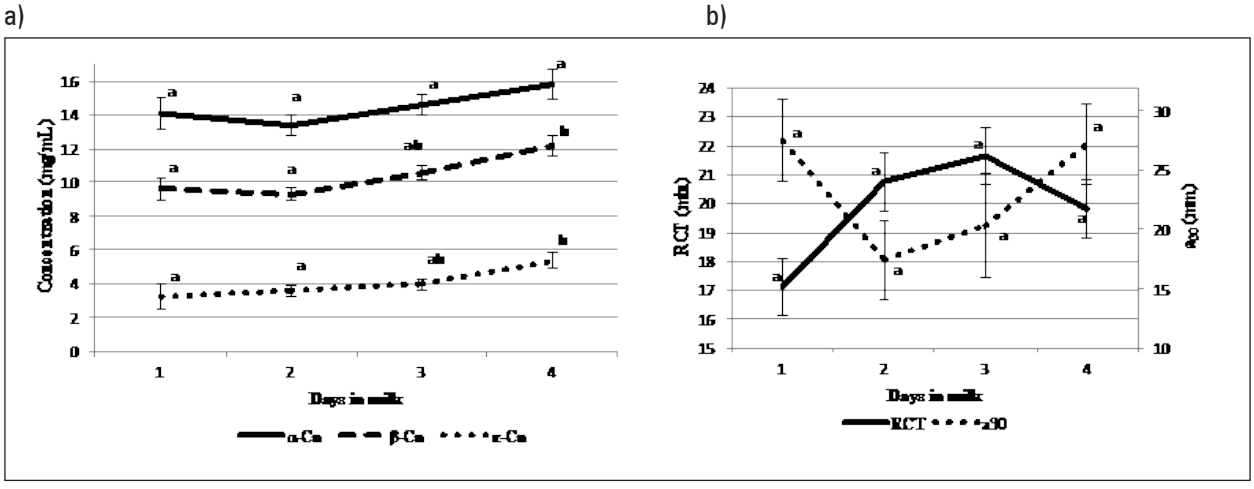
Table 2. F-value and significance of fixed effects included in the analysis of casein fractions and milk technological traits

Trait[(1)]	Herd	Parity	DIM[(2)]	R^2	RMSE[(3)]
κ-Cn, mg/mL	8.84***	1.13	4.12*	0.37	1.22
α-Cn, mg/mL	0.75	3.79*	2.10	0.23	2.65
β-Cn, mg/mL	3.56*	0.82	6.89***	0.32	1.76
RCT, min	0.68	1.10	1.82	0.15	4.77
a_{30}, mm	0.52	1.53	1.95	0.17	11.81
pH	6.66***	2.25	3.08*	0.27	0.09
SCS	4.20**	5.66**	1.73	0.35	1.50

[(1)] κ-Cn = κ-casein; α-Cn = α-casein; β-Cn = β-casein; RCT = rennet coagulation time; a_{30} = curd firmness; SCS = somatic cell score; [(2)] DIM = days in milk; [(3)] RMSE = root mean square error; Statistical significance is given as: *$P<0.05$; **$P<0.01$; ***$P< 0.001$

Least squares means

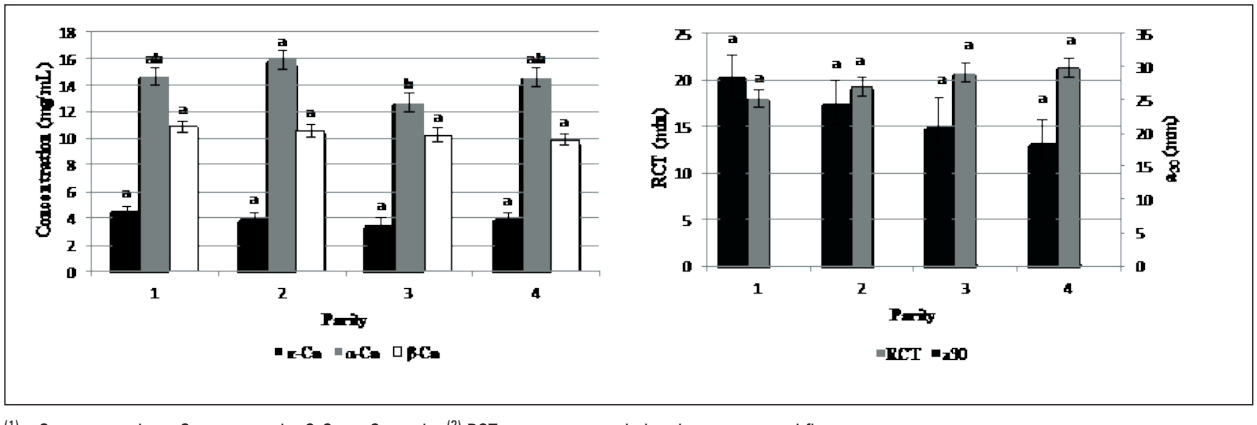
Figure 1 depicts the least squares means of milk casein fractions and MCP across DIM. In particular, **κ-Cn and β-Cn concentrations** were more abundant in late than early lactation stages ($P<0.05$). Despite being not significant, this result was confirmed also for α-Cn. Similar lactation variation of casein fraction contents was observed by Ng-Kwai-Hang et al. (1982). Rennet coagulation time resulted shorter in early lactation, and a_{30} exhibited the best values at the beginning and end of the lactation. Despite the trends for MCP across DIM that were not statistically significant ($P>0.05$), they were very similar to findings of Penasa et al. (2014) and Varotto et al. (2015) on milk of Holstein-Friesian cows.



[(1)] κ-Cn = κ-casein; α-Cn = α-casein; β-Cn = β-casein; [(2)] RCT = rennet coagulation time; a_{30} = curd firmness

Figure 1. Least squares means of (a) milk casein fractions[(1)] and (b) milk coagulation properties[(2)] across days in milk. Least squares means with different letters across days in milk for a given trait mean that they are significantly different according to Bonferroni's correction (P<0.05)

Figure 2 depicts the least squares means of milk casein fractions and MCP across parities. Concentration of casein fractions was quite stable and did not show particular trends across parities. Only α-Cn exhibited different concentration (P<0.05) in milk from the second and third parity cows. Albeit not significant (P>0.05), RCT and a_{30} deteriorated from the first to fourth and later parities, suggesting that MCP were more favourable in primiparous than multiparous cows. This trend was observed also in other studies (Tyrisevä et al., 2003; Penasa et al., 2014; Varotto et al., 2015). However, Ikonen et al. (2004) reported lower values of a_{30} in milk of primiparous than multiparous cows.



[(1)] κ-Cn = κ-casein; α-Cn = α-casein; β-Cn = β-casein; [(2)] RCT = rennet coagulation time; a_{30} = curd firmness

Figure 2. Least squares means of (a) milk casein fractions[(1)] and (b) milk coagulation properties[(2)] across parities. Least squares means with different letters across parities for a given trait mean that they are significantly different according to Bonferroni's correction (P<0.05)

CONCLUSION

The present study is the first contribution to the quantification of casein fractions in Burlina cattle population. Casein fractions showed the highest concentration in late lactation, and MCP showed better values in early lactation. Moreover, MCP deteriorated across parities. Overall, the comparison of results from the present study with the scientific literature suggests that Burlina has similar casein composition and MCP of other cattle breeds, such as Simmental and Holstein-Friesian, thus indicating that small-sized local breeds could be interesting for traits of economic importance.

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THE ENVIRONMENTAL IMPACT OF COW MILK IN THE NORTHEAST OF ITALY

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Original scientific paper

SUMMARY

This study presents a "from cradle to farm gate" Life Cycle Assessment on cow milk produced in Northeast Italy. System boundaries consider milk and meat delivered at farm gate, including all upstream emissions. All farm activities were considered. Inputs and outputs required in one year are counted and information about 34 dairy farms are used to represent the production area. Different allocation approaches were used to share resources and emissions between milk and meat. Functional unit was one kg of raw milk. The Ecoinvent v3.1 and Agri-footprint v1.0 database were used for secondary data, and SimaPro© 8 was the main software in the analysis. The following impact categories were investigated: Climate Change (CC), Terrestrial Acidification (TA), Freshwater Eutrophication (FE), Land Occupation (LO), Water Depletion (WD) and Cumulative Fossil Energy Demand (CFED). Purchased feed production was the first emitter, followed by on-farm crop production, animals and manure management emissions. Considering the most debated impact categories, 1.80-2.19 kg CO_2eq and 8.84-10.78 MJ represent, respectively, CC and CFED per kg of raw milk. This research could be applied in regional studies on environmental impact of Italian dairy production.

Key-words: LCA, dairy farm, milk, environmental impact

INTRODUCTION

Life Cycle Assessment is becoming a solid tool to identify and estimate main emission drivers in dairy production chain. Italy has a developed dairy industry, mainly based on traditional cheeses and PDO products (Cassandro, 2003). Considering the several kinds of Italian dairy products and the difference dairy farming systems existing in the Italian territory, an estimation of environmental impacts occurring in raw milk production at farm is advantageous in order to better represents each production areas, furthermore considering that in terms of overall environmental impacts, the majority emission drivers in dairy products are located to raw milk production at farm (Kim et al., 2013). Several environmental impacts such as Climate Change, Acidification, Eutrophication, Land Use, non-renewable energy use, and other impacts belong to dairy farms as shown by Italian (Guerci et al., 2013a,b) and international (Thoma et al., 2013) researchers. The aim of this study was to estimate environmental impact of one kg of raw milk production in the Northeast Italy.

MATERIAL AND METHODS

Life Cycle Assessment (LCA), ISO 14040-14044 (ISO 2006), was used to perform the study, adopting a *"from cradle to farm gate"* perspective and an attributional approach (Thoma et al., 2013).

The functional unit used in this study was 1 kg of raw milk delivered at farm gate. Meanwhile 1 kg of Live Weight delivered at farm gate was the functional unit to express meat production. Six allocation methods were considered to allocate inputs and final emissions to milk and meat: *biological* (IDF, 2010), *economic* (using annual economic revenue derived from product sales), *mass*, *fat* and *protein content* of delivered products; moreover a *No-Allocation* approach is performed attributing all emissions to milk.

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System boundaries considered milk and meat delivered at farm gate, including all upstream emissions. All farm activities were considered. Main product was raw milk, but meat production was a relevant co-product in dairy farms. Meat derived from: culled cows, exceed heifers, male calves, male and female animals breed as beef, and reproduction bulls. Manure produced was spread in the on-farm land and it was not considered as co-products, and emissions from manure were part of the system.

Thirty-four dairy farms (75% of annual milk production) were selected among the 65 dairy farms that conferred milk to the dairy cooperative. Milk produced by members was collected and processed by a unique dairy plant in order to produce Italian PDO (Protected Designation of Origin) cheeses. Annual presence of animals in farm and feed rations were investigated in all 65 dairy farms. During 2014, data were collected throughout personal interviews with farm owners, covering all farm processes during 2013. The study pursues the idea to obtain the best realistic representation of dairy area emissions, then all data collected were considered valid data, and only limited adaptations and supplements were applied, where lack of data were presented.

All resources incoming in the whole dairy area during one year were counted and used to assess environmental impact, except emissions related to building, machinery, medicines and refrigerant gases due to lack of data or due to their low importance on the total impact (Thomassen et al., 2008). Data collected regard: land, water, electricity, fuels (diesel and LPG), plastic (PP, HDPE, LLDPE) and paper (cardboard, kraft paper and tissue paper) packaging and related waste, fertilizers, chemicals, pesticides, bedding materials, purchased feeds, crops produced on farm. Raw material compositions and active ingredients, and their related emissions, were considered for fertilizers, chemicals, pesticides, bedding materials, purchased feeds. Transport to farm and from farm was associated to all resources. Emissions on-farm and off-farm were estimated using different methods: Ellis et al. (2007) for enteric CH_4; IPCC (2006), with updated conversion factors (IPCC, 2013), is used for CH_4 and N_2O emissions from manure management, and NO_3 leaching and run-off at field level; Mikkelsen et al. (2006) for CH_4 from bedding materials; EEA (2013) for NH_3, NO_X, NMVOC, PM_{10}, $PM_{2.5}$, NO and pesticides emissions from on-farm crop production at field level; Nemecek et al. (2007) for PO_4^{3-} leaching and run-off at field level; UFE/UFAM (2014) for diesel and LPG burning emissions. The Ecoinvent v3.1 and Agri-Footprint v1.0 database were used for secondary data; where possible databases were implemented with local data to increase the precision on the results, such as local-real transport for all resources, except for fertilizers, chemicals and pesticides. Specific Italian recycling unit processes were adopted for paper waste (Arena et al., 2004) and plastic waste (Ferrari et al., 2005; Perugini et al., 2005).

SimaPro© 8 was used as the main software in the analysis (PRé Consultants, The Netherlands 2014).

Environmental impact estimation includes the following impact categories: Climate Change (CC), Terrestrial Acidification (TA), Freshwater Eutrophication (FE), Land Occupation (LO) and Water Depletion (WD) according ReCiPe Midpoint (H) v1.11 (Goedkoop M.Jet al., 2009), and Cumulative Fossil Energy Demand (CFED) according to Frischknecht R. et al. (2007) v1.09, excluding infrastructure processes and long-term emissions. Only classification and characterization LCA steps (ISO 14040-14044, 2006) are considered in the study.

RESULTS AND DISCUSSION

Results per kg of raw milk delivered at farm gate, throughout impact categories and allocations, are shown in Table 1. Considering impact drivers, purchased feed production was the main contributor on overall impact categories and allocations. Among allocation methods, the biological approach (IDF, 2010) is taken into account to explain the results: purchased feed production was the main emission driver in FE (83%), CFED (71%), LO (63%), AC (62%) and CC (53%), while on-farm crop production was the first contributor in WD (94%) and the second emitter in all impact categories, except in CC where animal emissions counted for 37% of the total CC. Contemplating CC category, CO_2, CH_4 and N_2O emissions represented, respectively, 55%, 38% and 7% of total CC impact: enteric CH_4 and manure management CH_4 are, respectively, 80% and 20% of CH_4 derived from animals. The highest CO_2 contribution of decreases when CO_2 from land transformation (51% of total CO_2) is not counted: CH_4 becomes the first contributor with 49% of total emissions (97% from animals), CO_2 marks 43% (mainly from fuel combustion), and N_2O grows to 9% (55% from on-farm crop production). In AC, NH_3 composed 84% of the total emissions; meanwhile organic and synthetic fertilizers used in purchased feed production counted 78% of emissions in FE.

Table 1. Emissions per kg of raw milk delivered at farm gate and allocation factor to milk using different allocation methods

Impact category	Biological	Economic	Mass	Fat	Protein	No-Allocation
Climate Change, kg CO_2 eq	1.80	2.06	2.13	1.80	1.84	2.19
Terrestrial Acidification, g SO_2 eq	13.20	15.13	15.61	13.20	13.52	16.10
Freshwater Eutrophication, g P eq	0.16	0.18	0.19	0.16	0.16	0.19
Land Occupation*, m^2a	1.64	1.89	1.95	1.64	1.68	2.01
Water Depletion, m^3	0.47	0.54	0.56	0.47	0.49	0.58
Cumulative Fossil Energy Demand, MJ	8.84	10.14	10.46	8.84	9.06	10.78
Allocation to milk, %	82	94	97	82	84	100
*: Agricultural + Urban + Natural transformation						

Considering allocation approach to milk, our results were similar to those reported in the international methodology (IDF, 2010), while economic allocation values were similar to the results reported by Guerci et al. (2013a). Several *"from cradle to farm gate"* LCA have been performed for raw milk; these studies show results per kg of functional unit slightly lower than values estimated in the present study. Considering CC, an average value is 1.3 kg CO_2eq/kg milk (De Vrier and De Boer, 2010), although Guerci et al. (2013b) estimated values of 1.91 kg CO_2eq/kg ECM in Northern Italian dairy farms. Nevertheless, coherence is individualized in the main emission drivers. Italian authors (Fantin et al., 2011; Guerci et al., 2013a, 2013b) found on-farm emissions (mainly enteric, manure management and on-farm crop emissions) as the first emitter in CC, AC and FE, while purchased feed production as the second contributor in overall impacts and the first in CFED; moreover they underlined as enteric CH_4 was first contributor in CC, followed by CO_2 emissions. However, deep comparisons among studies are difficult due to different impacts under analysis, methods, functional units, system boundaries and emissions factors, such as the changing from IPCC (2006) to IPCC (2013). Reduction of impacts can achieve throughout rations for reducing enteric emissions, energy recovery technologies (such as manure anaerobic digestion), and the optimization in use as well as application of fertilizers.

CONCLUSION

In this assessment, purchased feed production deriving from the secondary data leads potential environmental impacts. This result derives by the choice to consider, and to break up, all concentrate feed used for each animal classes into singular raw materials. However, overlooking purchased feed impacts and CO_2 from land transformation, a general trend found in literature is recognized. An estimation of local emissions in raw milk production is better way to represent a specific dairy production. Comparison with other studies is made possible using international estimation methods for LCA and emissions. However, the specificity of region and data collected involves minor deep comparison with studies on national and international level.

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FATTY ACID PROFILE IN MILK OF BOVEC SHEEP UNDER TRADITIONAL FEEDING MANAGEMENT

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Original scientific paper

SUMMARY

The fatty acid profile in the milk of Bovec sheep fed total mixed ratio (TMR) and grazed natural pastures in the lowland (480 m altitude) supplemented with the second harvest (L) as well as grazed different altitude mountain pastures; M1 (1100-1300 m altitude), M2 (1600-1700 m altitude), M3 (1800 m altitude), M4 (1900 m altitude), M5 (2200 m altitude) were determined. There was an important effect when ewes were turned from the stable to the pasture on all fatty acids. The percentage of α-linolenic acid (ALA), arachidonic acid (ARA) and docosahexaenoic acid (DHA) increased significantly (P<0.001) with the diet. In the milk from M5 grazing the percentage of ALA was 2.5 times higher than in milk from L and 2.6 times higher than in milk from TMR. The percentage of ARA and DHA in milk was the highest when ewes were grazing on the M5 pasture (0.21±0.02 wt. %; 0.22±0.02 wt. %) respectively. Total n-3PUFA and n-6PUFA increased significantly (P<0.001) by the diet. Therefore, the n-6/n-3PUFA ratio was the best (1.2) in milk produced in the highest mountain pasture (M5), in terms of nutritional requirements.

Key-words: ewe milk, fatty acid, stable, mountain grazing

INTRODUCTION

Diet is the most important factor affecting ruminant's milk composition, particularly fatty acid composition. Appropriate feeding systems were implemented to increase the content of beneficial fatty acids in the most studies (Biondi et al., 2006; Morand-Fehr et al., 2007; Ostrovský et al., 2009). The milk composition of the grazing ewes is believed to be relatively healthy due to their richness in polyunsaturated fatty acids, mainly α-linolenic acid (ALA) and conjugated linoleic acid (CLA) compared to milk of ewes fed indoors with total mixed rations (TMR). Only a few studies dealing with milk fat quality of ewes raised on the pasture were published (Cabiddu et al., 2005; Mel'uchová et al., 2008). In Slovenia, all ewes' milk is processed into cheese. The Bovec sheep breed is reared in the local area (northwest Slovenia) under traditional extensive conditions to produce milk for Bovec cheese. Bovec cheese production starts from the stable and lowland produced milk and continues with mountain grazing produced milk. The aim of this study was to monitor the fatty acid profile of Bovec sheep milk produced under traditional feeding management, to investigate the effect of the diet.

MATERIAL AND METHODS

Fifteen randomly assigned ewes of the autochthonous Bovec sheep from a larger flock were observed during the lactation period started in the stable and finished in mountain pastures. The farm was located in Bovec (northwest Slovenia) and the flock is included in the national Breeding program for Bovec sheep. The rearing technology used is traditional for that area, whereby sheep are housed during the winter in lowland farms and, during the vegetation period, they are on all-day mountain grazing. Ewes started milking in the stable on April 21st, and were fed *ad libitum* with the total mixed ratio (TMR), consisted of grass silage, corn grain and the second harvest. Thereafter, from April 26th until June 16th ewes were grazing in lowland (480 m) and were supplemented by the second harvest (L). Then, after June 16th they were gradually moved to mountain pastures at the following altitudes: M1 (1100-1300 m altitude), M2 (1600-1700 m altitude), M3 (1800 m altitude), M4

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(1900 m altitude) and, finally to M5 (2200 m altitude), on the mountain Mangart. The observed flock grazed in the mountain pastures from June 17th to September 4th. Ewes were milked twice per day, in the stable by machine and after moving to the mountain pastures by hand. The milk yield was changing during lactation period and reached on the average1.7 kg, 1.67 kg, 0.90 kg, 1.08 kg, 1.15 kg, 0.84 kg and 0.60 kg per ewe/day in the TMR, L, M1, M2, M3, M4 and M5, respectively. Milk was used for Bovec sheep cheese production.

The chemical composition of grass was determined from clipped samples cut from randomly selected places at all pasture locations. Grass samples were cut using hand grass shears and collected as a composite sample from each location: L, M1, M2, M3, M4 and M5. Grass was cut on the same days as milk samples were taken and it was packed in polyethylene bags and stored at -20°C. The chemical composition of TMR components were determined from randomly selected places of grass silage, corn and second harvest depository and were taken on the same days as milk samples were.

Milk sampling was performed by the ICAR guidelines for AT4 milk recording (ICAR, 2011), with the recommended first (month) sampling during the morning and second (next month) sampling during the evening milking etc. The 50 ml milk samples were taken from each ewe for further fatty acid analysis. Milk samples were immediately packed in a cold chamber and transported to the laboratory. In the laboratory milk samples were shaken to homogenize the milk and then distributed into aliquots of 1.5 ml and stored at -20°C until the analysis started.

Milkoscan 6000FT was used for determining fat content in the milk. The method was based on the measurement of the absorption of mid-infrared radiation at wavelengths (International Standard, ISO 9622:1999). The fat content was expressed in percentages (g/100 g). Fatty acid methyl esters (FAME) from milk samples were prepared using the *in-situ* transesterification method of Park & Goins (1994). An Agilent 6890 series GC instrument equipped with an Agilent 7683 Automatic Liquid Sampler, a split injector, a flame-ionization detector (GC-FID) and a WCOT fused silica capillary column CP-select CB for FAME (Varian, 100 m x 0.25 mm i.d.) were used for separation of FAME. The Agilent GC ChemStation was used for data acquisition and processing. The Separated FAME were identified by retention time comparison and the results were quantified using response factors derived from chromatographic standards of the known composition (Nu Chek Prep, Nu Chek 85, Nu Chek 411, Nu Chek 68A). The fatty acid composition was expressed as a weight percentage (wt.%) of the total identified fatty acids. The accuracy and reliability of the method used was assessed with certified reference material (NIST 8435 whole milk powder). The repeatability of the method was tested with six injections of a composite milk sample. The detection limit was 0.005% and the limit of quantification was 0.05 wt.%. Fat content in the reference material was 21.3±2.4% for validating the material.

Data were analysed using the GLM procedure in the statistical package SAS/STAT (SAS Institute Inc., 2001) using the Model 1. All fatty acids with values above 0.01 wt.% of total fatty acids were included in the data processing.

$$y_{ij} = \mu + D_i + b_I (x_{ij} - \bar{x})^2 + e_{ij} \qquad \text{Model 1}$$

where: y_{ij}=trait; μ=mean; D_i=diet, i=total mixed ratio (TMR), lowland grazing supplemented with the second harvest (L), mountain grazing 1 (M1), mountain grazing 2 (M2), mountain grazing 3 (M3), mountain grazing 4 (M4), mountain grazing 5 (M5); x_{ij}=days in milk (DIM); b_I=regression coefficient, e_{ij}=residual

RESULTS AND DISCUSSION

The fatty acid composition in the TMR diet and grazing are shown in Table 1. Feed fatty acid analysis highlighted the difference between stable (TMR) and pasture (grazing) diet. Linoleic acid (LN) was predominant in the TMR. The percentage of LN was the highest in the corn (55.37 wt.%). Due to grass silage and the second harvest in TMR the percentages of α-linolenic acid (ALA) was high, 51.03 wt.% and 42.43 wt.% respectively. LN and ALA together accounted for more than 60% of fatty acids in the TMR diet. The ALA was predominant in grazing. The highest proportion was in grazing from the highest mountain pasture (M5; 61.08 wt.%).

Table 2 shows that the fatty acid composition in milk was changed with the diet transition from TMR to grazing. There was an interesting effect on all fatty acids when ewes were turned from the stable to the pasture. Saturated fatty acids as lauric (C12:0) and myristic (C14:0) were influenced ($P<0.001$) by the diet. When sheep started grazing in lowland the percentage of lauric acid decreased from 4.9 wt.% in milk from TMR to 3.8 wt.% in milk from lowland (L) grazing.

Table 1. Average fatty acid composition in total mixed ratio (TMR) and grazing (wt.%)

Fatty acid	TMR			L	M1	M2	M3	M4	M5
	Grass silage	Corn	Second harvest						
C12:0	0.28	/	0.44	0.30	0.22	0.73	0.57	0.32	0.61
C14:0	0.59	0.04	0.65	0.57	0.47	0.90	0.70	0.44	1.05
C16:0	16.91	12.47	20.74	15.56	15.33	16.69	17.33	13.82	13.69
C18:0	1.45	1.85	2.47	1.38	1.15	2.23	1.54	1.38	1.28
LN	20.35	55.37	19.15	20.30	17.33	16.71	17.27	13.27	11.48
ALA	51.03	1.66	42.43	54.69	57.89	47.41	48.53	60.66	61.08
DHA	/	/	/	/	/	0.32	0.25	0.05	/
SFA	24.50	15.21	31.34	22.29	21.08	27.69	26.86	21.49	22.55
MUFA	3.69	27.72	6.81	2.73	3.70	6.34	5.71	3.74	4.76
PUFA	71.81	57.07	61.85	74.99	75.22	65.97	67.43	74.77	72.69
n-6 PUFA	20.68	55.39	19.15	20.30	17.33	18.15	18.59	14.03	11.61
n-3 PUFA	51.12	1.66	42.70	54.69	57.89	47.82	48.84	60.73	61.08
n-6/n-3	0.40	33.40	0.45	0.37	0.30	0.38	0.38	0.23	0.19

LN: C18:2n-6 (linoleic acid); ALA: C18:3n-3 (α-linolenic acid); DHA: C22:6n-3; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; n-6 PUFA: n-6 polyunsaturated fatty acids; n-3 PUFA: n-3 polyunsaturated fatty acids; n-6/n-3: n-6/n-3 PUFA ratio; TMR: total mixed ratio; L: lowland grazing supplemented with second harvest; M1–M5: mountain pastures

ALA remained constant until sheep were grazing on the mountain pasture M2. After ewes were moved from M2 to the highest mountain pasture (M5) ALA increased. In the milk from M5 grazing the percentage of ALA was 2.5 times higher than in milk from L and 2.6 times higher than in milk from TMR. The percentage of arachidonic acid (ARA) in milk was the highest when ewes were grazing on M5 pasture (0.21±0.02 wt.%) and differed significantly only from TMR milk (0.12±0.02 wt.%). DHA increased significantly ($P<0.001$) with the diet, the lowest value were in milk from TMR (0.01±0.02 wt.%) and the highest in milk from M5 grazing (0.22±0.02 wt.%). The CLA percentage decreased after the transition from TMR to L and remained stable when grazing occurred in mountain pastures. The differences were significant between TMR and M3, M4 and M5. The highest percentage of CLA was in milk from TMR. This was probably due to grass silage and the second harvest of good quality as the main components of TMR. Total n-3 PUFA and n-6 PUFA increased significantly ($P<0.001$) according to the pastures altitudes. Therefore, the n-6/n-3PUFA ratio was the best (1.2) in milk produced in the highest mountain pasture (M5), in terms of nutrition.

Beside the effect of the diet, the fatty acid composition was influenced by the stage of lactation (days in milk) as well. ALA, LN, ARA, n-6 PUFA ($P<0.05$), n-3 PUFA ($P<0.01$) and DHA ($P<0.001$) increased significantly with the lactation stage.

Biondi et al. (2008) confirmed the diet effect when switching from stall to pasture feeding for all fatty acids in sheep milk, except C4:0 and C6:0. They reported a 2-fold ALA increasing within the first 2 days of transition to pasture. Nevertheless, the increasing of ALA was even higher after the transition from stall to herbage diet. Ostrovský et al. (2009) investigated the effect of ewes' diets on the milk fatty acids from a TMR to a pasture diet. They found similarities to our results with increased ALA values from a TMR period throughout a transition period to the beginning of the grazing season. They also found out a decreased CLA contents in July, which increased again in the middle of September.

Table 2. Fat content (g/100g) and fatty acid profile (wt. %) in ewes' milk from different diet

Trait	LSM ± SE							p-value	
	TMR	L	M1	M2	M3	M4	M5	D	DIM
Milk fat	5.5±0.8^{a}	8.3±0.4^{b}	6.8±0.3^{ab}	7.1±0.3^{ab}	6.5±0.3^{ab}	6.8±0.4^{ab}	7.7±0.8^{ab}	***	ns
C12:0	4.9±0.4	3.8±0.2^{a}	3.1±0.1^{ab}	2.9±0.1^{b}	2.7±0.1^{b}	2.8±0.2^{ab}	2.0±0.4^{b}	***	*
C14:0	12.4±0.7^{a}	11.4±0.4^{a}	10.4±0.3^{ab}	9.5±0.2^{bc}	9.2±0.3^{bc}	9.7±0.3^{ab}	7.0±0.8^{bd}	**	***
C16:0	22.8±1.2^{ab}	26.0±0.6^{a}	24.0±0.5^{ab}	23.0±0.4^{b}	23.2±0.5^{ab}	24.3±0.6^{ab}	22.3±1.4^{ab}	***	ns
C18:0	9.8±1.1^{a}	11.4±0.5^{ab}	12.6±0.4^{ab}	13.4±0.4^{ab}	13.8±0.4^{b}	12.8±0.5^{ab}	14.2±1.3^{b}	**	*
LN	2.5±0.3^{ab}	2.3±0.1^{a}	3.0±0.1^{b}	3.0±0.1^{b}	3.4±0.1^{b}	3.5±0.1^{b}	4.00±0.3^{b}	***	*
ALA	1.07±0.2^{a}	1.1±0.1^{a}	1.4±0.1^{a}	1.9±0.05^{b}	2.2±0.1^{bc}	2.4±0.1^{c}	2.8±0.2^{c}	***	*
CLA	2.1±0.2^{a}	1.6±0.1^{ab}	1.6±0.1^{ab}	1.7±0.1^{ab}	1.5±0.1^{b}	1.5±0.1^{b}	1.5±0.2^{b}	**	ns
ARA	0.12±0.02^{a}	0.14±0.01^{ab}	0.13±0.01^{ab}	0.13±0.01^{ab}	0.16±0.01^{ab}	0.17±0.01^{ab}	0.21±0.02^{b}	**	*
EPA	0.18±0.01^{a}	0.12±0.01^{b}	0.15±0.01^{a}	0.14±0.01^{ab}	0.16±0.01^{ac}	0.16±0.01^{ab}	0.18±0.02^{ab}	***	ns
DHA	0.01±0.02^{a}	0.05±0.01^{a}	0.09±0.01^{b}	0.09±0.01^{b}	0.10±0.01^{bc}	0.14±0.01^{c}	0.22±0.02^{d}	***	***
SFA	66.6±2.1^{a}	66.2±1.0^{a}	62.8±0.8^{ab}	60.7±0.7^{b}	60.3±0.8^{b}	61.9±0.9^{ab}	55.9±2.3^{ab}	**	*
MUFA	26.9±1.7^{ab}	27.8±0.8^{a}	29.9±0.6^{ab}	31.5±0.6^{b}	31.6±0.7^{ab}	29.4±0.8^{ab}	34.4±1.9^{ab}	**	*
PUFA	6.5±0.5^{ab}	5.9±0.2^{b}	7.2±0.2^{a}	7.7±0.2^{ac}	8.0±0.2^{ac}	8.6±0.2^{c}	9.6±0.6^{c}	***	ns
n-6PUFA	2.8±0.3^{ab}	2.7±0.1^{a}	3.5±0.1^{b}	3.5±0.1^{b}	3.9±0.1^{b}	4.0±0.1^{b}	4.6±0.3^{b}	***	*
n-3PUFA	1.4±1.2^{abc}	1.5±0.1^{c}	2.0±0.1^{ab}	2.4±0.1^{d}	2.6±0.1^{d}	3.1±0.1^{d}	3.6±0.2^{d}	***	**
n-6/n-3	1.8±0.1^{a}	1.7±0.1^{a}	1.7±0.03^{a}	1.4±0.03^{b}	1.5±0.03^{a}	1.3±0.04^{c}	1.2±0.1^{c}	***	ns

Means within a row with different letters differ significant (P<0.05);*:P<0.05; **:P<0.01; ***:P<0.001; ns: not significant. LN: C18:2 n-6 (linoleic acid); ALA: C18:3n-3 (α-linolenic acid); CLA: c9t11C18:2; ARA: C20:4n-6; EPA: C20:5n-3; DHA: C22:6n-3; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; n-6 PUFA: n-6 polyunsaturated fatty acids; n-3 PUFA: n-3 polyunsaturated fatty acids; n-6/n-3: n-6/n-3 PUFA ratio; TMR: total mixed ratio; L: lowland grazing supplemented with second harvest; M1–M5: mountain pastures; D: diet; DIM: days in milk

CONCLUSION

The results suggested that grazing on M1 pastures at 1100-1300 m above the sea level already improved the fatty acid profile of milk compared to lowland grazing supplemented with the second harvest. When ewes were moved to the highest pastures, milk fatty acid profile became even more beneficial probably due to the high ALA content in grazing from mountain pastures. The traditional Alpine grazing management of Bovec sheep could become a useful strategy to manipulate dietetic characteristics of milk and cheese.

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EFFECTS OF FEEDING SYSTEM AND BREED ON LAMB PRODUCTIVE AND CARCASS CHARACTERISTICS IN THE SOUTH MEDITERRANEAN REGION

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Original scientific paper

SUMMARY

The objective of this study was to evaluate the effect of feeding regimes and breed type on growth, non-carcass components and carcass characteristics of light lambs. Twenty four light lambs from the rustic fat-tailed Barbarine (BB) breed and 21 from the thin-tailed Sicilo-Sarde (SS) breed were used. For each breed, animals were divided into 3 groups (8 BB and 7 SS breed, each) by live weight (LW). Two groups were conducted on rotational grazing of barley grass (GB) or perennial rye-grass (GR) and received daily 350 g of concentrate per lamb. The last group was conducted on feedlot system (FL) with 450 g of ryegrass hay and 650 g of the same concentrate per lamb per day. The whole grass yield was 5 t DM/ha for ryegrass prairie and 4.2 for barley one. The final LW was higher for GB and GR lambs (28.3 kg) than for FL ones (26.9 kg). Irrespective to breed, the average daily gain was higher for both grazing groups than FL system, 144, 137 and 121 g for GR, GB and FL regimes, respectively. Slaughter LW was higher for BB (29.0 kg) than SS breed (26.5 kg). Barbarine lambs had more fat (23 vs. 17%) and less muscle (53 vs. 57%) than SS ones. FL lambs carcasses were more adipose (26%) than those of both grass groups (18%), while grass lambs had more muscle (57 vs. 51%). Grazing grass based diets increased carcass muscle and decreased fat proportions, which could be a useful feeding strategy to naturally manipulate lamb meat nutritional characteristics.

Key-words: grass, grazing, concentrate, growth, lambs, carcass traits

INTRODUCTION

In Southern Mediterranean area, sheep feeding is based on natural resources, range land and stubble. To satisfy nutrient needs, intensive husbandry systems are developed, given the availability of such resources is uncertain throughout the year. However, the humid and sub-humid regions present an important fodder potential and must play a more determining role in ruminants feeding. Grazed ryegrass has a relative superior nutritional value than grass silage (Kennedy et al., 2008) and grass hay (Nefzaoui and Chermiti, 1989). Also, dairy ewes grazing ryegrass or green barley compared with FL or green barley, conventional practice, engendered promising results (Atti et al., 2006). Moreover, lambs finished on feedlot (FL) were fatter than lambs grazed on perennial ryegrass forage (McClure et al., 1994), green barley or natural fallow (Atti and Abdouli, 2001). The objective of this study was to compare the growth and carcass characteristics of lambs by the feeding strategy, feedlot vs. grazing, and the breed. The considered breeds were the Barbarine (BB), the dominant Tunisian breed, and the Sicilo-Sarde (SS), the breed of the humid area of Tunisia.

MATERIAL AND METHODS

For this study, 24 light lambs from the rustic fat-tailed Barbarine (BB) breed and 21 from the thin-tailed Sicilo-Sarde (SS) breed were used. For each breed, animals were divided into 3 groups (8 BB and 7 SS breed, each) by live weight (LW), which initially averaged 14.8±2.9 kg. Two groups were conducted on

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rotational grazing with a stocking rate of 60 lambs per ha in separate pastures of barley grass (GB) or perennial ryegrass (GR). Both grazing groups received 350 g/lamb/day of concentrate at 18% crude protein (CP) content. The third group was conducted during the same period indoors on FL and fed individually ryegrass hay (8.5% CP) and concentrate *ad libitum*. Feed intake was daily and individually controlled indoors. In pasture, the whole herbage production was calculated after cutting and weighting grass in 15 quadrates of 0.25 m^2 before entering each paddock. The mean daily grass availability was calculated as the ratio of paddock grass production by lambs' number and by number of days spent in the paddock. For hay, concentrate, and grass, DM was determined by drying at 105ºC and nitrogen by Kjeldahl method (CP=N×6.25). Animals were weighed weekly and then slaughtered at the end of the growth trial (97 days). Lambs LW were recorded just before slaughter. After slaughter, weights of the different components of offal were determined (skin, head, feet, red organs and gut). Visual evaluations of carcass conformation and fat scores were determined by trained INRAT personnel according to photographic standards using a 15 points scale. After cooling for 24 h at +4°C, cold carcass were weighted (CW) and each carcass was split longitudinally in halves. The left half-carcass was cut into six joints (leg, lumbar region, flank, thoracic region, neck and shoulder). Every joint was weighed and dissected into muscle, fat, bone and waste (tendons, lymph nodes etc). For data analysis, animals were considered as experiment unit, the GLM procedure of SAS (SAS Institute, 2004) was used in a balanced 2x3 factorial experiment (2 breeds and 3 feeding regimes) and their interaction. Then, the Duncan test was used to compare diet mean effects (P=0.05). Animals grazed in groups; feed intake was not included in the data analysis.

RESULTS AND DISCUSSION

The grass amount produced by the whole ryegrass plot during all grazing period was higher than the barley one. The whole grass yield was 1240 and 1050 kg DM being equivalent to 4.96 and 4.20 tons of DM per ha of ryegrass and barley, respectively. By these amounts and experiment duration, the mean daily herbage availability was 0.850 and 0.720 kg DM per lamb of ryegrass and barley, respectively. For FL lambs of both breeds, mean daily DM intake was 0.450 kg of hay and 0.650 kg of concentrate. The mean CP content of grass for both species was 137 g/kg DM; it is higher than ryegrass hay CP content (Table 1).

Table 1. Chemical composition of experimental foods

	Ryegrass hay	Concentrate	Ryegrass grass	Green barley grass
Dry matter (g/kg)	830	835	310	210
Organic matter (g/kg DM)	937	960	871	877
Crude protein (g/kg DM)	85	143	120	125
Crude fiber (g/kg DM)	285	78	290	233
Ash (g/kg DM)	63	40	129	122

The average daily gain (ADG) was 145 g for BB breed compared to 121 g for SS one ($p<0.01$); it was 144, 137 and 121 g for GR, GB and FL treatments, respectively ($p=0.07$). Consequently, slaughter LW was higher for BB breed (29.0 kg) than SS one (26.5 kg) without significant difference. It was higher for GB and GR lambs (28.3 kg) than for FL ones (26.9 kg), but it did not differ among GB and GR treatments. The higher nutritive quality of grassland grazing system could engender higher ADG of the lambs in the grazing system. Many studies showed that daily gain of concentrate fed lambs was lower compared to grass ones (Atti and Abdouli, 2001; Nuernberg et al., 2008). For dairy cattle (Kennedy et al., 2008) and dairy ewes (Atti et al., 2006), milk yield and quality were higher for grass than FL system. For the Tunisian native sheep breeds, the higher growth performance for grazing than FL lambs was shown (Khaldi, 1989; Atti and Abdouli, 2001). So, grazing grassland sheep resulted in a higher growth than FL animals. Consequently, with the half amount of concentrate, grass feeding lambs reached the slaughter weight earlier than FL ones. Hence, this system constitutes an economic way of producing meat lamb. The differences in growth rate between animals from BB and SS breeds resulted from vocation of each breed. In fact, SS sheep is raised as a dual purpose breed, but its breeding program has neglected growth performances, placing more emphasis on milk production. However, BB breed is raised to produce meat lambs. The widely variation in breed performance in a common environment was known and differences in growth between dairy and meat breeds were mentioned (Emmans and Friggens, 1995).

Carcass weight and carcass fat score were similar for all the treatments (Table 2). These parameters depend on live weight at slaughter (Colomer-Rocher and Espejo, 1972; Mahouachi and Atti, 2005) which was similar for all the treatments. However, carcass conformation was higher for BB than SS breed ($p<0.05$) and GB than FL groups ($p<0.01$).

Head and feet weights were affected neither by breed nor by feeding system, while grazing animals (GB and GR) had heavier ($p<0.01$) red organs than the FL ones (Table 2). The weight of offal components high in bone content (head and feet) did not vary or varied slightly with diet since these components are early maturing parts (Wallace, 1948; Atti et al., 2003; Mahouachi and Atti, 2005). Conversely, the weight of red organs (liver, heart, lungs) increased for grazing

lambs compared to FL ones. Nutrients produced by fermentation of grass based diets are probably important factors in liver weight changes (Ortigues and Doreau, 1995). This phenomenon may explain the higher weight of these organs. The skin was higher for BB than SS lambs since lambs were unshorn and the BB wool production is higher than SS one.

Table 2. Live weight (LW), average daily gain (ADG), empty body weight (EBW), carcass weight (CW), carcass conformation score (C Score), fat score and offal weight

	Breed$^{\mu}$ (Br)		Feeding system$^{\mu\mu}$ (FS)			p value	
	BB	SS	GB	GR	FL	Br	FS
Initial LW (kg)	14.9	14.7	14.7	14.6	15.2	ns	ns
ADG (g)	145	121	137^{ab}	144^{a}	121^{b}	0.006	0.07
Slaughter LW (kg)	29.0	26.5	28.0	28.6	26.9	0.6	0.06
EBW (kg)	23.4	22.1	22.8	23.6	21.9	ns	ns
CW (kg)	12.2	11.4	11.6	12.4	11.4	ns	ns
C Score	8.2	7.0	8.8^{a}	7.5^{ab}	6.7^{b}	0.03	0.006
Fat score	6.7	6.5	6.1	7.1	6.5	ns	ns
Head (kg)	1.7	1.7	1.7	1.7	1.7	ns	ns
Feet (kg)	0.7	0.7	0.7	0.8	0.7	ns	ns
Red organs	1.2	1.2	1.3^{a}	1.3^{a}	1.0^{b}	ns	0.001
Gut	2.3	2.2	2.3^{a}	2.3^{a}	2.1^{b}	ns	0.06

$^{\mu}$BB: Barbarine; SS: Sicilo-Sarde; $^{\mu\mu}$GB: Green barley pasture; RG: ray grass pasture; FL: Feed lot; Means in the same line with different alphabets (a, b, c) are significantly different ($p<0.05$); ns: not significant ($p>0.05$).

As proportions of the carcass, joints had similar values for all groups (38.5, 21.1, 13.9, 8.5, 9.7 and 8.3 for leg, shoulder, thoracic region, lumbar regions, flank and neck, respectively). This results in constancy of joint proportions in the carcass concord with the anatomical harmony established by Boccard and Dumont (1960) and confirmed by several authors (Sents et al., 1982; Atti et al., 2003; Karim et al., 2007). So, neither feeding system nor breed affect the proportion of first category joints.

Lambs from BB breed had more fat proportion and less muscle and bone ($p<0.01$) than those from SS one (Table 3). The lean and bone weights were affected neither by the feeding system nor by the breed. Bone is a tissue with early development in all-animal species and does not depend on regimen at older ages (Wallace, 1948; Atti et al., 2003). Grass (GB and GR) regimens resulted in a decrease in the fat tissue weight and proportion (18 vs. 26%) and an increase in muscle proportion ($p<0.01$). So, carcasses of grazing grass lambs were leaner than carcasses produced on hay and concentrate diets. Fat mass of FL lambs represented 140% of fat mass recorded with grazing animals. This tendency confirmed our anterior results (Atti and Abdouli, 2001). Hence and for the similar amount of muscle, the mean fat weight of grazing sheep represented 0.7 times of fat weight for FL groups; which is equivalent to a reduction of 80 g of fat per kg of carcass. Increased fat deposition in concentrate fed animals compared to those on pasture has been reported by several authors (Borton et al., 2005; Nuerberg et al., 2008).

Table 3. Mean weights of tissue (g) and as a percentage of whole carcass

	Breed$^{\mu}$ (Br)		Feeding system$^{\mu\mu}$ (FS)			p value	
	BB	SS	GB	GR	FL	Br	FS
Bone (g)	1179	1174	1211	1219	1102	0.9	0.1
Bone (%)	21.6	23.2	23.3^{a}	22.7^{a}	21.1^{b}	0.01	0.01
Muscle (g)	2926	2889	2956	3115	2659	0.8	0.08
Muscle (%)	53.1	56.9	56.7^{a}	57.6^{a}	50.7^{b}	0.002	0.001
Fat (g)	1314	930	967^{b}	1019^{b}	1394^{a}	0.01	0.03
Fat (%)	22.9	17.9	17.7^{b}	17.7^{b}	26.0^{a}	0.002	0.001

$^{\mu}$ BB: Barbarine; SS: Sicilo-Sarde; $^{\mu\mu}$GB: Green barley pasture; RG: ray grass pasture; FL: Feed lot; Means in the same line with different alphabets (a, b, c) are significantly different ($p<0.05$). ns: not significant ($p>0.05$)

CONCLUSION

The results of this study showed that ryegrass forage production was higher than barley one suggesting the extension of humid and sub-humid South-Mediterranean areas reserved to ryegrass culture. Lamb's growth was higher for grazing system than feedlot diet (hay and concentrate). So, fattening lambs on cultivated prairie in sub humid areas with a relatively high stocking rate (60 lambs/ha) and low concentrate supply (350 g/head/day) is interesting. This management system can be recommended to reduce the concentrate feeds and feeding cost and, hence, increase farmer's income. Moreover, grazing system resulted in a similar amount of muscle with only 0.7 fat mass of that produced by lambs reared in FL. So, grazing grass could be a simple feeding strategy to naturally manipulate dietetic characteristics

of sheep products; the carcasses of grassland lambs, being leaner, will be demanded by the consumers and may be recommended by the nutritionists.

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ANALYSIS OF CONFORMATION TRAITS OF THE SLOVENIAN COLD-BLOODED HORSE

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Original scientific paper

SUMMARY

Slovenian Cold-Blooded horse is an autochthonous horse breed in Slovenia, traditionally reared in the North-Eastern and Northern parts of Slovenia. Today the breed is widespread all over the country. Breeding program for the Slovenian Cold-Blooded horse was accepted 2005 when the Association of Slovenian Cold-Blooded Horse Breeders was established, too. The aim of the study was to analyse conformation and gaits traits of the Slovenian Cold-Blooded horse. Likewise, we tried to evaluate fixed effect, affecting the included traits. Data were collected during the classifications of Slovenian Cold-Blooded horse performed from 1996 to 2011. In this study, 1920 horses were included, 52 of which were stallions and 1868 mares. The scoring system included 8 measured and 10 scored traits. Data were analysed by GLM procedure of statistical package SAS/STAT considering sex, age at scoring and birth year as fixed effects. Stallions of Slovenian Cold-Blooded horses were on the average 152.4±0.56 cm high at withers (stick), while mares were 151.22±0.11 cm. Body length (stallions 163.95 ± 1.48 cm; mares 164.28±0.17 cm) was on the average larger than the height at wither thus indicating the rectangular body frame.

Key-words: Slovenian Cold-Blooded horse, conformation trait, body measurements, gaits

INTRODUCTION

Slovenian Cold-Blooded horse is an autochthonous horse breed in Slovenia traditionally reared in the North-Eastern and Northern parts of Slovenia. Today the breed is widespread also throughout Slovenia. In the year 2014, the estimated population size was around 3000 Slovenian Cold-Blooded horses of all categories (Veterinary faculty, Institute for breeding and equine health). The breed was formed in the past based on local populations of cold-blooded horses like was Posavinja horse, Bohinj horse, Kobarid horse, Međimurje horse, Alpine horse and many others. Most of them are extinct today or were merged in the population of Slovenian Cold-blooded horse. Local mares were improved with cold-blooded stallions of the Belgian horse as well as Noric horse. Breeding program (Rus, 2010) was accepted in the 2005 for the first time, when the Breeders Association of Slovenian Cold-Blooded Horse was established, too. The Slovenian Cold-blooded horse is a medium body framed horse with a large head and convex nose profile, with a moderately long neck. The body is large, deep, wide and compact. The croup is low and often split up. The horse's legs are strong and have good gaits (Rus, 2010). The aim of the study was to analyse conformation traits in the Slovenian Cold-Blooded horse and to evaluate fixed effects affecting those traits.

MATERIAL AND METHODS

Data were collected during the Slovenian Cold-Blooded horse classifications, taken after the horses achieved breeding maturity around 30 months of age. Classification was performed for males and females prior to records in the Slovenian Cold-Blooded horse Stud book by only one classifier. The scoring system included 8 measured and 10 subjectively scored (1 to 10 point scale) traits.

This study includes only data of Slovenian Cold-Blooded horses, aged 30 to 60 months in the scoring day. Data of younger and older horses as well as outliers were excluded from further analyses. Data of conforma-

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tion traits used in this study belong to 1920 horses of Slovenian Cold-Blooded horse population, where 52 stallions and 1868 mares were born during the period 1999-2007. Likewise, four body indexes were computed from measured traits as follows, cannon bone circumference/ height at wither (ICBC), chest width/ height at wither (ICW), chest depth/ height at wither (ICD) and croup width/ height at wither (ICrW).

Data were analysed by GLM procedure of statistical package SAS/STAT (SAS User's Guide, 2001) considering sex, age at scoring and birth year as fixed effects (Model 1). The horses were divided into two groups by the age at scoring day, from 30 to 42 months (N=1444), and from 43 to 60 months of age (N=476).

$$y_{ijk} = \mu + S_i + A_j + B_k + e_{ijk} \qquad \text{Model 1}$$

where:

y_{ijk}–conformation trait; S_i–sex; i=1, 2; A_j–age at scoring day; j=1, 2; B_k–birth year; k=1,…11; e_{ijk}-residual

RESULTS AND DISCUSSION

Significant differences between stallions and mares of the Slovenian Cold-Blooded horse were recorded at three measured, four scored and two body indexes (Table 1). Stallions were higher in wither (stick measured) (152.40±0.56 cm), had larger cannon bone circumference (24.72±0.17 cm) and shorter chest girth (191.8±1.34 cm) than mares (151.22±0.11 cm, 22.62±0.03 cm, 196.43±0.26 cm), respectively. The average values for the measured traits especially height at wither measured by stick (152.40±0.56 cm) has shown that the Slovenian Cold-Blooded horse is a draft horse with smaller body frame compared to the well-known Noric draft horse (156–162 cm) (Druml, 2006). On the other side, stallions and mares of the Slovenian Cold-Blooded horse were higher at wither compared to stallions (142.8±0.56 cm) and mares (142.0±0.24 cm) of the Slovenian population of Posavje horse (Simčič et al., 2012). Dario et al. (2006) found lower chest girth (187.89±0.67 cm) in Murgese stallions as well as Simčič et al. (2012) in Posavje stallions (188.2±1.57 cm) compared to Slovenian Cold-Blooded horse.

However, height at wither measured by stick were not in accordance with a breeding goal of the Slovenian Cold-Blooded horse, which assumed 155 cm (148-160 cm) for stallions and 150 cm (146-158 cm) for mares (Rus, 2005).

The average value for each scored trait means also the average value of the population, regarding to breeding goals in the breeding program (Rus, 2010). The explanation what exactly each point meant is very subjective, while 1 means that the trait was the worst expressed, and 10 that the trait was the most expressed (Simčič et al., 2012).

Significant differences between stallions and mares existed also in the scored traits. Stallions expressed breed type significantly better (7.49±0.09) than mares (7.29±0.02). Also head, neck and rear legs were significantly better scored in stallions (7.43±0.09, 7.52±0.11, 6.59±0.09) compared to mares (7.06±0.02, 7.28±0.02, 6.29±0.02). No significant differences were found out between stallions and mares in gaits correctness as well as in gaits efficiency. The differences between sex were significant in the two (ICBC, ICrW) of four body indexes. ICBC and ICrW were higher in stallions (16.26±0.10% and 37.54±0.59%) compared to the mares (14.96±0.02% and 39.04±0.05%). The scored traits had higher scores at stallions with the exception of gaits efficiency and correctness where a more intensive selection is seen in stallions compared to mares.

Body indexes showed interdependence among the measured traits, compliance of the body and coherence with the breed standards (Ivanković, 2004). On the base of body index (chest depth/height at wither) we could determine which group, oriental (hot-blooded) (45.0-46.5%), half-blooded (warm-blooded) (46.5–48.5%), or cold-blooded (>50.0%), a horse or a population belong to (Brinzej, 1980 cit. by Ivanković, 2004). Consequently, the Slovenian Cold-Blooded horse belongs to half-blooded (warm-blooded) group with the index chest depth/ height at wither of stallions 47.22±0.66% and mares 47.75±0.06%. The Slovenian population of Posavian horse were also arranged in the same half-blooded group, where the index chest depth/height at wither of stallions were 47.5±0.41% and of mares 47.5±0.13% (Simčič et al., 2012).

Table 1. Least square means (LSM), standard errors (SE) and p-values of included effects

	Stallions			Mares			p-values		
	n	LSM	SE	n	LSM	SE	Sex	Age	Birth year
Measured traits (cm)									
Height at wither – stick (WH)	52	152.4	0.559	1868	151.22	0.108	*	**	***
Chest girth	50	191.8	1.339	1859	196.43	0.255	**	n.s.	***
Cannon bone circumference (CBC)	50	24.72	0.168	1822	22.62	0.032	***	*	***
Chest depth (CD)	10	72.07	1.125	1845	72.22	0.097	n.s.	n.s.	***
Croup height	13	154.09	1.591	1861	153.16	0.157	n.s.	n.s.	***
Chest width (CW)	10	49.31	1.369	1844	49.21	0.118	n.s.	n.s.	***
Croup width (CrW)	10	57.27	0.939	1843	59.03	0.081	n.s.	*	***
Body length	17	163.95	1.481	1835	164.28	0.167	n.s.	n.s.	***
Scored traits (1-10)									
Breed type	52	7.49	0.086	1841	7.29	0.017	*	*	***
Head	49	7.43	0.088	1777	7.06	0.017	***	n.s.	***
Neck	49	7.52	0.105	1777	7.28	0.02	*	n.s.	***
Front part	49	7.7	0.106	1776	7.52	0.021	n.s.	n.s.	***
Middle part	49	7.26	0.098	1775	7.12	0.019	n.s.	n.s.	***
Rear part	49	7.53	0.104	1775	7.37	0.02	n.s.	n.s.	0.004
Front legs	49	6.39	0.102	1773	6.43	0.02	n.s.	n.s.	***
Rear legs	49	6.59	0.091	1770	6.29	0.018	**	n.s.	***
Gaits correctness	34	6.41	0.109	1739	6.46	0.018	n.s.	n.s.	***
Gaits efficiency	34	6.91	0.101	1729	7.05	0.017	n.s.	n.s.	***
Total score of scored traits	35	70.15	0.975	1762	69.05	0.161	n.s.	n.s.	***
Body indexes (%)									
ICBC = (CBC/WH)*100	50	16.26	0.096	1821	14.96	0.019	***	n.s.	***
ICW = (CW/WH) *100	10	32.34	0.87	1844	32.53	0.075	n.s.	n.s.	***
ICD = (CD/WH) *100	10	47.22	0.663	1845	47.75	0.057	n.s.	*	***
ICrW = (CrW/WH) *100	10	37.54	0.591	1843	39.04	0.051	*	**	***

LSM–least square means, SE–standard errors, *-p<0.05, **-p<0.01, ***-p<0.001, n.s.-p>0.05

Differences in conformation traits between younger (30-42 months) and older (43-60 months) groups of horses were significant in height at wither, cannon bone circumference and croup width (Table 1). On the other side, differences in conformation traits among the birth years were significant for all the included traits. Within the effect of birth year, the sire effect could be expressed, because each sire did not have offspring in all the studied years (Table 1).

CONCLUSION

Stallions of Slovenian Cold-Blooded horses had on the average 152.4±0.56 cm height at withers (stick), while mares had 151.22±0.11 cm. Body length (stallions 163.95±1.48 cm; mares 164.28±0.17 cm) was on the average larger than the height at wither thus indicating the rectangular body frame. However, in this study it was realised for the first time, in the case of Slovenian Cold-Blooded horses, that at least fixed effects like sex, age and birth year need to be considered in the conformation traits evaluation since some traits were significantly affected by them. The total score of the scored traits is regarding to actual Breeding program scored immediately after the end of the scoring procedure. Such a scoring system does not allow to consider environmental effects, which in turns, could cause mistakes in the horse classification. To improve the classification of horses based on the conformation traits, estimation of breeding values need to be implemented. However, an analysis of conformation traits in Slovenian Cold-Blooded horse was the first step prior to the estimation of genetic variances of measured and scored traits, as well as body indexes which are basis for the evaluation of breeding values.

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INDIVIDUALITY OF CENTRE OF BODY MOVEMENT AT WALK AND TROT WITHIN THE HAFLINGER BREED

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Original scientific paper

SUMMARY

Kinematic measurements of fourteen Haflinger horses without lameness, walking and trotting on a treadmill were taken to document the location of the centre of the body (CB), defined as the centre between markers on the head, on the withers, on the sacral bone and on the lateral wall of all four hooves in relation to the sacral bone marker. During walk and trot, there are three dimensional CB position (x: forward-backward, y: side-to-side, and z: up and down). For each horse minimum of eight motion cycles were considered in walk as well as in trot. For all three axes, mean CB location, its standard deviation and its 95% confidence interval (CI) were calculated. For statistical analysis, Shapiro-Wilk test and Spearman's correlation test were carried out. Mean body mass was 463±42 kg, CI (439, 487); mean height at the withers was 131±5 cm, CI (128, 134); mean height at the sacrum was 128 ± 2 cm, CI (127, 130). Mean CBx was in front of the sacrum (walk 74±2 cm, CI (72, 75); trot 73±2 cm, CI (72, 74); walk vs trot p=0.008). Mean CBz was below the sacrum (-71±2 cm, CI (-73, -70) in walk; -69 ± 2 cm, CI (-70, -68) in trot; walk vs trot p=0.001). Positive correlations were found out between MeanCBx and trunk length in walk and trot, which could highlight the biomechanical importance of the trunk as it plays a crucial role in deceleration and acceleration. The analysis of the body location centre may be used to identify differences between horses of the same breed, and thus support evaluation of the quality of the horse during locomotion.

Key-words: motion analysis, conformation, centre of body, walk, trot, Haflinger horses

INTRODUCTION

In the horse, conformation traits include body measures as well as locomotion characteristics of limbs and different parts of the trunk. Such objective locomotion traits have been documented in a variety of horse breeds, and in the present study a single synthetic parameter, the centre of the body is documented, containing the information of all limbs and the head and trunk of the horse. Specifically, the Haflinger breed was investigated in the present study.

These horses have been developed from multipurpose, sturdy mountain horses, used mainly for carrying loads and driving, to a calm, robust, all-round leisure horse used mainly for riding and driving over the last century. In the Italian Haflinger breed a study was carried out to estimate variance components and breeding values for general conformation, and to develop an aggregate selection index including three conformation traits (breed type, general harmony and gaits) and one body measurement (height at the withers) based on more than 4500 records. Based on this the authors suggested a total merit index for breed selection process which has been approved by the Italian Haflinger Association (Miglior et al., 1998). Samore et al. (1997) estimated genetic parameters and breeding values for linear type traits in the Italian Haflinger population. They included the withers prominence and a tight upper line as being desired in their breeding program, whereas the correlation between these traits was 0.66.

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Three traits for the croup were also investigated, and while there was a strong correlation between width and length, the slope of the croup was only weakly correlated. The introduction of these objective measurements of horse's conformation, breeding values and gait characteristics offers the possibility of improving the classification quantitatively and qualitatively. This gives an additional system of measurements to the time-proven system of subjective evaluation by experts. However, the movement quality of the horse, probably its most important quality is not objectively assessed. A computerised motion analysis system enables such a locomotion parameters measurement in 3D (van Weeren, 2012). Therefore, the aim of the current study was to establish a single objective synthetic parameter, based on the locomotion of limbs, head and trunk, for the horse at walk and trot.

MATERIAL AND METHODS

Fourteen horses without clinical sign of back pain and lameness were used in this study (14 Haflingers mares, mean age was 8±3 years, CI (6, 9), range 4-14 years; mean body mass was 463±42 kg, CI (439, 487), range 396-526 kg; mean height at the withers was 131±5 cm, CI (128, 134), range 125-145 cm, mean height at the sacrum was 128±2 cm, CI (127, 130), range 124-135 cm and mean trunk length (considered as the distance between withers and sacral markers) was 76±4 cm, CI (74, 79), range 67-185 cm). Horses were warmed up and accustomed to the experimental set up on the treadmill. The following procedure was applied to reach the optimal measurement speed; the horses were walked and trotted on the treadmill until they were at ease with the situation, indicated by a rhythmic pattern of movement and the absence of overt signs of distress. The speed of the treadmill was gradually increased and the gait of the horse observed at which speed the horse showed the most rhythmical movement pattern with all strides at similar length and height, and then the horse was measured at that speed (Peham et al., 1998).

Seven reflective skin markers were positioned on each horse using adhesive tape; one on the forehead, one on the highest point of the withers, on the sacrum and lateral side of each hoof to identify motion cycles. Three-dimensional kinematic data in walk and trot were collected using ten high-speed cameras recording at 120 Hz. Three-dimensional coordinates of each marker during the time course of each experiment were calculated from the data using kinematic software. These time series were then smoothed by use of a Butterworth low-pass filtered (cut-off frequency, 10 Hz). The data was split to motion cycles by a custom-made MATLAB script starting with the left forelimb stance phase and the duration of the motion cycle was calculated. For each horse eight motion cycles were considered in walk as well as in trot. For the centre of body (CB) three dimensional marker positions CBx (forward-backward direction), CBy (side-to-side direction) and CBz (up-and-down direction) (as shown in Figure 1) were summed up and divided by the number of markers and normalized for the local position with the sacrum marker.

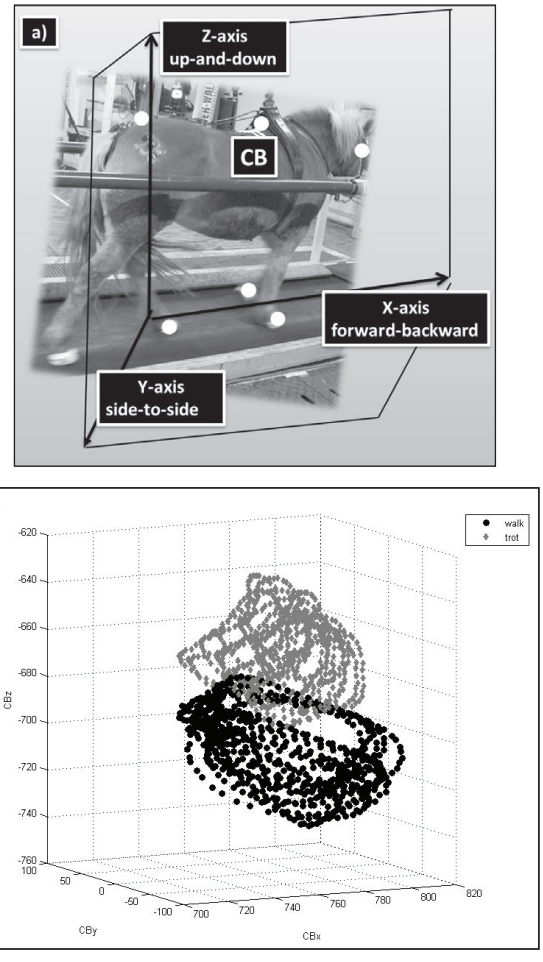

Figure 1. a) Horse with the reflective markers used for tracing of the movement of the limbs (markers placed on the hooves), the head (marker placed on the forehead), and the trunk (one marker was placed on the mid-thoracic area and one marker on the sacrum). The horse was walking on the treadmill. The right-handed coordinate system used is also shown, and the centre of body (CB) as determined as the centre of all of the markers. b) The position of the CB during 12 motion cycles in relation to the sacral bone marker in Horse 12 during walk (black circles) and during trot (grey diamonds). The axes of the coordinate system are: forward-backward (CBx), side-to-side (CBy) and up-and-down (CBz)

Statistical analyses were done in SPSS. Normality of distributions was tested with Shapiro-Wilk tests. The analysis of parametric data consisted of an independent T-test and the analysis of non-parametric data consisted of a Mann-Whitney U test. Correlations between the different conformation traits and centre of body parameters were sought with Spearman's correlation test. Alpha level (α) level of significance was set as 0.05. The results are expressed as mean±S.D. (standard deviation) CI (95% confidence interval for mean).

RESULTS AND DISCUSSION

The results of the present study are presented in Table 1, Table 2 and in Figure 2.

Table 1. Results of the position of the CB during walk and trot and statistically significant differences between walk and trot

		MeanCBx		MeanCBy		MeanCBz		Forward-backward movement		Side-to-side movement		Up-and down movement	
cm								SdCBx		SdCBy		SdCBz	
		walk	trot	walk	trot	walk	trot	walk	trot	walk	trot	walk	trot
Mean		74	73	0.6	0.5	-71	-69	0.6	0.8	2.6	2.0	0.7	0.9
Standard deviation		2	2	3.3	4.1	2	2	0.4	0.9	3.3	1.1	0.4	0.9
95% CI for Mean	Lower Bound	72	72	-1.3	-1.8	-73	-70	0.4	0.3	0.7	1.4	0.5	0.4
	Upper Bound	75	74	2.4	2.9	-70	-68	0.8	1.3	4.5	2.7	0.9	1.4
Minimum		70	68	-2.6	-7.3	-75	-73	0.2	0.2	0.5	0.6	0.4	0.2
Maximum		77	77	6.1	6.1	-66	-66	1.3	3.8	13.7	5.2	1.6	3.3
p		0.008				0.001							

Statistical significant correlation coefficients were found out to be from moderate to very strong positive correlations (Table 2).

Table 2. Statistically significant correlations between the body measures and centre of body values

		Spearman's rho	P
Heigth at the sacrum	Heigth at the withers	0.80	0.001
Trunk length	Body mass	0.72	0.004
	Heigth at the withers	0.59	0.026
MeanCBx(walk)	Body mass	0.73	0.003
	Heigth at the withers	0.60	0.022
	Trunk length	0.86	0.000
MeanCBx(trot)	Body mass	0.57	0.034
	Trunk length	0.65	0.012
	MeanCBx(walk)	0.78	0.001
MeanCBy(trot)	MeanCBy(walk)	0.75	0.002
SdCBx(trot)	SdCBx(walk)	0.71	0.004

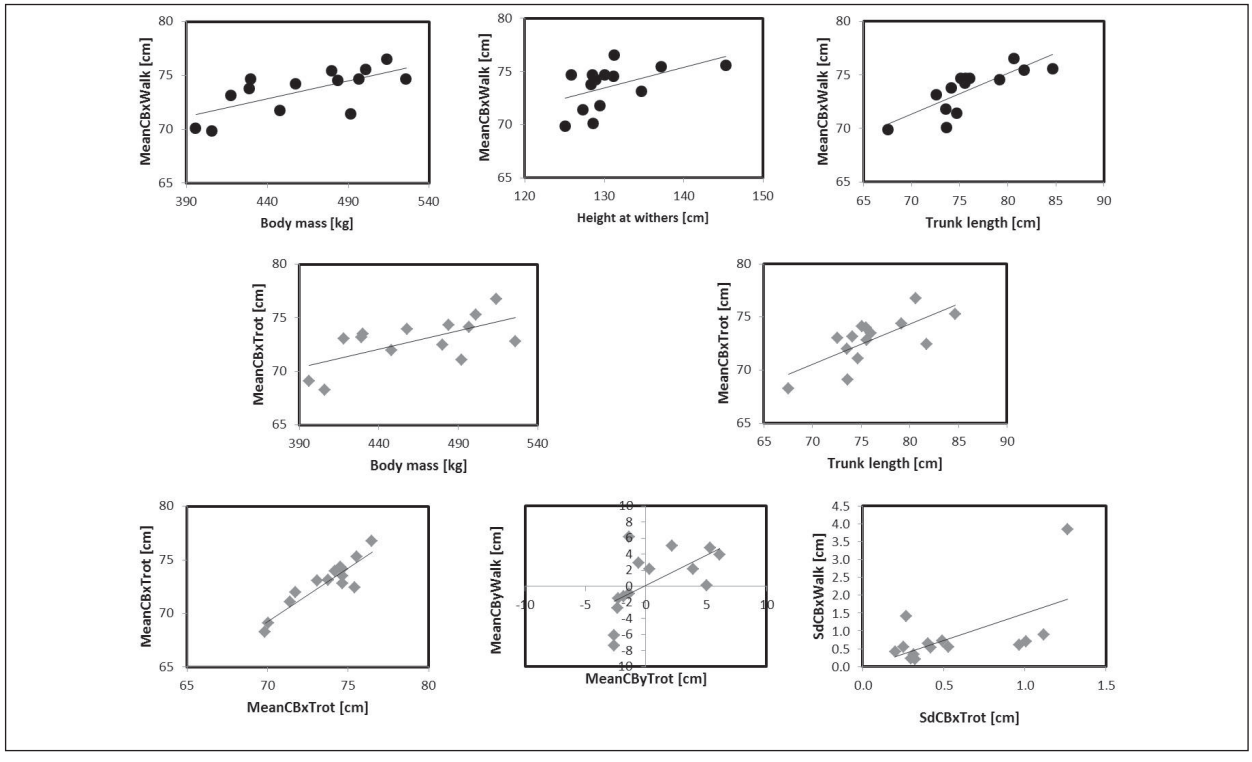

Figure 2. Scatter plot of statistically significant correlations showing all horses (n=14)

The present study was out to analyse within breed characteristics in order to highlight its potential use for selection within a breed. While the Haflinger breed developed originally as a working horse, now the use of these horses turned to leisure activity. Therefore, the conformation of these horses also needed to be adapted to this kind of use. These 14 mares show that the effect of this process is still visible and also measurable; as the current study showed highly variability between the horses' body measures. Haflingers used in this study were of a variety of shapes and body masses – within the breed specifications – e.g. body measures such as the height at the sacrum and the height at the withers are positively highly significantly correlated. The same is also true for the trunk length and the horse's height at its withers. In the present study, average mares of the Haflinger breed were used. They did not have an excellent breeding pedigree, conformation or gait, and therefore the method described could not be evaluated for its use in determining such breeding characteristics. Had such a population of Haflinger mares been used, the use of CB for determining excellence might have been tested.

In the previous studies (Nauwelaerts et al., 2009, 2013), the centre of body mass of horses was studied using a variety of methods and it is presumably closely related to the centre of body. However, body mass and its centre during locomotion are difficult to determine (Nauwelaerts et al., 2009) as segmental masses and segmental shapes change during motion. In the present study the centre of body was calculated relatively easily and accurately with the standard seven marker set-up used for many kinematic studies (Clayton et al., 2013). It is also important to consider during these calculations that the three top line markers and four hoof markers were used, giving more relevance to the relatively light limbs, and therefore the gait over the posture of the head and the trunk. This could easily be balanced out by adding one more marker along the top line. With the CB location of these horses, we are getting a general representative value of their overall movement picture, but not necessarily of the forces required or exerted on the body. In the breeding selection process the movement picture is one of the most important selection criterions. So, with the current method CB offers a way to objectively measure the overall movement picture of these horses and with these delivering also closer results to the subjective breeding evaluation process which is regularly done by breeding experts. The normalization of the data was done in relation to the sacral makers while it was the most stable of the markers in all directions including both gaits. Is is very suitable to document the limb movement in relation to the body. However, an important characteristic of gaits graded highly for the Haflinger breed is the vertical movement of the body. This characteristic is not represented by the CB, and probably limits its use as a single quality criterion for horse locomotion.

In the present study, there was a significant difference in the mean positions along the forward-backward axis (MeanCBx) and the mean positions along the up-and-down axis (MeanCBy) between walk and trot, with the walk showing a CB located more forward and lower in the body. However, there were no significant differences found between the forward–backward movement (SdCBx), side-to-side (SdCBy) and up-and-down

(SdCBz) centre of body movements between walk and trot, showing that the horses moved with similar stability in both gaits. MeanCBx marker position was in correlated with body mass at walk and trot, and also with height at the withers and trunk length, similarly in trot correlated with body mass and trunk length. The lack of correlation between MeanCBx and height at the withers at trot indicates that in this gait the variation in trot stride associated X axis positions of the relevant markers is larger between individuals than the variation of height at the withers – i.e. one small horse can throw its legs relatively further forward than a larger horse. This effect is gait specific; despite walk and trot MeanCBx marker positions of the centre of body were strongly positively correlated. This also supports the use of the trot as the main gait for breeding selection. Lateral position of the CB as indicated by MeanCBy was closely related between walk and trot but not to any of the body measurements of the horses. Also, this position was almost exactly in the midline of the horses, an indication that they were sound, and not favouring one side. The height of the body centre (MeanCBz) was not correlated to any of other body measures, nor was it correlated to either MeanCBy or MeanCBx. This was probably due to the rather smaller differences in height at the withers in the population studied, than in the other parameters.

The forward – backward movement of the body centre during motion, as indicated by the (SdCBx) was highly correlated between walk and trot, despite the difference in the gait characteristics, and the reported differences in back movement, with shortening of the back during the stance and extension of the back during the suspension phases in walk (Johnston et al., 2002).

No correlation was found out for side-to-side centre of body movements (SdCBy) between walk and trot, most likely due to the much larger lateral trunk movement at walk, which is a gait with single, two, and three limb stance phases occurring, and therefore asymmetric body positions during parts of the movement cycles (Dunbar et al., 2008). We have found out positive correlations between MeanCBx and trunk length in walk and trot, which could highlight the biomechanical importance of the trunk as it plays a crucial role in deceleration and acceleration. Similarly Williams et al. (2009) showed based on their pitch-avoidance model, that polo ponies during rapid deceleration moved their centre of mass towards the hip, which also increased the length between the shoulder joint and the centre of mass.

CONCLUSION

Analysing the body centre in 14 Haflinger mares in the current study it was shown that distinction was allowed between individual's gait characteristics, and their relation to commonly analysed body measures. Using this parameter, trot as a main breed selection criterion for the Haflinger breed was confirmed. Also, the soundness of the horse can also be easily determined using the same analysis.

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CHEMICAL COMPOSITION AND *IN VITRO* FERMENTATION OF SILAGES FROM DIFFERENT SORGHUM HYBRIDS CULTIVATED IN THREE PILOT FARMS

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Preliminary communication

SUMMARY

This experiment compared silages obtained from ten hybrids of sorghum grown in three farms of the Po Valley, in terms of in vitro degradability, gas production (GP), and energy value. Hybrids were sown on 30 experimental plots (three plots per each hybrid), harvested at late-milk stage of maturity and ensiled for 60 d into 30 mini-silos (3 silos×10 hybrids). After ensiling, silages were analyzed for proximate composition, pH, ammonia N, and fermentation acid profile. Degradability of NDF (NDFd) and of true dry matter (TDMd) was determined after 48 h of incubation using sequentially Daisy^{II} incubator and Ankom^{220} Fibre Analyzer. Two incubation runs (at 48 h) were carried out to evaluate in vitro GP of silages, and to estimate their energy content. All data were submitted to ANOVA considering "hybrid" and "farm" as sources of variation. The interaction between hybrid and farm was never significant and it was excluded from the statistical model. The contents of dry matter and NDF of silages were influenced by hybrid and farm (P<0.001). In contrast, the percentage of non-structural carbohydrates of silages was affected by hybrid (P<0.001) but not by farm. All chemical parameters were significantly affected by hybrid (P<0.01) and, except NCS, by farm (P<0.05). In vitro parameters (NDFd after 48 h and GP at 24 and 48 h of incubation) were influenced by hybrid and farm (P<0.001 and P<0.01), respectively. Among hybrids and farms, large differences (P<0.001) were also found out as regard to net energy content of sorghum silages. Because of this large variability, sorghum silages can be included successfully in ruminant diets considering the peculiarities of each hybrid with respect to the energy requirements of dairy cows.

Key-words: Sorghum hybrids, Sorghum silage, in vitro degradability, in vitro gas production, dairy cows

INTRODUCTION

Corn silage is the main ingredient of diets fed to dairy cows in farms of the Po plain (Italy), with the exception of those producing milk processed into some Protected Denomination of Origin cheeses. In the last years, corn production in the Po Plain has been complicated by the spread of mycotoxin contamination that reduces quantity and quality of corn silage and precludes its utilization for lactating cows. These concerns have given rise to the interest for alternative forages, which might partially or totally replace corn silage in diets. In this regard, sorghum (*Sorghum vulgare*) would represent an interesting crop, as plants adapt to different soils and are productive in conditions of water deficit (Sanchez et al., 2002). However, data about nutritional and energy value of sorghum hybrids commercially available are scarce. This experiment was aimed at comparing silages obtained from different sorghum hybrids grown in three farms of the Po plain, in terms of chemical composition, *in vitro* degradability, gas production (GP), and energy value.

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MATERIAL AND METHODS

Ten commercial hybrids of *Sorghum vulgare* spp., traded by four Italian seed companies, were analyzed (see Table 1). Hybrids were grown in three pilot farms of the Veneto Agricoltura Agency, Farm 1 located in the province of Treviso (Mogliano Veneto, latitude 45.3°N, longitude 12.1°E; 8 m a.s.l.), Farm 2 in the province of Rovigo (Ceregnano, latitude 45.0°N, longitude 11.9°E; 5 m a.s.l.), and Farm 3 in the province of Venice (Vallevecchia, latitude 45.6°N, longitude 12.9°E; 0 m a.s.l.). In all farms, sorghums were sown in 30 adjacent plots (three plots per each hybrid), with an area of about 28 m^2 each, at the first decade of June 2014. No fertilizers were applied; urea (100 kg/ha) was distributed at post-emergence phase in Farm 1, at pre-emergence phase in Farm 2 and 3. Herbicides were also distributed at post-emergence phase in Farm 2 and 3. Sorghums were harvested at the third decade of October 2014 in Farm 1, at the first decade of October 2014 in Farm 2 and 3, in order to collect plants being at late-milk stage of maturity. After harvest, three aliquots of chopped forage (10 kg each) were prepared for each hybrid, as representative sample of three experimental plots, homogeneously mixed, and ensiled into 30 laboratory mini-silos (3 silos per each hybrid) with a capacity of 20 l. The mini-silos were hermetically closed and stored for 60 d at 24±3°C. On the mini-silos opening, the upper layer (10-15 cm) of silage was discarded, to limit risk of anomalous fermentation. After that, two aliquots (about 1.5 kg each) were prepared for each sorghum silage, as representative sample of the three mini-silos. The same procedures were followed in all farms. The first aliquot was used to determine the proximate composition, pH, ammonia N ($N\text{-}NH_3$) content, and fermentation acid profile of silages. The analysis of proximate composition was conducted according to AOAC (2012). Values of pH were determined by a potentiometer equipped with a specific electrode (pH meter BASIC 20, Crison Instruments, Alella, Spain). The content of $N\text{-}NH_3$ was determined using a colorimetric method (Cataldo et al., 1975). Fermentation acids were measured using a Thermo Finnigan Spectra System AS3000 auto-sampler (Thermo Electron Corporation, Waltham, MA, USA), equipped with a H_2SO_4 0.0025 N Bio-Rad HPX-87H column (Bio-Rad Laboratories, Richmond, CA, USA). The second aliquot was used for *in vitro* tests. A pooled sample (about 500 g as fed) was dried in a forced-air oven at 60°C for 48 h for each silage to determine dry matter (DM) content, and then ground to 1 mm. Degradability of NDF (NDFd) and of true DM (TDMd) was determined after 48 h of incubation using sequentially Daisy^{II} incubator and Ankom^{220} Fibre Analyzer (Ankom Technology, NY, USA). Each filter bag was filled with 0.250±0.0010 g of feed sample (Cattani et al., 2009) and included in glass jars filled with rumen fluid and buffer solution (ratio 1:2). Rumen fluid was collected by an esophageal probe (Tagliapietra et al., 2012) from three intact dry Holstein-Friesian cows fed hay *ad libitum* and 2.5 kg/d of concentrates. Buffer solution was prepared according to Menke and Steingass (1988). The following experiment design was used: 1 run×10 hybrids×3 farms×2 replications, plus 8 blanks (bags without feed sample; 2 per each glass jar). Gas production was measured using Ankom^{RF} gas production (GP) system (Ankom Technology, NY, USA). This system is a kit of bottles (310 ml) equipped with a pressure detector and wireless connected to a PC. Each bottle was filled with feed sample (0.500±0.0010 g), 25 ml of rumen fluid, and 50 ml of buffer solution (ratio 1:2), collected as previously described. After filling, bottles were incubated at 39±0.4°C for 48 h and vented at 3.4 kPa, to avoid overpressure conditions (Cattani et al., 2014). Due to of the limited number of bottles, each silage was analyzed in two replicates, separately incubated in two successive runs. The resulting experimental design was: 2 runs×10 hybrids×3 farms, plus 10 blanks (bottles without feed sample; 5 per each run), giving a total of 70 bottles incubated (35 per each run). Metabolizable energy content (ME; MJ/kg DM) and milk forage unit (MFU; n°/kg DM) of silages were estimated according to Menke and Steingass (1988). Data of silage composition and of the various *in vitro* parameters were analyzed by the PROC GLM of SAS (SAS Institute Inc., Cary, NC, USA release 9.1), considering effect of hybrid and farm as sources of variation. The interaction hybrid × farm was excluded from the statistical model as it was never significant.

RESULTS AND DISCUSSION

The DM content and chemical composition of silages were influenced by hybrid and farm (Table 1). Within hybrids, the DM content ranged from 24.2 to 33.3%, respectively ($P<0.001$). The NDF content showed a great variability, ranging from 57.0 to 74.0% on DM ($P<0.001$). In turns, also the percentage of NSC (non-structural carbohydrates) was highly variable, being included between 14.9 to 27.1% on DM ($P<0.001$). Silages obtained in Farm 2 showed, on the average, the greatest DM ($P<0.01$) and NDF contents ($P<0.001$), whereas the mean content of NSC did not differ on the three farms. Chemical composition of silages was included in the expected ranges (NRC, 2001), even if the NDF content resulted, on the average, greater compared to the literature (Colombo et al., 2007; Colombini et al., 2010). Hybrids did not differ for final pH, which was always in the range (3.48-4.50) indicating proper fermentations during ensiling (Gallardo and Gagiotti, 2004). In contrast, final pH of silages resulted different on the three farms ($P<0.05$). The occurrence of correct fermentations in all hybrids was confirmed by high production of lactate, being the prevalent fatty acid (FA; on the average 77.1% on the total FA), followed by acetate (on the average 21.3% on the total FA); propionate was present only in traces, whereas n-butyrate was never detectable by HPLC (not shown by data). The ratio between $N\text{-}NH_3$ and total N was influenced by hybrid and farm ($P<0.01$) but it was always lower than the threshold of 7, which indicates a proper preservation of silages (Romero, 2004).

Table 1. Dry matter (DM, %), chemical composition (% on DM), pH, proportion of ammonia N on total N ($N\text{-}NH_3/N$), lactate and acetate (% on total fatty acids) of different sorghum hybrids

	DM	CP	NDF	ADF	ADL	Ash	NSC	pH	$N\text{-}NH_3/N$	Lactate	Acetate
Hybrid											
Argensil	24.9	5.0	58.1	35.4	4.4	9.1	25.8	3.82	2.48	78.6	20.5
Argensor	25.1	5.0	60.4	37.0	5.6	7.7	24.5	3.82	2.27	79.5	19.4
Biomass Mix	26.6	4.4	59.2	36.9	4.8	8.1	26.5	3.87	2.66	75.7	22.5
Buffalo Grain	24.2	5.6	62.2	36.1	4.0	8.1	22.1	3.84	3.30	78.5	20.5
Bulldozer	27.8	3.2	74.0	47.6	6.6	6.3	14.9	3.87	2.84	70.4	25.2
Freya	33.3	4.0	66.2	42.2	7.0	7.1	20.5	3.97	2.26	76.3	21.8
Hannibal	28.3	3.8	66.8	41.7	5.9	6.8	20.9	3.91	2.48	77.3	21.4
Little Giant	25.0	6.7	57.0	33.8	4.1	9.9	24.0	3.86	2.70	82.5	16.3
Mix Asolo Tris	25.1	5.1	58.4	35.2	4.4	7.3	27.1	3.79	3.64	76.0	23.3
Nectar	25.3	4.2	61.9	41.8	5.3	7.6	24.5	3.75	2.63	76.6	22.3
Farm											
Farm 1	27.0	4.7	62.7	40.5	0.7	7.1	23.4	3.80	2.12	77.1	21.7
Farm 2	27.5	3.4	65.3	38.9	0.3	7.7	21.9	3.83	2.19	74.8	23.4
Farm 3	25.1	6.0	59.3	36.9	0.7	8.5	24.1	3.92	3.70	79.6	18.8
P value											
Hybrid	<0.001	<0.001	<0.001	<0.001	<0.001	<0.01	<0.001	ns	<0.01	ns	ns
Farm	<0.01	<0.001	<0.001	<0.05	<0.001	<0.01	ns	<0.05	<0.01	<0.05	<0.01

CP=crude protein; NDF=neutral detergent fibre; ADF=acid detergent fibre; ADL=acid detergent lignin; NSC=non-structural carbohydrate

Values of *in vitro* NDFd were influenced by hybrid and farm ($P<0.001$) showing a great variability, ranging among hybrids from 39.6 to 61.8% on NDF, and among farms from 47.9 to 55.3% on NDF (Table 2). A large variability of *in vitro* NDFd among different hybrids has been also reported by Di Marco et al. (2009). As the main result of large differences in chemical composition, the ten hybrids produced different amounts of gas, both at 24 (GP24) and at 48 h (GP48) of incubation ($P<0.01$). Likewise, hybrids differed largely ($P<0.01$) for energy content. In terms of ME, hybrids ranged from 7.4 to 9.5 MJ/kg DM; in terms of MFU silages ranged from 0.59 to 0.80 MFU/kg DM. These values agree with data reported by main feeding systems for ruminants (INRA, 1988; NRC, 2001). No differences among farms were found out for GP24 and, consequently, for energy content (ME and MFU) of sorghum silages ($P>0.05$). However, large differences were observed as regard to biomass yield provided by the different hybrids, varying from 9.7 to 17.3 t DM/ha ($P<0.001$), and obtained on the three farms varying from 9.6 to 14.4 t DM/ha ($P<0.001$). However, such yields are in line with ranges declared by seed companies. As consequence, also the MFU produced per hectare showed a great variability, ranging from 6731 to 11250 MFU/ha among hybrids ($P=0.001$), and from 6563 to 10040 MFU/ha among farms ($P<0.001$).

Table 2. *In vitro* degradability of NDF (NDFd, % on NDF) and of true dry matter (TDMd, % on DM), gas production at 24 h (GP24; ml/g DM) and 48 h of incubation (GP48; ml/g DM); metabolizable energy (ME; MJ/kg DM) and milk forage unit (MFU[1]; expressed as n°/kg DM); biomass yield (BY; t DM/ha) and milk forage unit produced per hectare (MFU[2]; expressed as n°/ha) of different sorghum hybrids

	NDFd	TDMd	GP24	GP48	ME	MFU[1]	BY	MFU[2]
Hybrid								
Argensil	51.9	74.3	231	289	8.9	0.74	10.6	7829
Argensor	48.7	71.4	213	276	8.4	0.69	11.5	7961
Biomass Mix	49.8	72.3	224	283	8.6	0.71	12.4	8772
Buffalo Grain	61.8	78.3	253	312	9.5	0.80	10.9	8647
Bulldozer	48.9	64.6	191	269	7.7	0.62	14.7	8882
Freya	39.6	62.8	179	244	7.4	0.59	11.5	6731
Hannibal	48.3	67.8	203	266	8.0	0.65	17.3	11250
Little Giant	53.4	75.4	219	271	8.7	0.72	9.7	7028
Mix Asolo Tris	55.6	76.1	233	295	9.1	0.76	12.6	9507
Nectar	49.1	70.9	222	286	8.6	0.70	15.8	11166
Farm								
Farm 1	48.6	69.7	222	280	8.7	0.68	14.1	10040
Farm 2	55.3	72.7	216	289	8.3	0.68	9.6	6563
Farm 3	47.9	71.4	212	268	8.4	0.69	14.4	9729
P value								
Hybrid	<0.001	<0.001	<0.01	<0.01	<0.01	<0.01	<0.001	0.001
Farm	<0.001	<0.05	ns	<0.05	ns	ns	<0.001	<0.001

CONCLUSION

The results of the study provide evidence that sorghum silages can largely differ for chemical composition, *in vitro* parameters, energy value, and biomass yield. Thus, cultivation and inclusion of this forage in dairy cow diets must inevitably consider peculiarities of each hybrid in relation to the energy requirements of animals (sorghum silages with lower energy content could be successfully used for animals with low energy requirements; i.e. heifers or dry cows). However, preliminary results presented in this paper should be validated by *in vivo* experiments.

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USE OF FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPY TO PREDICT VFA AND AMMONIA FROM IN VITRO RUMEN FERMENTATION

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Original scientific paper

SUMMARY

The aim of the present study was to develop a FTIR method to quantify amounts and proportions of volatile fatty acids (VFA) and ammonia nitrogen (N-NH_3) in fermentation fluids collected in vitro using innovative Bayesian models as chemometric technique. A set of 170 fluids, collected before and after 4 in vitro incubations of 8 diets in 5 replication plus 5 blanks, were analysed for VFA, N-NH_3 and scanned using the MilcoScan FT2 (Foss Electric, Hillerød, Denmark) in the spectral range between 5000 and 900 cm^{-1}. A Bayes B model was used to calibrate equations for each fermentative trait. The calibration equation predicts well VFA and N-NH_3 amounts in calibration and also in validation (R^2_{VAL} ranged from 0.93 to 0.83 for iso-valeric and n-butyric acid, respectively). However, the prediction of VFA expressed as proportions of total amount was much less accurate (R^2_{VAL} ranged from 0.81 to 0.52 for iso-valeric and n-butyric acid, respectively). In conclusion, FTIR and Bayesian models can be used as tools to accurately predict VFA amounts in vitro.

Key-words: mid infrared spectroscopy; in vitro rumen fermentation; Bayesian regression model

INTRODUCTION

Volatile fatty acids (VFAs) and ammonia nitrogen (N-NH_3) are the mean products of microbial fermentation and their production reflect the diet degradation in the rumen (Tagliapietra et al., 2011). The Fourier Transform Infrared (FTIR) spectroscopy has been applied in many different fields because it is simple, rapid, economic and doesn't require sample pre-treatments. For these reasons, FTIR can be a useful tool to evaluate fermentative parameters of samples collected both in *in vitro* and *in vivo* (Bhagwat et al., 2012). In our knowledge, few attempts have been done to predict VFAs expressed as proportions of total VFA amount with FTIR (Udén and Sjaunja, 2009). Different statistical approaches were used to calibrate FTIR equipment like the Partial Least Square Regression (PLS) being a popular multivariate calibration technique used to analyse spectra data. Recently, Ferragina et al. (2015) compared the traditional PLS approach with diverse Bayesian regression models, commonly used for genomic selection, founding the "Bayes B" model as a powerful predictor of milk properties. Therefore, the present work aims to develop a FTIR method to quantity amounts and proportions of VFA and N-NH_3 in fermentation fluids collected *in vitro* using the Bayes B regression model.

MATERIAL AND METHODS

Data of 4 *in vitro* incubations were used to calibrate the FTIR equipment. Two incubations were stopped at 24 h, whereas the other two lasted for 48 h. In each incubation 8 diets were tested in 5 replication plus 5 blanks, where the rumen fluid (RF) was incubated without any substrate. At the beginning of the incubation 3 samples of RF, of buffer and of their mix were also collected. A total number of 54 samples per incubation were collected and stored for chemical and FTIR analysis. The tested diets were formulated for lactating cows and differed for fibre, crude protein, lipids and starch

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content to be representative of Italian intensive dairy system (Dal Maso et al., 2009). Of each test diet, 1 g of sample was incubated with 100 mL of buffer (Menke and Steingass, 1988) and 50 mL of RF collected from 3 dry cows as described by Tagliapietra et al. (2012). The fermentations were monitored using a fully automatic gas production system described by Tagliapietra et al. (2010). RF, buffer and buffered RF at the beginning of incubation and fermentation fluid at the end of incubation were sampled for the immediate infrared (IR) analysis and others two aliquots were stored at −20°C with metaphosphoric acid (25%, w/v). The N-NH3 content was measured using a FIAstar 5000 analyzer FOSS (FOSS Analytical, Hilleroed, Denmark) following the internal procedure (Method Cassette Ammonium). The VFA profile was determined by GC with flame ionization detection (7820A GC system, Agilent Technologies, Milan, Italy) using a 30-m stainless steel column (J&W DB-FFAP, Agilent Technologies, Milan, Italy) and H_2 as a carrier gas (flow rate: 30 mL/min; isothermal oven temperature: 150°C). The Fourier transform equipment, designed for milk analysis (MilcoScan FT2, Foss Electric A/S, Hillerod, Denmark), was used for scanning the fresh samples within 3 h from collection over the spectral range of 5000 to 900 waves $\times$ cm^{-1}. Two spectral acquisitions were carried out for each sample and the results were averaged before data analysis. For technical reasons, for 1 incubation, 3 of 5 sample replications and also the samples collected at the beginning of incubation were not scanned. The Mahalanobis distances were used for the detection of spectra outliers, and those showing a distance higher than three times the standard deviation were discarded. Data editing was done in R environment (R Core Team, 2013). Finally 170 spectra were available for the study. A Bayesian model (Bayes B), implemented in R-software BGLR (Pérez and De Los Campos, 2014), was used to calibrate equations for each fermentative traits as recently described by Ferragina et al. (2015). The calibration was performed on a random dataset of 80% of data values and the remained 20% of values were used in validation. This guarantees that calibration and validation sets were independent. The procedure was repeated 20 times for each trait. As index of prediction accuracy of calibration was used the determination coefficient was calculated as square of the correlation between observed and predicted values in the calibration dataset (R^2_{CAL}). Similarly, the R^2 of validation was computed as square of the correlation between the observed and predicted values in validation dataset (R^2_{VAL}).

RESULTS AND DISCUSSION

The mean values and the standard deviation of the fermentative parameters used in the study are given in Table 1. On the average the concentration of total VFA in fermentation fluid was about 3.06 g/L with a large variability (SD $\pm$1.06 g/L) reflecting the different degradability of diets incubated. Also the inclusion in the calibration set of samples were collected at the beginning of incubation: RF (on the average 3.77 g/L of total VFA), buffer (without VFA) and buffered RF (1.39 g/L of total VFA). The variability of VFA parameters expressed as a proportion of total VFA was much lower. The SD of VFA proportions expressed as percentage of mean values ranged from 6.2% to 22.3% respectively for acetic acid and N-valeric acid. Also the $N\text{-}NH_3$ concentration in the calibration set showed a large variability both for different CP content of fermented diets and the inclusion in the data set of RF (74 mg/L), buffer (172 mg/L) and buffered RF (132 mg/L). Finally, the pH was on the average close to 6.8 and rather stable among samples for the high concentration of bicarbonate in the medium and for the ammonia produced throughout the fermentations. Therefore, except for pH, the variability of measurements was comparable to the reported by previous studies (Udén and Sjaunja, 2009) and allows the development of robust calibrations. The sample spectra were homogeneous but some outliers were identified probably for the presence of small particles in suspension that could interfere with the instrument sensors designed for milk analysis.

The coefficients of determination between the measured and predicted values obtained in calibration (R^2_{CAL}) and validation (R^2_{VAL}) datasets are given in Table 1. The amounts of VFAs were accurately predicted as shown by the R^2 that on the average ranged between 0.97 to 0.93 of respectively acetic acid and n-butyric acid. Moreover, the individual calibration, repeated 20 times, always exceeded 0.90 R^2_{CAL}. In validation the performance of prediction remain high with mean R^2_{VAL} values always greater than 0.90 with the only exception of n-butyric acid (R^2=0.84). Also the values of $RMSE_{VAL}$, suggest an expected error analysing an external sample of about 0.15, 0.08 and 0.06 g/L for acetic acid, propionic acid and n-butyric acid respectively. Udén and Sjaunja (2009) reported comparable performance of calibration working with semi artificial rumen fluids, obtained removing the VFA naturally present in the samples by acidification and adding defined amounts of acetic, propionic, n-butyric acid and bicarbonate.

Table 1. Statistics of samples used for the calibration and prediction R-squared in calibration (R^2_{CAL}) and validation (R^2_{VAL}) and root mean square error in validation ($RMSE_{VAL}$)

	Mean	SD	R^2_{CAL}			R^2_{VAL}			$RMSE_{VAL}$
			Mean	Max	Min	Mean	Max	Min	Mean
VFA amounts, g/L									
- Acetic acid	1.76	0.60	0.97	0.98	0.95	0.92	0.97	0.83	0.15
- Propionic acid	0.71	0.26	0.96	0.96	0.95	0.90	0.96	0.76	0.08
- Iso-butyric acid	0.04	0.01	0.96	0.97	0.95	0.91	0.97	0.83	0.00
- N-butyric acid	0.43	0.17	0.93	0.94	0.91	0.84	0.91	0.65	0.06
- Iso-valeric acid	0.07	0.03	0.97	0.98	0.97	0.93	0.98	0.87	0.01
- N-valeric acid	0.06	0.02	0.96	0.97	0.95	0.91	0.96	0.81	0.01
Total VFA	3.06	1.06	0.97	0.98	0.97	0.93	0.97	0.85	0.26
VFA proportion, g/100 g									
- Acetic acid	58.1	3.60	0.87	0.91	0.85	0.64	0.80	0.43	2.1
- Propionic acid	22.9	2.27	0.81	0.87	0.76	0.55	0.82	0.36	1.5
- Iso-butyric acid	1.2	0.19	0.87	0.89	0.84	0.72	0.84	0.55	0.1
- N-butyric acid	13.7	1.92	0.69	0.75	0.65	0.52	0.75	0.36	1.4
- Iso-valeric acid	2.4	0.51	0.92	0.93	0.90	0.81	0.92	0.70	0.2
- N-valeric acid	1.8	0.39	0.92	0.95	0.90	0.77	0.89	0.55	0.2
Ammonia nitrogen, mg/L	176	47.3	0.86	0.89	0.84	0.73	0.86	0.50	23
pH	6.85	0.16	0.54	0.59	0.47	0.39	0.50	0.30	0.12

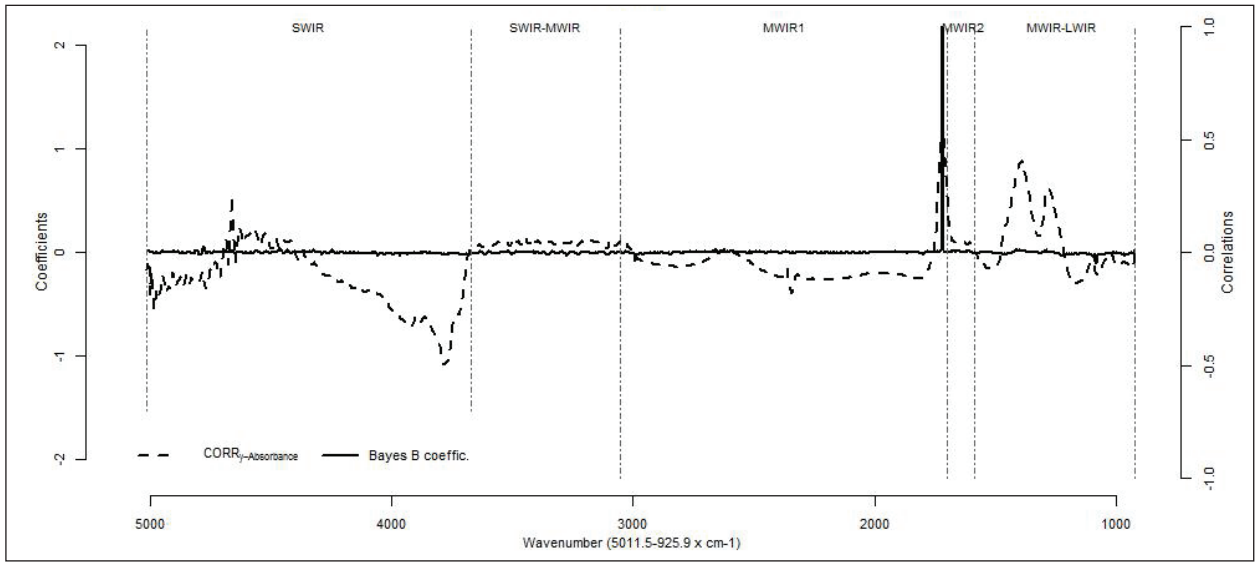
R^2_{CAL}= coefficient of determination calculated as the square of the correlation between observed and predicted values in calibration (80% of entire data set); Mean= mean of the R^2 of 20 replicas; Min= minimum value of R^2 obtained in 20 replicas; Max= maximum value of R^2 obtained in 20 replicas; R^2_{VAL}= coefficient of determination calculated as the square of the correlation between observed and predicted values in validation (20% of entire data set); Mean= mean of the R^2 of 20 replicas; Min= minimum value of R^2 obtained in 20 replicas; Max= maximum value of R^2 obtained in 20 replicas; $RMSE_{VAL}$= mean of the root mean square errors in validation of 20 replicas

The prediction accuracy of VFA values, expressed as proportion of total amounts, was acceptable in calibration with R^2 that ranged between 0.92 to 0.69 for valeric acid and n-butyric acid respectively. However, the correlation between measured and predicted values decreased in validation but with large differences among VFAs. The ability of the model to predict the $N\text{-}NH_3$ in fermentation fluids was slightly lower compared to VFA amounts as evidenced by the lower R^2_{VAL} and the higher $RMSE_{VAL}$.

In Figure 1 the correlation coefficients between the trait and each wavelength absorbance, and the estimated coefficients of the Bayes B equations for the prediction of acetic acid are shown. For the prediction of the amount of acetic acid (Figure 1a), it could be seen that the absorbance recorded at several wavelength is correlated with the measured VFA, but only in two cases the correlation coefficient approached 0.50.

In both cases, the spectral areas more correlated with VFA measurements were the spectral regions characterized by the large variability of absorbance due to water bonds: SWIR-MWIR and MWIR2 regions as classified by Bittante and Cecchinato (2013). The SWIR region and the area between MWIR1 and MWIR2 regions were negatively and positively correlated with VFA data, respectively. The Bayesian method B selected one wavelength in this last area (1721 cm^{-1}) as the most important one for predicting acetate quantity. This specific wavelength corresponds to the absorption peak of C=O bond of the carboxylic group (Bittante and Cecchinato, 2013). This result clearly evidences a direct relation between the prediction model and the chemical-physical properties of acetic acid. A similar condition was observed for the others VFAs and for $N\text{-}NH_3$. A different behaviour has been observed when VFA were expressed as proportion of their total amount. The pattern of correlations between absorbance at individual wavelengths and the acetate proportion is similar in shape, lower in extension and often opposite in sign than when acetate is expressed in g/L (Figure 1b). To predict the acetic acid proportion, the Bayes B model attributed a high coefficient to the wavelengths 4356, 3989 and especially 1644 waves $\times$ cm^{-1} with a negative, positive and negative sign respectively. These wavelengths are not directly related to the absorbance properties of acetic acid and the prediction depend on the correlation between acetic acid proportion and other chemical compounds in the fermentation fluid. *In vitro* experiments aimed to evaluate the effects of different feed combination or additives (Cattani et al, 2012) would take benefits from contemporary measurements of FTIR predicting changes in VFA composition.

A) Acetic acid, g/L



B) Acetic acid, g/100 g VFA

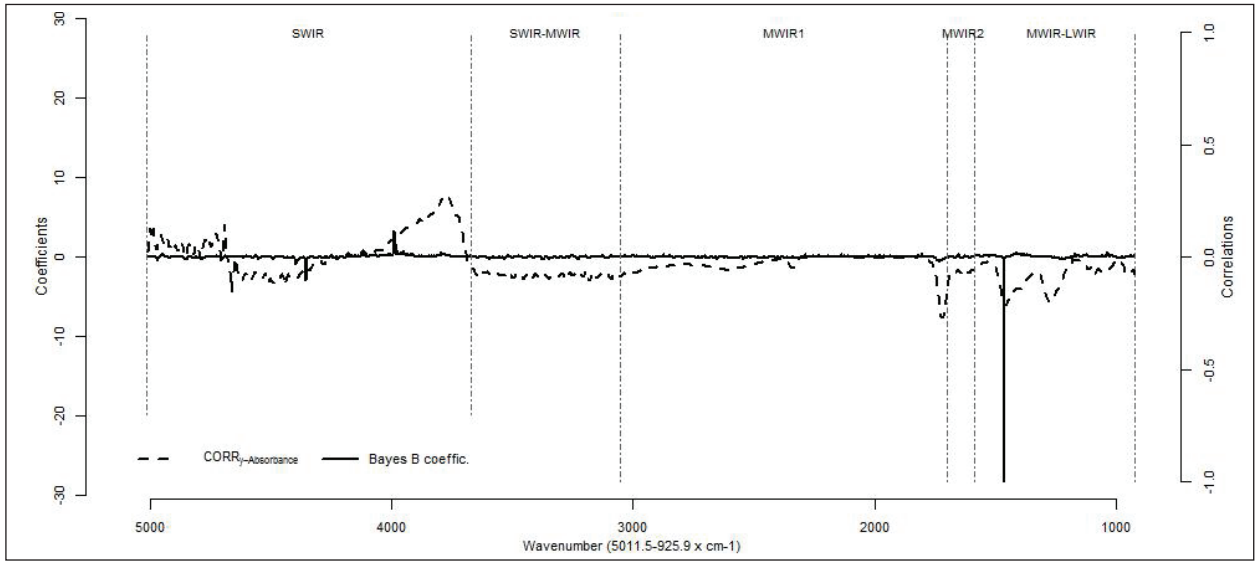


Figure 1. Graphs of correlation coefficients (r) between *in vitro* chemical compounds (A: acetic acid mg/L; B: acetic acid, % VFA) and NIR spectrum wavenumber absorbance (dot line), and prediction equation coefficients of each spectrum wavenumber (solid line) between 5000 and 900 waves × cm^{-1}

CONCLUSION

The FTIR technique, calibrated using a Bayesian regression approach, was able to predict accurately the VFA and ammonia amounts in fermentation fluids. However, the prediction of VFA expressed as proportions of their total amount was much less accurate. This is due to the fact that FTIR absorbances are mainly related to the concentration of specific chemical bonds in the fluid and not to their proportions. VFA proportions seem to be predicted only in an indirect way on the bases of correlations with the amount of some compounds present in the sample. For these parameters a greater number of samples in calibration set could be needed for a good prediction accuracy.

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PREVALENCE OF CAPRINE ARTHRITIS ENCEPHALITIS VIRUS IN ASSOCIATION WITH CLINICAL ARTHRITIS IN SIX PRODUCTION FARMS OF FRENCH ALPINE GOATS IN NORTH-WESTERN CROATIA

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Professional paper

SUMMARY

Prevalence of Caprine Arthritis Encephalitis Virus (CAEV) and occurrence of clinical arthritis were investigated on 543 goats of French Alpine breed on six intensive production farms in North-Western Croatia. The aim of the study was to determine seropositivity to CAEV and to examine the occurrence of clinical arthritis in relation to CAEV seropositive goats. All goats were examined clinically and presence of arthritis was noted. The blood samples were tested for antibodies against CAEV using the immunoenzyme test. All collected data were cross-classified in two-way contingency tables. Of the total number of goats, CAEV was serologically confirmed in 50.8% and 31.6% of all goats were diagnosed with clinical arthritis. CAEV seropositive goats were 21.9% and they also expressed clinical signs of arthritis. Statistical tests confirmed positive association between clinical arthritis diagnosis and seropositivity to CAEV with Phi coefficient of 0.25 (P<0.01). Results suggest that serious eradication programs should be introduced in north western Croatian goat herds, but also that further investigations in all Croatian herds should be conducted and measures should be applied on all herds.

Key-words: seropositive, ELISA, lentivirus, small ruminants, health

INTRODUCTION

CAEV is a slow, progressive and incurable disease of goats spread around the world (Peterhans et al., 2004). Because of its ability for cross-infection between goats and sheep, the virus is classified in Small Ruminant Lentiviruses (SRLV) group (Leurox et al., 1995). Infected goats may stay in a state of unapparent infection for life and spread the virus, or develop various clinical forms such as: arthritis, synovitis, neurological dysfunctions, indurations of udder, chronic interstitial pneumonia, and general wasting (Blacklaws et al., 2004). CAEV is considered to be an immunological disease causing indurations of target organs and the udder is one of the main routes of virus spreading (Desport, 2010). Virus spreads primarily through herds by vertical transmission from dames to kids by colostrum and milk or via feeding kids with unpasteurized colostrum//milk from infected goats. Also, virus can be spread by horizontal transmission through cohabitation of infected and uninfected goats (Rowe and East, 1997), transmission less investigated: in utero, contact with the vagina of an infected doe during parturition, via infected blood, with equipment and milking machines or iatrogenic (Adams et al., 1983; Rowe et al., 1991, 1992; Rowe and East, 1997). French Alpine goat production is dominant in Croatia and is concentrated in North Western Croatia. First herds of French Alpine breed were imported to Croatia two decades ago. Historically it was present in this area for two decades, when first herds were imported. CAEV was previously investigated in herds of North-Western Croatia by Čač et al. (1996) who noted prevalence of 4.7% on sample of 1290 goats. We wanted to determine CAEV prevalence in the studied farms today, and evaluate if clinical

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arthritis occurring in herds of French Alpine goats is connected exclusively to CAEV infections.

MATERIALS AND METHODS

We conducted the research on 543 goats of French Alpine breed from six production herds in North Western Croatia during the year 2013. Chosen farms were large (more than 50 goats), operating continuously from the first French Alpine goat import, enclosed without animal introduction from other herds, and registered in breeding program. Because of that fact we consider that it was possible to investigate how CAEV prevalence raised in separate enclosed production units. All animals were kept under intensive milk production conditions, had similar diet, with access to open area and were milked twice a day by machines. All of the goats from each farm were studied, and the sample did not include goats culled due to low production, illness or infertility. Kids in all six examined herds were not separated from dames, so they fed by suckling. All goats were clinically examined. The examination included assessment of general conditions and gait, as well as inspection of the carpal joints and the presence of clinical arthritis (cold swelling and stiffness with lower mobility) and it was noted bilaterally. Animals with a score of >7 cm between carpal circumference and metacarpal circumference in at least one joint were considered clinically affected. Animals with a score of <5 cm were considered clinically healthy (asymptomatic) (Bertoni et al., 1994). We collected samples of blood from the jugular vein during the first half of the lactation. The virus was diagnosed using the serological immunoenzyme test CHEKIT CAEV/MVV (IDEXX, Switzerland) by the official method for diagnostic of CAEV in Croatia and suggested as method by OIE (2007). The data was cross-classified in two-way contingency tables. Association between CAEV seropositivity and the occurrence of clinical arthritis were measured using Chi-square and Fisher's exact test in FREQ procedure of SAS programme (SAS, 2004).

RESULTS AND DISCUSSION

CAEV prevalence

Recorded total CAEV prevalence of 50.8% of the all examined French Alpine goats is even higher than the CAEV prevalence of 6–47% reported in other countries (Peterhans et al., 2004). CAEV was recorded in all six examined herds. Prevalence of CAEV recorded in a preceding study by Čač et al. (1996; 2000) at the same area on the sample of 1290 goats, was substantially lower, only 4.7%. This research showed that it had significantly increased during the last two decades. Horizontal transmission of the CAEV virus might lead to cross - species transmission. Although CAEV is typical for the goats in intensive production systems, possible horizontal transition could also endanger the valuable autochthonous goat breeds (Croatian white and Croatian spotted goat), especially in conditions of cohabitation in mixed flocks of goats and sheep (Pisoni et al., 2005).

Clinical arthritis

Out of the entire clinically examined population, clinical arthritis was diagnosed in 31.6% of the animals. Large percentage (21.9% out of 31.6%) was CAEV seropositive and also expressed clinical signs of arthritis. Such a high prevalence of clinical arthritis in CAEV seropositive goats was not found in other research (Grewal et al., 1986; Torres-Acosta et al., 2003). Importance of the vertical transmission and the lack of serious eradication measures resulted in rapid increase of the CAEV prevalence during the period with no CAEV control program. Furthermore, genetics of the animals or the virus strain could explain this result (Bertoni and Blacklaws, 2010).

Positive association between clinical arthritis occurrence and seropositivity to CAEV was confirmed in the examined number of samples, with Phi coefficient of 0.25 ($P<0.01$). This result indicates that CAEV could be the main reason for the clinical representation of the arthritis (Smith and Sherman, 2009). Clinical arthritis was found in 9.6% of the whole sample but CAEV was not confirmed. The reason for this may be that not all goats express detectable serum antibodies (Contreras et al., 2003; Leitner et al., 2008), or due to other possible different cause of arthritis such as mycoplasmas infection (Naglić and Šoštarić, 1995).

CONCLUSIONS

High percentage of serologically confirmed arthritis in the examined number of samples suggests that a serious CAEV eradication program should be introduced as obligatory measure in goat herds in Croatia. Additionally, these results indicate that the occurrence of clinical arthritis could also be reduced by this program. Furthermore these results indicate that the occurrence of clinical arthritis could also be reduced by this program because 21.9% of seropositive goats manifested clinical arthritis.

The high representation of clinical arthritis in the CAEV seropositive cases implies that further investigation of the virus strain is needed to explain it. Moreover, the presence of clinical arthritis in goats without CAEV reveals the need for further investigation of other possible causes.

Although the production of French Alpine goats in Croatia developed rapidly over the last two decades, replacement feeding or pasteurization of the colostrum and the milk for kids as well as the eradication of CAEV through separation of kids from the infected dames will be expensive and labour intensive. Control system should consider additional economic analysis based on specific production values of CAEV positive and negative goats such as lifetime lactation amount, lactation

per parity, protein or fat content and productive years in the herd or cull rate.

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WOLF (*CANIS LUPUS*) PREDATION ON DAIRY CATTLE IN EASTERN ITALIAN ALPS

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Original scientific paper

SUMMARY

Natural wolf recolonization of the Alps brings the challenge to reduce livestock losses and social conflicts. The uncommon impact of a wolf pack on the cattle farming systems of the "Lessinia", in the eastern Italian Alps was examined in this study. Dairy cattle farming predominates there using summer pastures (June-September) and grazing on lowland meadows out of summer. Grazing is organized with aim to minimize labour and costs. Animals are usually left unattended during the day and night in unprotected pastures. Since the return of the wolf in 2012, which formed a pack in 2013, attacks to livestock increased rapidly. Predations peaked during the summer, and they also were extended into the preceding and following months, especially during 2014. Cattle were the predominant species predated (79% of events and 71% of individual losses), with a strong selection towards young age classes. To prevent attacks, livestock should be grouped and kept protected by electric fences or in stables during the night, but this is in contrast with the free-grazing management that farmers have adopted for reducing costs. We suggest that management costs and introduction of protection measures changes should be taken into account for a future economic valorisation of the cattle farming sector.

Key-words: dairy cattle, mountain, wolf, livestock systems, depredation

INTRODUCTION

The recent natural recolonization of many European areas by wolf has increased the conflicts with humans (Linnel and Boitani, 2011; Reinhardt et al., 2011). Conflicts arise particularly where farmers have lost the habit to protect their livestock, which are often left grazing unattended and unprotected, even at night (Reinhardt et al., 2011). Reducing the conflicts due to predation on livestock will therefore require changes in the farming practices and the adoption of protection methods (Dalmasso et al., 2011; Linnel and Boitani, 2011). In fact, damages compensation alone fails to reduce animosity towards wolves (Dalmasso et al., 2011; Reinhardt et al., 2011). Sheep and goat are the most frequently livestock species killed by wolves in Europe (Reinhardt et al., 2011), but predation on cattle may also occur (Dalmasso et al., 2011). In order to assess the feasibility of adoption of prevention methods on cattle herds it would be useful to focus on recently recolonized areas strongly committed to cattle farming. This is the situation of "Lessinia", in the eastern Italian pre-Alps, where a wolf pair settled in 2012 and formed a reproductive pack in 2013. Predations on livestock raised in the farmers a strong objection and the willingness to get rid of wolves again. In this study, conducted in the context of the A7 action of the LIFE Wolfalps Project (LIFE 12 NAT/IT/000807), co-financed by the EU, we present the farming and grazing systems in Lessinia and describe the patterns of predations on livestock by the recently formed wolf pack. We then discuss the changes in farming practices, with the appropriate protection measures for reducing the impact of predations, and the cultural and economic difficulties to implement them.

MATERIAL AND METHODS

Study area

The Lessinia is located in the eastern Italian pre-Alps. It includes 18 municipalities of the Verona province in the Veneto region and one municipality in the Trento

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Province, with a total surface of 689 km^2. Almost 100 km^2 are included in the Lessinia Natural Regional Park established in 1990 by the Veneto Region. The area is mostly mountainous; the main villages are located on the slopes below 1200 m a.s.l., where forest patches and meadows are predominant land cover. Above this elevation, wide areas of grassland are used for livestock summer grazing. The potential wild prey for wolves are mainly roe deer (*Capreolus capreolus*) and wild boar (*Sus scrofa*). Alpine chamois (*Rupicapra rupicapra*) is present in habitats where predation is difficult, and red deer (*Cervus elaphus*) still has a very low abundance (Calderola S., personal communication).

Data collection and analysis

To describe the livestock farming and grazing systems in Lessinia we used data from Official Agricultural Censuses (ISTAT) and databases produced for previous studies (Mrad et al., 2009; Sturaro et al., 2013; Sturaro et al., 2014). We gathered information on predation events collected by the Veneto Region. Predation events were assigned to wolf based on an *in situ* inspection by trained personnel (at least two persons per event, in total 11 persons during 2012-2014) of the State Forestry Corp and the Lessinia regional Park. In the study area there are no other large carnivores, and stray dogs are absent. Information about the date, location, the owner of the farm/livestock, species of the prey, age and number of individuals injured or killed was organized and analysed. We georeferenced the predation events (open-GIS software Quantum GIS) and calculated the size of the area where attacks occurred by the use of minimum convex polygon method (ArcGIS® software by ESRI). The frequencies of predation events among periods were compared using the Chi square test.

RESULTS AND DISCUSSION

Livestock farming and grazing systems

Dairy cattle farming largely predominates sheep and goat farming in Lessinia (Table 1). Many small traditional farms have been abandoned and the intensification of production systems has led to an increase in the herd size in the period 1980-2010 (Table 1). Despite these changes, cattle farming in the area is still based on the use of meadows and pastures (Sturaro et al., 2014), especially during summer. Summer farms are located at an average elevation of 1462 ± 128 m, allowing long usage period (124 ± 9 days). The average size of pastures is 68 ± 38 ha and stocking rate is 0.96 ± 0.36 LU/ha (LU=Livestock unit). Composition of herds/flocks in summer farms is 43% dairy cows, 38% heifers and calves, 6% beef cattle (suckler cows with calves), and 12% sheep and goats (only 2 flocks). Summer farms are managed to reduce labour and costs as much as possible: the animals are left unattended and free to graze in unfenced areas during day and night, without guarding dogs (Mrad et al., 2009; Sturaro et al., 2013). Thanks to the very good accessibility (it is possible to reach 84% of summer farms by normal car), farmers make only short visits once or twice per day to check the animals or to milk them, but farmers usually (86% of the units) do not stay permanently with them. Many farmers use lowland meadows for a period of grazing, also unattended, before and after the summering season.

Table 1. Trend of the livestock sector (permanent farms) in Lessinia from 1982 to 2010 (ISTAT) (na = not available)

Farming systems	1982	1990	2000	2010
Cattle farms	2256	1661	983	656
Cattle heads	38952	40683	34335	26668
Dairy cows	16108	18558	15234	12072
Sheep and goat farms	na	na	237	142
Sheep and goat heads	na	na	2229	3117

Wolf Predation

Wolf predations on livestock first occurred during the winter 2011/2012, and since then increased rapidly (Figure 1). In 2014, 42 events and 64 livestock losses were observed. In addition, in this year the permanent farms, previously never attacked, suffered 10 predations on lowland meadows after the summer period, revealing that wolves began to follow the herds on their return to the villages. The total surface (minimum convex polygon) affected by predation events was 26 km^2 in 2012, 33 km^2 2103, and increased to 105 km^2 in 2014. The distributions of predation events and of livestock losses (Figure 2) differed significantly between months ($\chi^2=37.5$, $P<0.001$, and $\chi^2=54.3$, $P<0.001$). Summer was the most dangerous period, although predations extended over the previous and following months. The median number of days between subsequent attacks decreased from 11 days in 2012 and 2013 to 3 days in 2014. The strong increase of predations after the wolf return happened in the area where livestock is managed without protection practices and the temporal pattern of predations peaking in summer are similar to those observed in other areas (Dondina et al., 2014).

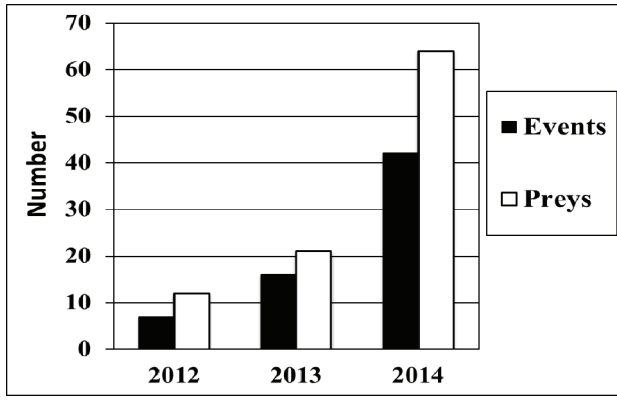

Figure 1. Total number of predation events and livestock losses in the Lessinia from 2012 to 2014

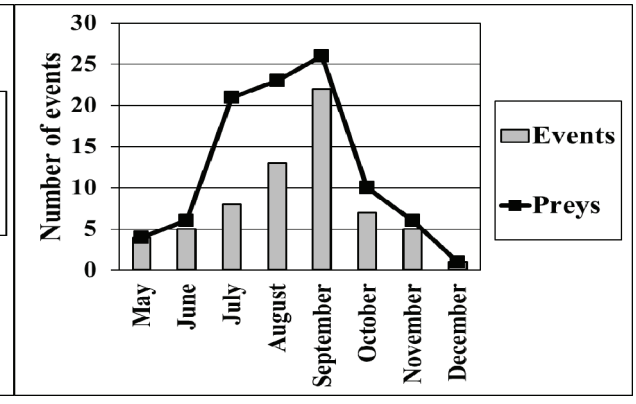

Figure 2. Monthly distribution of predation events and livestock losses in Lessinia from 2012 to 2014

Attacks mostly occurred on cattle (79% of events and 71% of individual losses, secondly on equids (15% and 18%), and lastly on sheep and goats (6% and 11%). This pattern is unusual, since sheep and goats are the preferred domestic prey of wolf (Reinhardt et al., 2011), but it can be related to the very low availability of small ruminants combined with the lack of protection measures for grazing cattle. In such situations, predation on cattle may be remarkable (Álvares and Blanco, 2014). Wolf clearly exerted a selection for age classes of cattle predated: most of the attacks (77%) were on calves <1 year old (42 % on calves < 6 month of age). Yearling cattle were attacked less (21%), whereas individuals older than 2 years were avoided (2%), although they were almost half of the cattle grazing. This pattern is consistent with the observed in other areas (Dondina et al., 2014).

In the farming and grazing systems of Lessinia there are many problems to be addressed in order to reduce the impact of the wolf predation. The most effective protection tools for livestock are electric fences and guarding dogs, especially for sheep and goat (Marucco and Boitani, 2012; Reinhardt et al., 2011). Dissuasive methods, acoustic or visual, are effective only temporarily and in specific situations (Reinhardt et al., 2011). Delimitation with permanent anti-predator fences of the rugged and wide pastures is impossible, because of the cost and impact on wild animal biodiversity and touristic attractiveness of the regional park. Experience of using guarding dogs with cattle is very limited in Italy, and in any case dogs may work only if livestock are not dispersed over wide pastures. Therefore, the only option to protect cattle in Lessinia is the night gathering within appropriate electric fences or stables. For making this feasible, however, farmers should abandon the practice of continuous free-grazing, adopting instead rotational grazing, making easier to group and protect the animals. However, single farmers cannot afford the additional costs of providing fences and water troughs to create pasture sections, and especially the salary for a shepherd to move the animals and to gather them before the night. An improvement in pasture productivity through a better management would not create a benefit for the farmers, because actual stocking rates are already lower than the pasture capacity and/or animal requirements are compensated with concentrate supplementation. These difficulties increase the negative attitude of farmers and other local stakeholders against wolf. Although the livestock losses are refunded by the regional administration and can be estimated at <1% of the number of cattle present in Lessinia, intolerance towards the wolf is growing (WOLFALPS, 2015). Most of the farmers do not accept the idea of implementing livestock protection measures, even if publicly supported, because if applied they would implicitly accept the presence of wolf. In this context, we suggest that the mitigation of wolf predation should be integrated into a comprehensive plan aimed at re-valuing the cattle farming sector in Lessinia, which is weakened (Sturaro et al., 2014) by the limited attitude of the owners towards innovating the farming structures and practices, the inadequacy of buildings and equipment in summer farms (Sturaro et al., 2013), and the low price paid for the milk sold to private dairies. For this purpose, opportunities are good (Sturaro et al., 2014), since the area has a high touristic attractiveness and the "Monte Veronese" local cheese is protected by a PDO, that could be used as a marketing tool. Therefore, the mitigation of the human-wolf conflict needs an effort of farmers and local stakeholders, supported by the regional agricultural policies, for a structural and technical innovation of the farms, a cooperative processing and milk marketing to increase its value, and a diversification of incomes through agro-touristic activities. This might greatly increase the economic viability of farming, and then justify the complication in management and the increased costs of grazing management for protection against wolf attacks.

CONCLUSION

This study examined the uncommon case of predations concentrated on dairy cattle by a wolf pack recently established in an area with a high density of livestock. In protection measures absence, predations

are increasing, and this has shaped a strongly negative attitude of the local communities against wolf. The farmers are unwilling to modify the practices that they consider traditional in order to adopt adequate prevention measures for which they cannot afford the costs. Simply compensating the direct costs of such measures would not be acceptable in front of the indirect costs of the modified management practices. Therefore, the solution of the human-wolf conflict must be integrated into a global approach to innovate and sustain the livestock sector, taking advantage of the synergies with tourism and marketing that are now undervalued.

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GENETIC DIVERSITY OF BELA KRAJINA PRAMENKA COMPARED TO THREE CROATIAN SHEEP BREEDS – A PRELIMINARY STUDY

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Original scientific paper

SUMMARY

In the Balkan Peninsula, different populations of Pramenka were the most common sheep breed. Due to different ecosystems and climate environment, these sub-populations have evolved genetic differences. The largest influence on the local genotype of Bela Krajina Pramenka in Slovenia based on the literature data could have Lika Pramenka from the neighbouring Croatia and some other Pramenka breeds from Bosnia and Herzegovina. Herein, genetic diversity within Slovenian Bela Krajina Pramenka sheep (BP) compared to three Croatian breeds, Cres island sheep (CRE), Lika Pramenka sheep (LIK), and Istrian Pramenka (IST) was studied using ten microsatellite markers. Bela Krajina Pramenka had relatively high genetic diversity shown by the mean number of alleles per microsatellite locus (7.20±2.04) and expected heterozygosity (0.72±0.03). The delta K method reviled four clusters as the most appropriate fit. The STRUCTURE software formed four unique distinct clusters equal to the actual number of analysed populations. Therefore, Bela Krajina Pramenka was found to be an authentic breed based on ten microsatellite markers and compared to three geographically closest sheep breeds. Some admixture among the included populations was found as well.

Key-words: Bela Krajina Pramenka, microsatellite markers, genetic diversity

INTRODUCTION

In the countries of the former Yugoslavia, different populations of Pramenka were once the most common sheep breeds. In the central Europe it was known also as »Zackel« (Drăgănescu & Grosu, 2010). These sub-populations have evolved genetic differences due to different ecosystems and climate environment and they were usually named the region or village (Mitić, 1984). In the sixties of the previous century Muck (1956) described three major sheep breeds in Slovenia; Pramenka (Bovška, Istrian, Bela Krajina), Jezersko-Solčava and Bergamasca. Zagožen (1984) stated that the Pramenka was a descendant of the Balkan mouflon crossed with the wild Asian steppe sheep.

The largest influence on the local genotype of Bela Krajina Pramenka in Slovenia could have Lika Pramenka from the neighbouring Croatia and some other Pramenka populations from Bosnia and Herzegovina (Grabrijan, 1997). Today, Bela Krajina Pramenka is one of the four Slovenian autochthonous sheep breeds, besides Jezersko-Solčava sheep, Bovška sheep and Istrian Pramenka. The Bela Krajina Pramenka is widespread in the Southeast part of Slovenia near the river Kolpa, mainly for lamb production. Total population was estimated at 900 purebred animals in the year 2014. Therefore, the breed is endangered by the national rules. Lika Pramenka, the most similar to Bela Krajina Pramenka by type traits and purpose, is reared across the border, in Croatia, on the pastures of Lika and Gorski Kotar. Istrian Pramenka is an indigenous breed of the Northern Adriatic area. Today, the initially transhumant Istrian Pramenka population is fragmented in separate populations in Slovenia, Croatia and Italy. Cres island sheep is a dual-purpose breed used mostly for meat production (Salamon et al., 2015).

The knowledge on the genetic diversity of breeds such as Bela Krajina Pramenka is of high importance for conservation of endangered populations. However, other

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three Slovenian autochthonous sheep breeds (Jezersko-Solčavska sheep, Bovška sheep, Istrian Pramenka) were included in genetic analyses before (Kavar et al., 2002; Dalvit et al., 2008). Istrian Pramenka from Slovenia was precisely compared with Croatian population of Istrian Pramenka (Salamon et al., 2015) as well. Numerous studies describe different sheep breeds using microsatellite markers to assess genetic diversity. Peter et al. (2007) explained genetic diversity of 57 European and Middle-Eastern sheep breeds, while Ćinkulov et al. (2008) studied genetic diversity and structure of seven West Balkan Pramenka population. Bela Krajina Pramenka has never been included in such genetic analyses. Therefore, the aim of the present study was to provide a preliminary research in understanding genetic diversity of Bela Krajina Pramenka compared to the three Croatian sheep breeds.

MATERIAL AND METHODS

Animals and DNA extraction

Blood samples of Bela Krajina Pramenka sheep (BP; n = 29) from Slovenia as well as blood the ones of the three Croatian breeds, Cres island sheep (CRE; n = 25), Lika Pramenka sheep (LIK; n = 25), and Istrian Pramenka (IST; n = 35) were collected. Animals of BP and IST breed were unrelated within the sampled group and originated from 12 and 18 flocks, respectively while animals of CRE and LIK originated from one flock each. A total of 114 animals were included in the analysis. The DNA was isolated from blood samples using Blood Genomic DNA Kit (GenEltueTM; Sigma-Aldrich®, St. Louis, MO, USA). For the initial selection of microsatellite markers, multiplex reactions were optimized using fluorescent-labelled primers and hot-start polymerase (JumpStart™ REDTaq® ReadyMix™; Sigma-Aldrich®, St. Louis, MO, USA). Ten markers had been selected from the sheep diversity list recommended by the Food and Agriculture Organization of the United Nations (FAO, 2011). Diluted PCR products were processed in a 16-capillary electrophoresis ABI3130XL Genetic Analyser, with two of the PCR-multiplex reactions. Genetic diversity parameters were estimated for ten microsatellite loci.

Data analysis

Allele frequency, the mean number of alleles (MNA), polymorphic information content (PIC), observed heterozygosity (Ho) and heterozygosity expected (He) under the Hardy-Weinberg (HWE) equilibrium assumption across the markers and the populations were calculated using the Excel Microsatellite Toolkit v. 3.1 (Park 2001). Genetic variation and the distribution of genetic diversity among and within the groups were determined by the analysis of molecular variance (AMOVA) using the GenAlEx 6.501 Software (Peakall and Smouse, 2012). Individual assignment in the populations was investigated using the STRUCTURE software 2.3.1 (Pritchard et al., 2000). We performed 10 runs to choose the appropriate number of inferred clusters (K), tting K from 1 to 5. The burn-in period for all runs was 35 000 iterations, and data were collected during the period of 15 000 iterations. To choose the optimal K, the delta K method was used (Evanno et al., 2005).

RESULTS AND DISCUSSION

A total of 106 different alleles were found in 114 genotyped individuals. The average number of alleles per locus was 10.60. The highest number of detected alleles recorded was 18 for marker HUJ616. The PIC values per marker varied from 0.543 for MAF214 to 0.821 for HH47 (Table 1).

Table 1. Genetic diversity parameters estimated for ten microsatellite loci

Marker	A	Ho	He	HWE	Fst	Fis	PIC
CP34	6	0.735	0.763	n.s	0.002	0.050	0.724
JMP58	10	0.770	0.840	p=0.0008	0.140*	-0.016	0.818
JMP29	15	0.763	0.787	n.s	0.106*	-0.056	0.753
BM8125	8	0.696	0.682	n.s	0.109*	-0.078	0.647
DYMS1	11	0.726	0.723	n.s	0.059*	-0.035	0.698
VH72	8	0.737	0.793	n.s	0.125*	-0.028	0.763
MAF214	7	0.477	0.598	p=0.0053	0.133*	0.194*	0.543
MCM140	10	0.781	0.789	n.s	0.055*	-0.033	0.757
HH47	13	0.761	0.843	p=0.0017	0.109*	0.027	0.821
HUJ616	18	0.639	0.737	n.s	0.023*	0.191*	0.704
Overall	106	0.708	0.756		0.087	0.020	

A - number of alleles per locus, Ho - average observed heterozygosity, He - average expected heterozygosity, HWE – significant deviation from the Hardy-Weinberg equilibrium (n.s. - not significant), Fst - genetic difference among populations, PIC - polymorphic information content, Fis = coefficient of inbreeding (estimates and signi cance of the deviation of HW equilibrium per population across the 10 loci),* P<0.01

In the global population, and accounting for the multiple tests performed (ten loci, four populations), three loci were found to be in Hardy-Weinberg disequilibrium. The AMOVA analysis showed a significant and higher source of variation within (89.52%) than among (8.67%) the populations. Estimated inbreeding coefficients (Fis) were estimated for each locus in the global population. The Fis values for the markers ranged from -0.078 (BM8125) to 0.194 (MAF214). Moreover, two markers (MAF214 and HUJ616) showed positive and significant ($P<0.01$) Fis values.

Genetic diversity of the Bela Krajina Pramenka was relatively high (Table 2), as shown by the mean number of alleles per microsatellite locus (7.20 ± 2.04) and mean expected heterozygosity (0.72 ± 0.03). The observed heterozygosity was very similar between Bela Krajina Pramenka (0.72 ± 0.03) and Lika Pramenka (0.72 ± 0.02), followed by Istrian Pramenka (0.71 ± 0.028), while Cres Pramenka (0.66 ± 0.06) had the lowest Ho. The largest difference between Ho and He was found for Cres island sheep.

Table 2. Within-population genetic diversity parameters derived from ten microsatellites

Population	n	Ho	He	MNA
Bela Krajina Pramenka	29	0.72 ± 0.03	0.72 ± 0.03	7.20 ± 2.04
Cres island sheep	25	0.66 ± 0.06	0.71 ± 0.03	5.90 ± 2.02
Lika Pramenka	25	0.72 ± 0.02	0.71 ± 0.03	7.60 ± 1.71
Istrian Pramenka	35	0.71 ± 0.03	0.69 ± 0.03	6.00 ± 1.15

n - sample size, Ho – average observed heterozygosity (± SD), He - average expected heterozygosity (± SD), MNA - mean number of alleles

Expected heterozygosities obtained in this study were lower than reported by Ćinkulov et al. (2008) based on 15 microsatellites in Pramenka populations, where the lowest He was 0.74 in Karakacanska Pramenka from Macedonia and the highest 0.81 for Recka Pramenka from Albania. The aforementioned study reported higher Ho in Istrian Pramenka as well (0.76). We found lower expected heterozygosity value for Bela Krajina Pramenka than it was reported for Bovška (0.74) and Jezersko-Solčava sheep (0.76) in the study of Alpine sheep breeds (Dalvit et al., 2008). Much lower population size of Bela Krajina Pramenka compared to Jezersko-Solčava sheep could explain this result.

This result concur with geographical diversity pattern reported for 57 European and Middle-Eastern sheep breeds (Peter et al., 2007). As expected, Swiss high production breeds (Glowatzki-Mullis et al., 2009) showed lower diversity compared to Pramenka sheep populations (Ćinkulov et al., 2008). Expected heterozygosity values of Bela Krajina and Lika Pramenka were obtained between the two aforementioned groups.

To choose the most appropriate number of clusters the method of Evanno et al. (2005) was used. Based on the highest value of ΔK (0.544), the most likely number of clusters (K) was ascertained to be four, being equal to the actual number of the analysed populations. Using $K = 4$ in the STRUCTURE software, four unique, distinct clusters were formed (Figure 1). Each colour represents one cluster and the length of the bar represents the individual's estimated proportion of membership in the cluster. Therefore, Bela Krajina Pramenka was found to be an authentic breed based on ten microsatellite markers and compared to three geographically closest sheep breeds (Lika, Cres and Istrian Pramenka). However, we found some of the animals in all included populations to be admixed, which could be a consequence of geographical proximity.

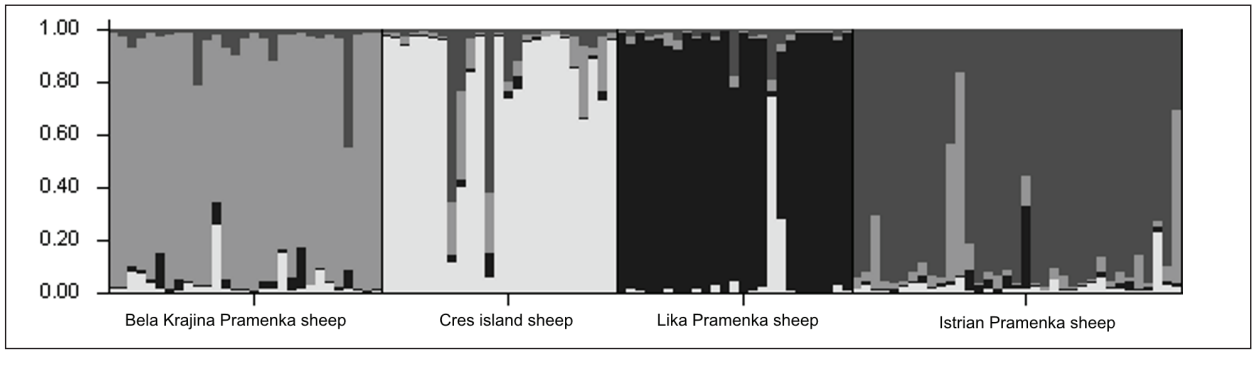

Figure 1. The population structure of four sheep breeds

CONCLUSION

Due to large influence of neighbouring Pramenka populations, genetic diversity of Bela Krajina Pramenka was studied. This study found considerable genetic diversity in the population of Bela Krajina Pramenka, very similar to Lika Pramenka. Despite the geographical proximity and similar type traits, Bela Krajina Pramenka and Lika Pramenka in this study, were considered as two separated breeds. To confirm results from this preliminary study, further analysis with larger number of markers and sheep populations are necessary and recommended.

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ESTIMATION OF GENETIC PARAMETERS FOR PRODUCTION TRAITS IN PIG BREEDS IN CROATIA

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Original scientific paper

SUMMARY

Genetic parameters for backfat thickness and test time were updated and used in genetic evaluation for field tested pigs in Croatia. Data consisted of 9,406 animals with measurements for production traits recorded from 2000 to 2014. The number of animals in pedigree was 10,728. Production traits were modelled using a single trait animal model including the following fixed class effects: breed, sex, classifier, season, and herd. Weight at the end of the test was included as linear regression in the model for backfat. Direct additive genetic effect, interaction herd-year-season of testing and common litter effect were included as random effects. Variance components were estimated using REML method as implemented in the VCE-6 program. The estimated heritabilities were 0.28±0.03 for backfat thickness and 0.12±0.02 for test time. Litter effect accounted from 15 to 24% of phenotypic variation, while herd-year-season of testing explained additional 24 and 28% of variability for analysed traits.

Key-words: genetic parameters, pig, breeding, heritability, genetic trend

INTRODUCTION

Genetic evaluation approaches had huge impact on the efficiency of pork production in the last decades. Since 2005, mixed model methodology known as Best Linear Unbiased Prediction (BLUP, Henderson, 1973) has been used as standard procedure for genetic evaluation (Vincek et al., 2004) of production traits used in Croatian pig breeding programme. This approach predicts the genetic potential of the animal based on its own performance and of all phenotyped relatives. Genetic progress can be achieved for traits that are heritable such as growth rate, backfat thickness (BF) (Ferraz et al., 1993), feed efficiency, muscle thickness and hind leg mass (Hermesch et al., 2000) which can be measured directly. Those traits emphasize the performance traits associated with efficient muscle development. The most important of them are minimum backfat and maximum growth rate. Both traits are of economic importance and since they are also highly heritable, they can be improved by selection. Improvement of these traits through breeding will likely be of use in the form of better feed efficiency, heavier weaning weights and more rapid development of gilts for breeding. The objective of this study was to update genetic parameters for production traits: backfat thickness and test time (TT) for field tested pigs in Croatia.

MATERIAL AND METHODS

Data used for the estimation of genetic parameters and breeding value prediction were collected on family farms by Croatian Agricultural Agency employees. Data were taken from database of Croatian Agricultural Agency. Backfat thickness was measured on alive animals at the end of the test with ultrasound (Renco® ultrasound). Data were edited and records were deleted if: a) test date was unknown, b) herd was unknown and c) animals were from different breed than those included in the analysis. Additionally, animals were excluded from the analysis if they had less than 75 and more than 140 kg and were younger than 120 and older than 360 days at the end of the test. Backfat thickness of analysed animals was limited within the range from 3.5 to 25 mm. The animals were grouped by herd and season and groups having less than 3 animals were excluded.

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Average values for BF and TT are shown in Table 1. As expected, Pietrain has lowest BF (7.06 mm), while Landrace had the greatest value for BF (10.28 mm). Breed with the shortest TT was Duroc (180.97 days) and breed with the longest duration of the test was Large White. Correction was made for TT where weight at the end of the test was set to the 105 kg representing the average weight of the tested gilts in Croatia. Correction was made in a way that average weight of 105 kg is multiplied with number of days in the test for an animal divided with real weight of the animal. After data editing out of total number of data (15,296) for production traits (BF, TT) 9,406 records were used in further analysis.

Table 1. Average backfat thickness and testing age for analysed breeds

Breed	Backfat thickness					Time on test				
	N	Mean	Std	Min	Max	N	Mean	Std	Min	Max
Large White	736	10.19	2.59	4.95	20.68	736	202.50	42.77	120.00	362.00
Landrace	6,782	10.28	2.25	4.95	23.98	6,782	182.21	26.56	119.00	365.00
Duroc	306	9.86	2.38	5.28	19.36	306	180.97	30.08	126.00	356.00
Pietren	1,582	7.06	1.17	4.29	13.31	1,582	187.60	22.66	128.00	305.00
Total	9,406	9.72	2.45	4.29	23.98	9,406	184.66	28.25	119.00	365.00

All animals with records and their relatives tracing back for three generations were included in the pedigree file (Table 2). The total number of animals involved in the pedigree was 10,728 and it was tracking back three generations. There were 87.7% animals with production records (generation 0) in the pedigree. Sires and dams, parents of those animals, represented additional 9.6% of the animals. Proportion of animals in the second and third generation decreased (2.5% and 0.2%) due to poor structure at the beginning of data collection. Animals were descendants of 1,030 parents in total. Time span in which data were collected relates to the animals born from beginning of 2,000 and animals tested until end of 2014.

Table 2. Structure of the pedigree

Item	Number of generations in pedigree								All	
	0		1		2		3			
	n	%	n	%	n	%	N	%	n	%
Male	3,566	33.2	374	3.5	171	1.6	17	0.2	4,128	38.5
Female	5,840	54.4	656	6.1	97	0.9	7	0.1	6,600	61.5
All	9,406	87.7	1030	9.6	268	2.5	24	0.2	10,728	100.0

Choice of the effects in the fixed part of the model was made by the effect significance, as well as coefficient of determination (R^2) and degrees of freedom for the model. Random part of the model included effects frequently used in the model according to literature review. It consisted of genetic part referred to direct additive genetic effect and environmental effects. The environmental effects were further partitioned to a permanent environmental effect within the parity and contemporary group.

The model [1] that best fit BF and TT [2] was determined with fixed effects as follows: breed (B_i), sex (S_j), classifier (C_k), season (M_l), and herd effect (O_m). Additionally, weight at end of test ($x_{ijklmno}$), nested within breed was included in the model [1] as a covariable.

$$y_{ijklmno} = \mu + B_i + S_j + C_k + M_l + O_m + b_{1i}\left(x_{ijklmno} - \bar{x}\right) + h_n + l_i + a_{io} + e_{ijklmno} \quad [1]$$

$$y_{ijklmno} = \mu + B_i + S_j + C_k + M_l + O_m + h_n + l_i + a_{io} + e_{ijklmno} \quad [2]$$

Random part was same in both models. It consisted of contemporary group defined as interaction of herd-year-season of testing (h_n), common litter environmental effect (l_i) referred to permanent environmental effect within the parity, direct additive genetic effect (a_{io}) and residual error ($e_{ijklmno}$).

The GLM procedure (SAS Inst. Inc., 2001) based on Least Square Method was used to define the fixed part of the model. Covariance components were estimated by Residual Maximum Likelihood method as implemented in the VCE 6 (Kovač et al., 2002) software.

RESULTS AND DISSCUSION

The proportion of variation accounted for fixed part of the model for BF was 53.21%. On the other hand, fixed part of the model for TT explained lower proportion of variation (33.25%). All listed effects in the model were significant ($p<0.0001$) as shown in Table 3.

Table 3. Coefficients of determination, degrees of freedom (DF), standard deviation for residual (σ_e), p-values of fixed effects

Model	Trait	
	Backfat	Test time
R^2	0.53	0.33
DF for model	195	191
σ_e	2.87	544.07
B	<0.0001	<0.0001
S	<0.0001	<0.0001
C	<0.0001	<0.0001
M	<0.0001	<0.0001
O	<0.0001	<0.0001

R^2- coefficient of determination, DF for model- degrees of freedom for model, σ_e- standard deviation for residual, B - breed effect; S - sex effect; C - classifier effect; M – season effect; O - herd effect

Estimated genetic parameters for BF and TT are shown in Table 4. Additive genetic variance for BF and TT was in the range of estimations observed for BF and TT in analysis of family farms (Škorput, 2013). Additive genetic variance for BF was higher compared to estimates of Malovrh and Kovač (1999) for German Landrace (0.23 mm^2) and Large White (0.25 mm^2), but lower than Swedish Landrace (0.38 mm^2). Estimated heritability was 28% for BF and 12% for TT. Vincek et al. (2004) reported similar heritability estimates being in the interval from 0.02 to 0.29 for BF and from 0.04 and 0.20 for TT based on data from three Croatian farms for Swedish and Dutch Landrace, Large White, Pietrain and their crosses. Malovrh and Kovač (1999) obtained heritability from 0.11 to 0.35 for BF of gilts and from 0.23 to 0.40 for boars estimated for Swedish landrace, Large White, and German Landrace on big farms in Slovenia. However, heritability in this paper was lower in comparison to estimated heritabilities by Škorput (2013), where the same breeds were used. Time span of analysed animals in Škorput (2013) was from 1998 to 2008.

Table 4. Covariance component estimates ±standard error for backfat thickness and test time at family farms

Trait	V_a	V_l	V_{hys}	V_e	h^2	l^2	hys^2	e^2
BF*	0.80±0.08	0.69±0.04	0.69±0.06	0.97±0.05	0.28±0.03	0.15±0.01	0.23±0.02	0.33±0.02
TT	56.58±10.53	134.64±7.48	134.64±8.00	172.93±7.43	0.12±0.02	0.24±0.01	0.28±0.01	0.36±0.02

BF*- backfat thickness; TT- time on test; V_a- additive genetic variance ; V_l– variance of common litter environment; V_{hys}- variance of herd-year-season interaction; V_e- residual variance, h^2- heritability; l^2- ratio for common litter, hys^2- ratio for herd-year-season interaction, e^2- ratio for residual

Heritability estimated by Imboonta et al. (2007) for BF and TT was higher than in our analysis (0.61±0.02 BF and 0.38±0.02 for average daily gain which can be compared to TT). For the analysis they used Landrace sows from Thailand coming from one nucleus herd. This can explain better connectivity of their data. Similar to previously compared studies, Bidanel et al. (1994) estimated higher heritability for TT and BF in Large White and French Landrace populations (0.25, 0.45 and 0.23 and 0.55) compared to the current study. Generally, heritability estimated on field test data are lower in comparison to data collected in stations (Peškovičová et al., 2002). Selection practices have shortened TT and together with this improved the average daily gain, BF thickness and other traits of pig carcass (Imboonta et al. 2007). However, correlation between selection for production traits and decreased reproductive performance has been reported. Production traits are necessary to combine in selection programme.

Interaction herd-year-season of testing explained 23% of phenotypic variance in BF and higher proportion (28%) for TT. Common litter effect explained 15% of total phenotypic variance for BF, whereas for TT common litter variance obtained 24% of phenotypic variance. Malovrh and Kovač (1999) reported common litter variance for BF to be lower in smaller breeds for Slovenian Swedish Landrace, Large White, and German Landrace. In their case, common litter variance explained 14% of phenotypic variance, being similar to our findings. On the contrary, common litter variance explained higher proportion of phenotypic variance (23% for BF) in the study of Škorput (2013). Similar proportion of common litter variance (26%) was obtained for TT.

Genetic trends of BV for BF and TT were calculated as the linear regression of the average annual predicted breeding values on the birth year (Figure 1). For each trait, genetic trend was shown for all animals. Genetic trend for BF was positive until 2009, with small drop in 2010 and stagnation thereafter. Genetic trend for TT was positive with peak in 2006 and then drop until 2011 and oscillation afterwards. Grey bars in both genetic trends represent number of animals tested per year and reduction of animals tested was obvious.

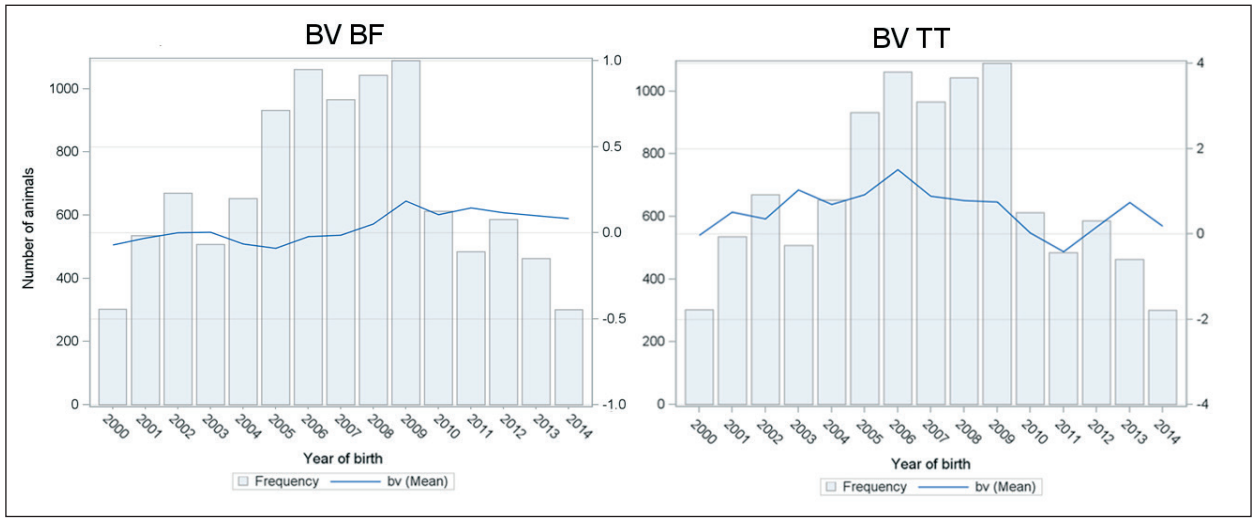

Figure1. Genetic trends for backfat and time on test with number of animals per year

CONCLUSION

Data structure affected the estimation of genetic parameters and prediction of breeding values. Heritability estimates for BF (0.28) and for TT (0.12) were lower compared to literature estimates for those traits due to specific data structure and low connectivity between the farms. Future perspective for genetic evaluation of pigs included in National pig breeding programme is to include additional production trait measures and estimate breeding values for fertility traits.

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IMPACT OF SNPS IN CANDIDATE GENES ON ECONOMICALLY IMPORTANT TRAITS IN PINZGAU CATTLE

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Original scientific paper

SUMMARY

The aim of this work was to evaluate the impact of polymorphisms in bovine genes encoding leptin (LEP), leptin receptor (LEPR) and pituitary transcription factor (Pit-1) on economically important traits in Pinzgau cattle. The genomic DNA samples were taken from the total of 85 Pinzgau cows. The detection of LEP/Arg2059Cys, LEPR/Thr945Met, and Pit-1/g.1178A>G single nucleotide polymorphisms were performed in order to evaluate their effect on long-life milk production traits. The genotyping of analysed animals was carried out using PCR-RFLP method. The observed allele frequencies showed that the LEP^A, $LEPR^C$ and $Pit\text{-}1^B$ allele were the most distributed in the analysed population. The high proportion of homozygous genotype in case of each locus was transferred in to the relatively lower level of total observed heterozygosity which also confirmed the positive values of F_{IS} index. The results of statistical analyses indicated the significant effect ($P<0.05$) of the evaluated polymorphisms in LEP and LEPR genes on milk, protein and fat yield. The LEP^{AA} and $LEPR^{CC}$ genotypes seem to be a desirable for milk production improvement. For the Pit-1 gene only tendency to increase the milk production for AA homozygous animals was found out. Our study was biased mainly by sample size and therefore the analysis of greater population is recommended.

Key-words: LEP, LEPR, milk production, Pinzgau, Pit-1, SNP genotyping

INTRODUCTION

The economically important production traits in cattle are influenced by many genes controlling the biological and physiological processes in organisms involved in formation of phenotypic traits. The candidate gene approach is based on a prior knowledge including the physiological mechanisms. Candidate genes are generally the genes with known biological function directly or indirectly regulating the developmental processes of the investigated traits, which could be confirmed by evaluating the effects of the causative gene variants in an association analysis (Zhu and Zhao, 2007). In practice, identification of the trait genes is achieved using a combination of genetic mapping, to localise the QTL region on a chromosome, and candidate gene or positional cloning approaches, to identify the trait gene within QTL region (Williams, 2005).

In dairy cattle many genes were evaluated as candidate for milk performance. In this study single nucleotide polymorphisms (SNPs) of three genes encoding leptin (*LEP/Arg2059Cys*), leptin receptor (*LEPR/Thr945Met*) and pituitary transcription factor (*Pit-1/g.1178A>G*) were analysed in relation to the variability of milk production traits. The genes have been selected firstly for their biological function and secondly based on previous published association studies that confirmed their impact on milk production in different cattle breeds. Leptin as hormone synthesized and secreted primarily in the adipose cells is involved in the feed intake, energy expenditure, and reproductive performance (Liefers et al., 2005). Leptin is supposed to be a signal to the reproductive system that enough total energy is present to support the added energy demands of a successful conception and pregnancy (Lindersoon et al., 1998). Polymorphisms in the *LEP* gene in cattle have been associated with somatic cells count (Kulig et al., 2010), milk performance (Anton et al., 2012), reproduction traits (Almeida et al., 2003) and growth traits (Kulig and Kmieć, 2009). Effects of leptin are provided

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through six receptors which are glycoproteins with a single transmembrane - spanning region. The longest and fully active isoform (*LEPR-b*) is expressed mainly in hypothalamus, the centre of the energy homeostasis and the regulation of secretory organs activity (Matteis et al., 2012). The polymorphisms identified in *LEPR* gene were significantly associated with milk performance (Komisarek and Dorynek, 2006), reproduction traits (Clempson et al., 2011) and growth traits (Silva et al., 2012). The pituitary cells development and hormone secreting genes expression in mammals is provided by specific pituitary transcription factor gene (*Pit-1*). The *Pit-1* is responsible for activating growth hormone, prolactin and thyrotropin genes in the anterior pituitary gland (Tuggle and Trenkle, 1996). The effect of Pit-1 gene polymorphisms was studied in relation to the body growth (Yang et al., 2010), milk composition and production (De Mattos et al., 2004) and reproduction traits (Edriss et al., 2009).

The aim of the present study was to determine *LEP*, *LEPR* and *Pit-1* genes allele and genotypes frequencies, population's genetic indices and evaluate the significance of their impact on long-life milk production traits in Pinzgau cattle that belongs to the animal genetic resources of the Slovak Republic.

MATERIAL AND METHODS

The genomic DNA for genotyping animals was obtained from total of 85 hair roots samples of Pinzgau cows. The analysed individuals were selected based on stratification method. Genomic DNA from each sample was extracted according to Gábor (2009). After extraction of DNA the concentration was controlled by the spectrophotometry measurement using NanoPhotometer™ (IMPLEN). The genotyping of each individual was carried out using PCR-RFLP methods. A 422 bp fragment of *LEP* gene was obtained by PCR with time and temperature conditions in accordance with Liefers et al. (2002). The amplification of a 197 bp fragment of *LEPR* gene was performed with appropriate reaction condition proposed by Almeida et al. (2008) and a 260 bp fragment of the *Pit-1* gene was amplified according to Ozdemir (2012). Subsequently, the PCR products of *LEP*, *LEPR* and *Pit*-1 genes were digested at 37°C for 10 min with 1 μl of FastDigest restriction enzymes *Sau*3AI, *Bse*GI and *Hin*fI, respectively. The PCR products and restriction fragments were separated and visualised by horizontal electrophoresis in 3% agarose gels in 0.5 x TBE (130 V for 50 min) stained with dye GelRed.

The determination of population structure in order to estimate the allele and genotype frequencies were done using Popgene32 software (Yeh et al., 2000). The differences between observed and expected genotype frequencies were tested using Chi-square (χ^2) analysis. The population genetic parameters, including observed and expected heterozygosity (H_e) and homozygosity (H_o), effective allele numbers (N_e), polymorphic information content (*PIC*), and Wright's fixation index (F_{IS}) were calculated using the PowerMarker software according to Liu and Muse (2005). The impact of analysed single nucleotide polymorphisms in *LEP* (*Arg2059Cys*), *LEPR* (*Thr945Met*) and *Pit-1* (*g.1178A>G*) genes on the long-life milk production traits (milk, protein, and fat yield) in analysed population were determined using SAS software. The normality test showed non-significant results and therefore the significance of genotypes effect on production traits were tested by parametric statistic.

RESULTS AND DISCUSSION

For the *LEP* gene (SNP *Arg2059Cys*), across analysed population, the prevalence of heterozygous animals was found. The lowest proportion was observed for homozygous BB animals. The high distribution of AA and AB animals in population was reflected in the A allele frequency increase. In the *LEPR* gene (SNP *Thr945Met)*, CC genotype gained absolute ascendancy because the observed frequency of CC genotype was high (82.4%). This indicated that the observed frequency of C allele is much higher than of T allele (Table 1). In case of *Pit1/g.1178A>G* locus heterozygous AB genotype was the most frequent. The B allele reached higher frequency in population than A allele. The differences between the observed and expected genotype frequencies were non-significant and in the analysed population the Hardy-Weinberg equilibrium was found for each locus. The heterozygosity, effective allele numbers and polymorphism information content of analysed bovine loci are shown in Table 1.

Table 1. The allelic and genotypic frequencies of *LEP*, *LEPR* and *Pit-1* genes in the analysed population

Locus	Genotypes			Alleles		χ^2 test	H_o	H_e	N_e	PIC	F_{IS}
	AA	AB	BB	A	B						
LEP/Arg2059Cys	0.447	0.494	0.059	0.694	0.306	2.234-	0.57	0.43	1.73	0.33	0.16
LEPR/Thr945Met	0.071	0.565	0.364	0.353	0.647	4.745-	0.84	0.16	1.19	0.15	0.09
	CC	**CT**	**TT**	**C**	**T**						
Pit1/g.1178A>G	0.824	0.176	-----	0.9118	0.0882	2.28-	0.54	0.46	1.84	0.35	0.24

H_o – observed homozygosity, H_e – observed heterozygosity, N_e – effective allele number, PIC – polymorphic information content, F_{IS} – fixation index

Observed Wright's fixation indexes showed positive values across all loci indicating slight deficiency of heterozygote animals compared to the Hardy-Weinberg equilibrium expectations. The F_{IS} reflects the average deviation of the population genotypic proportions from Hardy-Weinberg equilibrium for a locus. The F_{IS} can be considered also as the inbreeding coefficient of an individual with respect to the local subpopulation. In the analysed population the positive value of F_{IS} may indicate the increased homozygosity resulting from inbreeding level. According to the classification of *PIC*, the analyses showed low (*LEPR/Thr945Met*) or median (*LEP/Arg2059Cys* and *Pit1/g.1178A>G*) level of polymorphic information content across loci (Table 1). The effectiveness of loci allele impact in populations has been expressed by effective allele numbers. Comparison of loci N_e showed the highest effective allele numbers in *Pit1/g.1178A>G* locus. The observed N_e indicates a good level of genetic variability in Pinzgau cows population at the considered loci.

In the analysed population of Pinzgau cows comparable average value of milk production traits to the results of milk recording control in the Slovak Republic for years 2013/2014 was observed. The statistical analysis of *LEP/Arg2059Cys* locus impact showed significant effect on evaluated long-life milk production traits, with A as a desirable allele (Table 2). But the statistically significant differences ($P<0.05$) were found only in comparison of cows with AA and AB genotypes. Milk, protein and fat yield were significant by higher in homozygous AA cows. Similarly the analysis of *LEP/Arg2059Cys* genotype effect showed its significant influence on the evaluated traits ($P<0.05$). The CC homozygous cows had significantly more milk, protein and fat yield in long-life production compared to heterozygous individuals. The *Pit1/g.1178A>G* locus affected the average values of milk, protein and fat yield in relation to the each genotype group only non-significant. The cows with AA genotype had only slight tendency to produce more milk in the analysed production season.

Table 2. Average values of the evaluated production traits in relation to the different *LEP/Arg2059Cys*, *LEPR/Thr945Met* and *Pit1/g.1178A>G* genotypes

Genotypes	N	Traits (average)						
		Milk yield, kg	P	Protein yield, kg	P	Fat yield, kg	P	n
LEP/Arg2059Cys								
AA	38	20408.0	+	711.2	+	776.0	+	36
AB	42	14919.6	+	520.9	+	565.2	+	42
BB	5	15199.4		552.2		610.2		5
LEPR/Thr945Met								
CC	70	18384.8	+	642.6	+	699.9	+	69
CT	15	12054.1	+	422.0	+	459.4	+	14
Pit1/g.1178A>G								
AA	6	23844.2		789.9		868.1		6
AB	48	17386.8		607.1		658.9		46
BB	31	15950.0		567.0		619.5		31

+ significance of difference at $P<0.05$

The impact of selected loci on the economically important traits was evaluated in many recently published studies. On the contrary to our results, Liefers et al. (2002) reported in study of 613 Holstein cattle the association between the leptin genotype and milk production when the AB genotype was associated with higher milk yield. Heravi et al. (2006) also evaluated the association of genetic differences in *LEP* gene, milk production and reproduction traits. A significant association in total of 230 Iranian Holstein cows was detected between the *LEP/Arg2059Cys* polymorphism and 305-d milk yield. Javanmard et al. (2010) found in Iranian cattle significant association between *LEP/Arg2059Cys* polymorphism and milk fat. Significant effect of SNP *Arg2059Cys* on calving interval and weight at first calving was reported by Almeida et al. (2003) in study of 160 beef cows. Significant association between fat and protein content in milk and *LEPR/Thr945Met* genotype in 219 Jersey cows was confirmed by Komisarek and Dorynek (2006), when animals with TT genotype were characterized by the lowest values of both traits. On the contrary, Giblin et al. (2010) reported no significant effect of the analysed SNP on milk production and reproduction performance in 848 Holstein cattle, but significant associations were found between other polymorphisms in *LEPR* gene and energetically expensive process of lactogenesis, energy storage and fertility performance. Clempson et al. (2011) reported only a weak association of *LEPR/Thr945Met* polymorphism with milk yield and days to first service in 509 Holstein cows. On the contrary to our results the significant impact of *Pit-1* gene polymorphism was confirmed by Edriss et al. (2009) for reproduction performance and by Yang et al. (2010) for body growth. The effect of *Pit1/g.1178A>G*

locus on milk production was found by De Mattos et al. (2004) when the heterozygous AB genotype was superior for fat milk production. Viorica (2007) reported associations between *Pit1/g.1178A>G* allele A and better milk performance in Simmental cattle. Negative influence of BB genotype on fat and protein yield and positive on birth weight was found in the study by Edriss et al. (2009).

CONCLUSION

The selection of animals based on the *LEP/Arg2059Cys*, *LEPR/Thr945Met* or *Pit1/g.1178A>G* genotypes could result in production traits improvement in dairy cattle. It is generally accepted that the leptin and its receptor has important role during pregnancy, in feed intake, energy expenditure, growth and reproduction and therefore can be considered as a strong candidate gene for economically important production traits. The results of present study confirmed mainly the significant impact of *LEP* and *LEPR* genes on milk production traits. Since our study was biased by small sample size, the analysis of bigger population is recommended. That would increase the significance of the statistical analysis and also the reliability of the obtained results.

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POPULATION ANALYSIS OF THE LOCAL ENDANGERED PŘEŠTICE BLACK-PIED PIG BREED

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Original scientific paper

SUMMARY

The pedigree analysis of the local endangered Přeštice Black-Pied pig breed (n=19 289) was performed. Animals born within the period 2012-2014 were assumed as the reference population (n=1 374). The pedigree completeness index reached 100% for four generations back. The 100 % of the genetic pool was explained by 66 ancestors. Although all animals of the reference population were inbred, 57% of them had inbreeding less than five percent. Average inbreeding, co-ancestry coefficient and rate of inbreeding reached 4.93%, 13.48% and 1.29% in reference population, respectively. The effective population size calculated by four different methods varied from 32 to 91 animals in 2014. Average generation interval, average family size for sire and dam parents was 2.5, 17.46 and 6.5 animals, respectively. Total number of founders, effective number of founders, effective number of founders' genomes and effective number of non-founders genomes reached values 299, 98.05, 21.92 and 28.23 founders, respectively. The average genetic diversity (GD) loss was 13.71% in reference population. The GD loss has increased within the last three year period mainly due to the random genetic drift (77.6%) and by unequal contribution of founders (22.4%). The Preštice Black-Pied breed is highly endangered with GD loss. Mating of closely related animals has to be prevented in breeding and mating program of this breed.

Key-words: pig, endangered, pedigree analysis, inbreeding, genetic diversity

INTRODUCTION

The Přestice Black-Pied breed (PBP) is a local Czech breed, whose origin comes to the end of the 19th century (Fiedler et al., 2004). The PBP breed is registered by the UN FAO as threatened with extinction and it is classified as Animal Genetic Resource – AnGR since 1992 (Vaclavková et al., 2012). The breed is kept in situ as AnGR in closed population from 1996. It is typical with the good reproduction and high adaptation to the environmental conditions. The mature weight of boars is 260-280 kg; whereas sow's mature weight varies from 215 to 235 kg. The objective of the presented study was to analyze the trend of genetic diversity using the parameters of probability of identity by descent and by gene origins in the pedigree datasets.

MATERIAL AND METHODS

The Czech Pig Breeders association provided the historical data. Pedigree information of the Přeštice Black Pied breed (PBP) contains 19 289 animals. Animals born between 1990 and 2014 with known sex were used in the study only. The animals born within years 2012-2014 were assumed as the reference population. Quality of the pedigree was calculated by pedigree completeness index (PCI) with algorithm explained by MacCluer et al. (1983) and by maximum number of generations traced back. The average generation interval was assumed as average age of parents at their offspring birth expressed in years. Different methods were used to compute the effective population size (N_e). Based on the above mentioned, the N_e was calculated as $N_e = \frac{1}{2 \times \Delta F}$, where ΔF is rate of inbreeding. The firstmethod was based on the average inbreeding of offspring and their direct parents (N_e-ΔFp). The average inbreeding of offspring and average inbreeding of average parent's generation was the next method

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(N_e-ΔFg). Both methods were described by Falconer and MacKay (1996). Pérez-Ensico (1995) algorithm, based on logarithmic regression of *ln(1-F)* on the year of birth (N_e-*ln*), was used as the further method. The last method (N_e–*Ecg*) was based on rate of individual inbreeding coefficient calculated as $1-\sqrt[t-1]{1-F_i}$, where parameter *t* is computed as the sum of overall known ancestors of the term $(1/2)^n$ and was defined by Gutierrez et al. (2009).

Number of ancestors with unknown parents was assumed as total number of founders (f_t). Effective number of ancestors (f_a) was calculated as the minimum number of ancestors necessary to explain the total genetic diversity of the studied population by algorithm of Boichard et al. (1997). Effective number of founders f_e (Lacy, 1989), was defined as the number of equally contributing founders that would be expected to generate a similar amount of genetic diversity as in the population under the study. Effective number of founder genomes (f_{ge}) was defined as the number of equally contributing founders with no random loss of founder alleles that would give the same amount of genetic diversity as is presented in the population under the study and was calculated using the algorithm of Caballero and Toro (2000). Effective number of non-founders genomes (f_{ne}) was derived from difference of inverted values of f_{ge} and f_e. The genetic diversity loss was derived from f_e, f_{ge} and f_{ne}. Total genetic diversity (GD) of the reference population was calculated according to Lacy (1995). The loss of the genetic diversity due to unequal number of founders (GDLf) was expressed as 1 – GD*, where GD* was calculated by Caballero and Toro (2000) as $1-\frac{1}{f_e}$. The loss of GD due to the random genetic drift (GDLd) was calculated as the inverse of $2f_{ne}$ (Caballero and Toro, 2000). Detailed description of the presented methods can be found in Krupa et al. (2015). The POPREP package (Groeneveld and Lichtenberg, 2010), the CFC software package (Sargolzaei et al., 2006) and the PEDIG software (Boichard, 2002) were used for the analysis.

RESULTS AND DISCUSSION

The accuracy of the computed inbreeding and of consequently derived parameters depends mainly on the quality of analyzed datasets. The calculated pedigree completeness index (Figure 1) obtained maximum values (100%) up to four generations deep within the last five years. The PCI declined when four generations and more were considered. As mentioned by Gutierrez et al. (2003), a pedigree completeness level has sizable effect on the estimation of inbreeding coefficient, because the change of finding of common ancestors increases together with level of pedigree completeness.

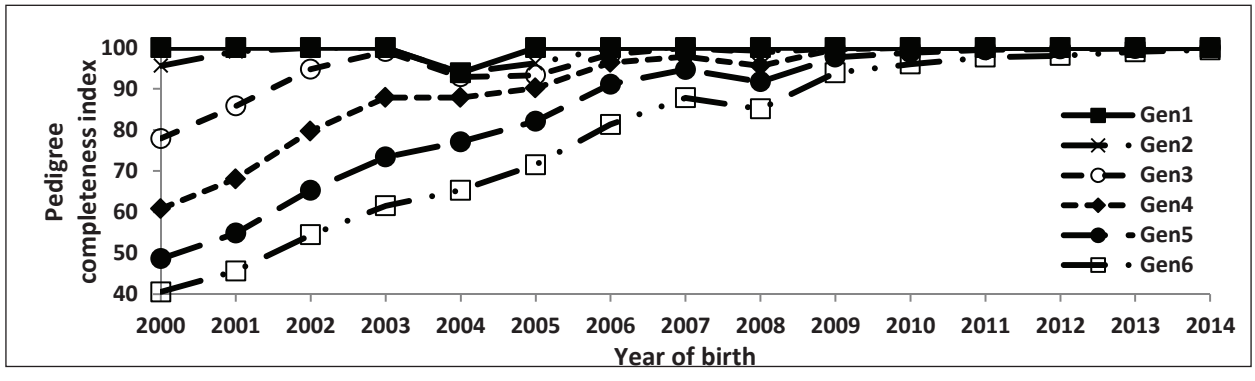


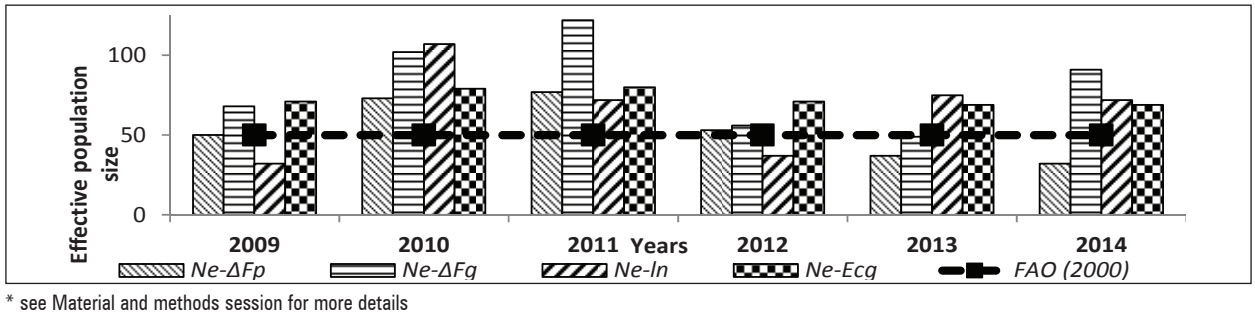
Figure 1. Pedigree completeness index

The basic characteristics of the reference population along with calculated inbreeding and co-ancestry coefficients are summarized in Table 1. The number of active animals (reference population) increased in the last years. The number of inbred animals, sires and dams was equal to hundred percent in reference population. Similarly, in our previous study (Krupa et al., 2015) high proportion of inbred individuals in reference population of five pig breeds was found, and exceeded 50% from all of the evaluated animals and 70% of sires. The average rate of inbreeding reached relative high value (1.29%) and exceeded the limits recommended by FAO (2000). The fact that PBP breed is kept as closed population could be a main factor of the prevalence of inbred animals in reference population. It is also important to mention that in reference population 57% of inbred animals had inbreeding coefficient up to five percent. The average inbreeding coefficient in reference population was 4.9% (±2.4%) and varied from 1.1% (minimum) to 17.3% (maximum). The average co-ancestry coefficient was 13.5% whereas the average rate of inbreeding in reference population was 1.3%.

Table 1. Base characteristics and inbreeding of the reference population

Trait	Value
Number of animals in pedigree	19289
Number of animals in reference population	1374
Maximum number of generations traced back	13
Proportion of inbred animals / Sires / Dams (%)	100/100/100
Average inbreeding (%)	4.93
Average co-ancestry (%)	13.48
Average increase of inbreeding - ΔF (%)	1.29

The effective population size (N_e) is one of the main parameter pointed to genetic variability of the population. The results of N_e computed by four different methods are shown in Figure 2. As Groeneveld and Lichtenberg (2010) mentioned, the choice of the best method depends on various conditions like used time window, PCI and also on changes in N_e from year to year. The decision tree in detail can be found in the population structure report as a result of the POPREP software (Groeneveld and Lichtenberg, 2010). Generally, the N_e values obtained in our study were relatively low and varied from 32 to 91 animals in 2014. As the most appropriate methods seems to be N_e-*ln* and N_e-*Ecg*. The N_e -*Ecg* method was the most stable, with minimal changes between the years. The N_e-*ln* method used the shortest time window, which allowed quickly responding to changes in population size and structure. It should be used as the default method.



* see Material and methods session for more details

Figure 2. Trends in the effective population size computed by different methods*

Parameters derived from analysis of gene origin of reference population are summarized in Table 2. The total number of founders and effective number of founders was 299 and 98, respectively suggesting the excessive use of certain animals in the reference population. Moreover, the ratio between the above mentioned parameters points to disequilibrium among founders in the analyzed population. On the other side, this ratio is not such high as the one observed for other Czech pig breeds (Krupa et al., 2015). It is probably caused by the fact that PBP breed is not under so high intensive selection compared to commercial pig's populations. Whilst the f_e / f_t ratio explained the GD loss caused by unequal contribution of founders, the f_{ge} / f_e ratio can be used to quantify GD loss due to random genetic drift. In generally, the lower values refer to the higher prevalence of GD loss. In our study, impact of random genetic drift on GD loss was higher compared to unequal contribution of founders when value for this ratio was lower than for f_e / f_t ratio (Table 2). The most influential ancestor explained 10.54% of genetic diversity. The trend in GD loss together with the average inbreeding coefficient is shown in Figure 3. The GD loss increases continuously within the last three year period with the average value of 13.71% in the reference population. The loss of GD was caused mainly by random genetic drift (77.6%) and unequal contribution of founders (22.4) in the reference population. Furthermore, the value of GD loss due to random genetic drift over the years increased whereas the value of the GD loss due to unequal founder contribution diminished.

Table 2. Parameters of gene origin for the reference population

Parameter	Value
Total number of founders(f_t)	299
Effective number of founders (f_e)	98.05
Effective number of founders genomes (f_{ge})	21.92
Effective number of non-founders genomes (f_{ne})	28.23
f_e / f_t and f_{ge} / f_e ratio	0.33/0.22
Number of ancestor explaining 50/75/100 % of genetic variability	7/13/66
Average generation interval (years)	2.5
Average family size for male / female parents	17.46/6.5

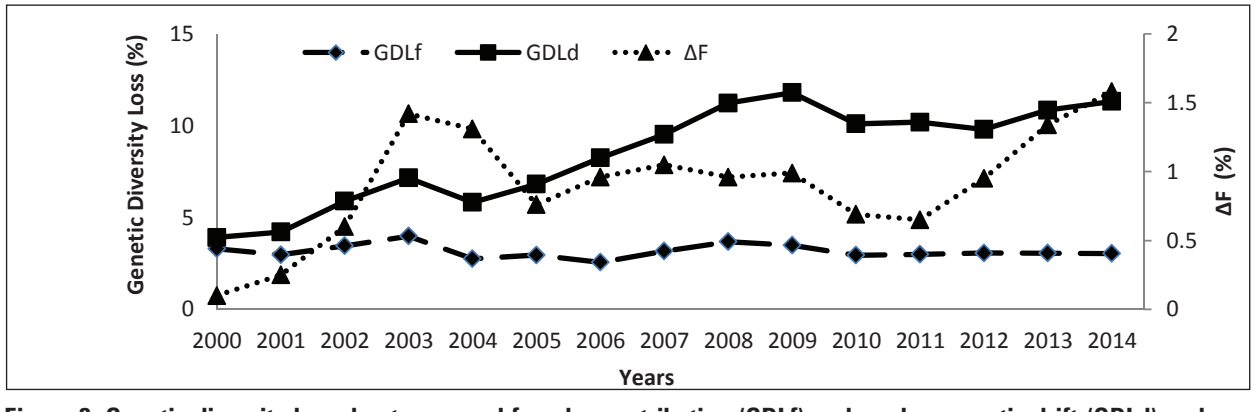


Figure 3. Genetic diversity loss due to unequal founder contribution (GDLf) and random genetic drift (GDLd) and average rate of inbreeding (Δ*F*)

CONLUSION

The comprehensive pedigree analysis of the reference population of Přeštice Black-Pied pig breed was performed. The breed has been characterized by adequate quality of pedigree. Values of the average inbreeding and co-ancestry coefficients are high and increase from one generation to other. Parameters derived from the gene origin analysis are pointing to the continuous genetic diversity loss, caused mainly by random genetic drift. It is very important to avoid next genetic diversity loss. The breeding must be focused on mating the animals with lowest relationship with the each other in order to prevent increasing the inbreeding in next generations.

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GENETIC CONTRIBUTION OF RAM ON LITTER SIZE IN ŠUMAVA SHEEP

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Original scientific paper

SUMMARY

The objective of the present study was to quantify the service sire effect in terms of (co) variance components of born and weaned lambs number and to propose models for the potential inclusion of this effect in the linear equations for breeding value estimation. The database with 21,324 lambings in Šumava sheep from 1992-2013 was used. The basic model equation for the analysis of variance of litter size contained effects of ewe´s age at lambing, contemporary group, permanent environmental effect of ewe and direct additive genetic effect of ewe. Two modifications of the basic model were used for estimation of service sire effect. The proportions of variance for the service sire effect for number of born and weaned lambs were 2.1% and 2.0%, when service sire was not included into relationship matrix; while included into the relationship matrix and dividing effect into genetic contribution and permanent environment effect refer that nongenetic effect seems to be bigger than genetic (0.013 vs. 0.009 for number of born and 0.017 vs. 0.004 for number of weaned). Changes in other variance components were relatively low, except of contemporary group. Model including service sire effect as a simple random effect without genetic relationship matrix inclusion is recommended for genetic evaluation of litter size traits.

Key-words: service sire effect, genetic parameters, reproduction

INTRODUCTION

The contemporary Šumava sheep is the successor of autochthonous landrace of sheep kept in Šumava Mountains in the South Bohemia and plays a crucial role in environmental system of Šumava National Park. Gradual regeneration of this local breed led to rams and ewes selection with similar phenotype to original population (Jandurova et al., 2005). Šumava sheep belongs to breeds of medium body size and general utilization. Single lambs were preferred in the past time, because they needed to walk for long distances at low quality grazing pasture. However, according to increasing economic value of meat relative to wool and the increased importance of lamb and sheep meat and milk production in recent years (Krupova et al., 2013) it mean that improving reproductive traits has high economic significance (Wang and Dickerson, 1991; Wolfova et al., 2011a, 2011b).

Litter size is a complex trait influenced by a paternal, maternal and fetal component (Hamann et al, 2004). Usually breeding schemes in sheep only include the maternal component of litter size as fertility trait. Service sire can influence both fertilization rate and prenatal survival rate.

Until recently, the service sire effect has not been studied in sheep breeds in the Czech Republic (Schmidova et al., 2014; Vostry and Milerski, 2013), and no information on this effect has been available for Šumava sheep. Therefore, the objective of the present study was to quantify the service sire effect in terms of (co) variance components of litter size and number of weaned lambs and to propose models for the potential inclusion of this effect in the linear equations for breeding value estimation.

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MATERIAL AND METHODS

Data

Performance test data from 1992-2013 were provided by the Sheep and Goat Breeders Association of the Czech Republic. The database with records of 29,401 lambings in Šumava sheep contained information on: animal (lambing ewe), herd, date of lambing, parity, ewe age at lambing, interval between successive lambings, service sire, number of born and number of weaned lambs. Sire and dam identification were added from the pedigree database. Four generations of the known ancestors were used for the estimation of genetic parameters. Number of born lambs was recorded on the day of lambing as total number of lambs born. Number of weaned lambs was recorded as number lambs weight at 80-120 days. Only ewes, that had lambed, were in the database (at least one lamb independently if it was alive or dead). The following records were deleted from the database prior to analysis: ewes lambing at younger than 10 months or older than 150 months of age, lambing ewes whose sire had less than 4 daughters with performance. Ewe age at lambing time was categorized into 6 classes divided according to age (10-18 months, 19-30, 31-42, 43-78, 79-102 and 103-150 months of age. Contemporary group (CG) effect was created with ewes lambed within successive 40-day intervals in a given herd and year constituting the CG's (Schmidova et al., 2014). Those CG's with fewer than 7 ewes were excluded from variance component estimation analyses. Also herds using only one ram and rams acting only in one herd-year were excluded.

The database adjusted in this way contained data on 21,324 lambings from 5,984 ewes and 396 rams bred in 35 herds.

Statistical methods

The basic model equation for the analysis of litter size variance was determined based on the single-trait repeatability model (Schmidova et al., 2014):

Model 1: $LS_{ijk} = A_i + CG_j + Ew_k + Epe_k + e_{ijk}$

where LS_{ijk} is the litter size of animal k (number of born or weaned lambs); A_i is the age class at lambing; CG_j is the random effect of contemporary group; Ew_k is the random direct additive genetic effect of ewe k; Epe_k is the random permanent environmental effect of ewe k; e_{ijk} is the random residual.

Two modifications of the Model 1 were used for estimation of service sire effect:

Model 2: $LS_{ijkl} = A_i + CG_j + Ew_k + Epe_k + S_l + e_{ijkl}$

Model 3: $LS_{ijkl} = A_i + CG_j + Ew_k + Epe_k + SG_l + Spe_l + e_{ijkl}$

S_l is the random effect of service sire l (Model 2); SG_l is the random direct additive genetic effect of service $sire_l$ (Model 3); Spe_l is the random permanent environmental effect of service sire l (Model 3).

Variance components were estimated by the Gibbs sampling method using the GIBBS1F90 program (Misztal et al., 2002). After some exploratory analyses one chain of 700,000 samples was used, rejecting the first 80,000 samples and saving every 100 thereafter.

RESULTS AND DISCUSSION

Distributions of number of lambs, means and standard deviations of lambs born and weaned are presented in the Table 1.

Table 1. Distribution of the number of lambs in litter, total number of records, mean and standard deviation (SD) of litter size

	Litter size					Total number of records	Mean	SD
	0	1	2	3	4			
No. at lambing	*	14,867	6,261	193	3	21,324	1.31	0.48
		69.72%	29.36%	0.91%	0.01%			
No. at weaning	1,908	14,400	4917	98	1	21,324	1.15	0.56
	8.95%	67.53%	23.06%	0.46%	0.00%			

* Only ewes, that had lambed, were in the database (at least one lamb independently if it was alive or dead)

Table 2 documents variance components and genetic parameter estimations for both litter size traits, as computed from repeatability models. The basic model (Model 1) shows low heritability and repeatability estimates. Similar heritability and repeatability for litter size in Šumava sheep was reported in Schmidova et al. (2014), the study also showed these values as the lowest ones in comparison of seven breeds.

Table 2. Variance components and genetic parameters for number of born and number of weaned lambs in Šumava sheep for different models

	σ_e^2	σ_p^2	σ_{Ew}^2	σ_{Ewpe}^2	σ_{CG}^2	σ_S^2	σ_{Spe}^2
Born							
model1	0.187	0.226	0.014	0.004	0.022		
model2	0.186	0.225	0.013	0.005	0.017	0.005	
model3t	0.186	0.225	0.013	0.005	0.016	0.002	0.003
Weaned							
model1	0.257	0.315	0.014	0.002	0.049		
model2	0.255	0.313	0.013	0.003	0.034	0.006	
model3t	0.255	0.313	0.014	0.003	0.036	0.001	0.005
	h^2(SE)	r_{rep}^2	Ew_{pe}^2	e^2	CG^2	S^2(SE)	S_{pe}^2
Born							
model1	0.061(0.007)	0.080	0.019	0.825	0.096		
model2	0.057(0.008)	0.078	0.021	0.827	0.075	0.021(0.007)	
model3t	0.058(0.008)	0.078	0.020	0.826	0.073	0.009(0.005)	0.013
Weaned							
model1	0.045(0.006)	0.053	0.007	0.815	0.133		
model2	0.042(0.007)	0.051	0.008	0.813	0.116	0.020(0.005)	
model3t	0.043(0.005)	0.052	0.008	0.812	0.115	0.004(0.004)	0.017

$\sigma_e^2\sigma_e^2$ = residual variance; $\sigma_{Ew}^2\sigma_{Ew}^2$ = additive genetic variance of ewe´s (maternal) performance; $\sigma_{Ewpe}^2\sigma_{Ewpe}^2$ = ewe´s (maternal) permanent environmental variance; $\sigma_S^2\sigma_S^2$ = additive genetic variance of sire´s (paternal) performance; $\sigma_{Spe}^2\sigma_{Spe}^2$ = sire´s (paternal) permanent environmental variance; $\sigma_{CG}^2\sigma_{CG}^2$ = contemporary group variance; $\sigma_p^2\sigma_p^2$ = phenotypic variance; h^2 = ($\sigma_{Ew}^2\sigma_{Ew}^2$/ $\sigma_p^2\sigma_p^2$) = maternal heritability; $r_{rep}^2 r_{rep}^2$ = (($\sigma_{Ew}^2\sigma_{Ew}^2$ + $\sigma_{Ewpe}^2\sigma_{Ewpe}^2$)/ $\sigma_p^2\sigma_p^2$) = maternal repeatability; $Ew_{pe}^2 Ew_{pe}^2$ = ($\sigma_{Ewpe}^2\sigma_{Ewpe}^2$/ $\sigma_p^2\sigma_p^2$) = permanent environmental variance as a proportion of phenotypic variance; e^2 = ($\sigma_e^2\sigma_e^2$/ $\sigma_p^2\sigma_p^2$) =residual variance as a proportion of phenotypic variance; CG^2 = ($\sigma_{CG}^2\sigma_{CG}^2$/ $\sigma_p^2\sigma_p^2$) = variance of contemporary group as a proportion of phenotypic variance; S^2=($\sigma_S^2\sigma_S^2$/ $\sigma_p^2\sigma_p^2$) = paternal heritability; $S_{pe}^2 S_{pe}^2$= ($\sigma_{Spe}^2\sigma_{Spe}^2$/ $\sigma_p^2\sigma_p^2$) = paternal permanent environmental variance as a proportion of phenotypic variance

The proportions of variance for the service sire effect for number of lambs born and weaned were 2.1% and 2.0%, when service sire was not included into relationship matrix (Model 2). While included into the relationship matrix dividing effect into genetic contribution and permanent environment effect (Model 3) refer that nongenetic effect seems to be bigger than genetic (0.013 vs. 0.009 for number of born and 0.017 vs. 0.004 for number of weaned). Changes in other variance components were relatively low, except of contemporary group. This is probably due to low number of rams in one flock.

Hagger (2002) found out a small influence of service sire effect on litter size in four breeds (0.7%-2.9%). Also the proportion of variance for service sire effect for litter size traits in pigs was in range from 2 to 3% (Wolf and Wolfova, 2012). Mohammadi et al. (2012) found out service sire effects to be important only for litter weight traits.

Nevertheless, it is well known that rams with health problems or deficiencies in sperm production can be the reason for insufficient litter sizes in a flock. Serious reproduction problems can arise if rams show sperm deficiencies or suffer from handicaps in locomotion, e.g. foot rot during time of joining. Also less severe disorders of rams could affect litter size (Hagger, 2002). The social relationships that an animal has with others of the same species can affect many aspects of the reproductive process too (Rosa and Bryant, 2002). Rams with high scores for sexual behaviour can improve flock fertility during breeding (Perkins et al., 1992).

CONCLUSION

Litter traits are generally considered as ewe traits. The results show that the service sires in Šumava sheep have a small, but nevertheless a clearly detectable influence on the litter size under the management systems practised. Model including service sire effect as a

simple random effect without inclusion of the genetic relationship matrix is recommended for genetic evaluation of litter size traits.

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GENETIC DIVERSITY IN CZECH HAFLINGER HORSES

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Original scientific paper

SUMMARY

The Haflinger as a small moutain horse breed originated from the South Tyrol district as a cross of Alpen Mountain breeds with Araber. This breed was expanding to Czech Republic during the last 25 years. The aim of this study was to analyse genetic diversity within the population using microsatellite markers. A total of 95 alleles have been detected. The highest frequency 88.18% showed allele 101 (HTG 6). The heterosigosity varied from 0.25 (HTG 6) to 0.84 (VHL 20), genetic diversity reached 0.6–0.8. The heterozygosity of the whole population studied is F_{IS}= -0.013. The average effective number of allele per locus was 2.93 with standard deviation 1.54, with minimal and maximal level 1.30 and 7.83, respectively. Average polymorphism information content per locus was 0.608 with standard derivation 0.146, with minimal and maximal level 0.208 and 0.824, respectively. The results showed that breeding program of Czech Haflinger is optimal, including optimized mating strategies. The diversity of the population Czech Haflinger, based on a small number of microsatellites, seems to be sufficient.

Key-words: microsatellite data, homozygosity by loci, genetic diversity

INTRODUCTION

The inbreeding coefficient is defined as the probability that, in a locus sampled randomly in a population, a pair of alleles is identical by descent with respect to a base population where all alleles are independent (Wright, 1922). The consequences of inbreeding are the loss of genetic variation, accumulation of recessive lethal genetic mutations and worsening of performance in production traits and fertility. Therefore, evaluating genetic diversity and relationship within and amongst populations of animals is a prerequisite for developing meaningful breeding programmes.

Inbreeding coefficients usually have been calculated from a pedigree, and the probability that a pair of alleles is identical by descent is estimated from statistical expectations. However, the recent availability of methods of molecular genetics has opened opportunities for using genomic information in animal breeding.

The development of tools for the analysis of DNA taking place in the last few decades has increased enormously the capacity to characterise variation within breeds. The microsatellites have been markers of choice to study genetic variation in the recent years. Based upon sites in which the same short sequences is repeated multiple times, they present a high mutation rate and codominant nature, making them appropriate for the study of both within- and between –breed genetic diversity.

The Haflinger horse is a breed of horse developed in the South Tyrol region during the late 19th century. This breed is a product of Alpen Mountain breeds with Araber cross. Until recently, the molecular genetic diversity has not been studied and no information on this diversity has been available for Czech Haflinger horse. The objective of this study is to determine the genetic diversity in the Czech Haflinger horse based on microsatellite markers.

MATERIAL AND METHODS

Blood samples were collected randomly from 369 horses in Czech Haflinger population within a 12-year period (2000-2012). Genomic DNA was isolated from the whole blood using the NucleoSpin Blood Kit (Clontech Laboratories, Palo Alto, CA, USA). Genotyping included 13 microsatellite loci (VHL20, HTG4, AHT4, HMS7, HTG6, AHT5, HMS6, ASB2, HTG10, HTG7, HMS3, HMS2 and HMS1) scattered at 8 chromosomes.

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Allele frequencies, observed heterozygosity, genetic diversity [heterozygosity expected assuming Hardy-Weinberg equilibrium (HWE)], an HWE test and genetic distances were estimated across the different loci and lines using the TFPGA 1.3 software package (Miller, 1997).

Heterozygosity in the whole studied population was evaluated by within-population inbreeding estimate also known as fixation index (F_{IS}) at each microsatellite locus. F_{IS} were computed by the FSTAT program (Gaudet, 2001) according to the following formula:

$$F_{IS} = 1 - \frac{H_O}{He}$$

where: H_0 – observed heterozygosity, H_e - expected heterozygosity. Effective number of allele were calculated by the following formula:

$$effective\ numberof\ \ alllele = \frac{1}{1 - D_j}$$

where: D_j – genetic diversity of locus *j*. The polymorphic information content was calculated using PICcalc (Nagy, 2012).

RESULTS AND DISCUSSION

The total number of alleles detected at 13 microsatellite loci in the Czech Haflinger population was 86. Microsatellites were highly polymorphic. The average number of alleles per microsatellite locus was 8.25 (±2.6) with a range of 4 to 13. Higher number of alleles for each locus suggested that all the markers used were appropriate to analyse genetic diversity. A more appropriate measure of genetic variation within a population was gene diversity (Nei, 1987). An estimated average for the observed heterozygosity across microsatellite loci was relatively high 0.656, while the estimated mean value of genetic diversity was 0.663. The heterozygosity observed for each of the microsatellites ranged from 0.217 for the HTG6 microsatellite to 0.844 for the VHT20 microsatellite. As with heterozygosity, the lowest value of genetic diversity was found out in the HTG6 microsatellite (0.228) and the highest value of genetic diversity was achieved via the VHL20 microsatellite (0.872). This corresponds to the value of the effective size of the alleles. Big difference was noticed between the total number of alleles and effective number allele by all loci. This difference indicates again the increase of homozygosity in population. These values are relatively high and therefore the population appeared sufficiently heterogeneous. Tekezaki and Nei (1996) determined that for markers to be useful measuring genetic variation, they should have an average heterozygosity ranking form 0.3 to 0.8 in the populations. General information on differences and aggregate statistics is shown in Table 1. High level of polymorphism is also the average value of polymorphic information content (PIC=0.61). PIC is calculated with the total number of alleles and allele frequencies in a population. If PIC value is higher than value 0.75 the locus becomes much more informative. This level is not exceeded at most loci. Statistically conclusive deviation from HWE was detected only at VHT20 ($P<0.01$) loci. Other loci were in agreement with HWE. Similar values of the observed heterozygosity and genetic diversity were also found out in Spanish Celtic horses (Canon et al., 2000), Lipizzaner horses (Achmann et al., 2004), German draft horses (Aberle et al., 2004) and Biłgorai horses (Zabek et al., 2005). Conversely, Iwanczyk et al. (2006) reported that the values of heterozygosity and genetic diversity of Polish heavy horses were considerably lower.

The heterozygosity of the whole population studied is F_{IS} =-0.013±0.031 whereas the average value of F_{IS} reached a negative number. According to Hamilton (2009) it can be concluded that there was no reduction of heterozygosity. However, this value is close to zero. This detected nonsignificant values indicating genetic variability increase in the population. The increased value of heterozygosity corresponds to reality that Czech Haflinger is open population for gene flow from other Haflinger populations. However, the utility of molecular marker information is rather limited, especially if high-quality pedigrees are available (Toro et al., 2009). The value of estimated populations parameters, which are considered as measure of inbreeding based on marker data, are very different from inbreeding coefficient estimated by Majzlík et al. (2012) based on pedigree information (Fx=0.84% with variation from 0 to 4.69%). The estimated inbreeding coefficient based on pedigree analysis in the 2012 does not correspond with molecular data. The molecular inbreeding measured with a handful of molecular markers is not necessarily a good predictor of the genealogical or genomic inbreeding, that there are problems in estimating genomic heterozygosity using only a few molecular markers (Toro et al., 2009).

Table 1. Characteristics and summary statistics for microsatellite loci analysed in the population of the Czech Haflinger Horses

Locus	No. of alleles	Observed heterozygosity	Genetic diversity	Chromosomal Location	Effective number of allele	F_{IS}	PIC	Hardy Weinberg equilibrium
VHL20	11	0.8442	0.8723	30	7.83	-0.031	0.824	p = 0.0000
HTG4	7	0.6112	0.6005	9	2.50	0.011	0.535	p = 0.8347
AHT4	9	0.7571	0.7826	24	4.60	-0.036	0.718	p = 0.1918
HMS7	8	0.7059	0.6658	1	2.99	0.057	0.653	p = 0.2794
HTG6	8	0.2171	0.2283	15	1.30	-0.051	0.208	p = 0.4496
AHT5	8	0.7378	0.7391	8	3.83	-0.002	0.700	p = 0.3903
HMS6	5	0.6749	0.7092	4	3.44	-0.054	0.611	p = 0.1040
ASB2	13	0.7198	0.7391	15	3.83	-0.024	0.675	p = 0.9142
HTG10	9	0.7278	0.7147	21	3.51	0.019	0.687	p = 0.5908
HTG7	4	0.6079	0.6250	4	2.67	-0.031	0.560	p = 0.5263
HMS3	9	0.6993	0.6984	9	3.32	-0.001	0.653	p = 0.1698
HMS2	11	0.6599	0.6630	15	2.97	-0.006	0.610	p = 0.4054
HMS1	13	0.5642	0.5788	15	2.37	-0.029	0.475	p = 0.5319
Mean	8.25	0.6559	0.6628	-	2.97	-0.013	0.608	-
SD		0.150	0.152	-	1.54	0.031	0.149	-

CONCLUSION

The chosen set of microsatellite markers has confirmed the high polymorphism and its usefulness in estimating genetic diversity by Czech Haflinger. The results of the analysis based on microsatellite data show a high heterozygosity in the population. The diversity of the population Czech Haflinger, based on a small number of microsatellites, seems to be sufficient.

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LIFETIME PRODUCTION OF SLOVENIAN LOCAL GOAT BREEDS

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Original scientific paper

SUMMARY

The objective of the study was to analyze lifetime production data for two Slovenian locally adapted dairy breeds: Slovenian Saanen goat (334) and Slovenian Alpine goat (1105) and for the dairy type of Dreznica goat (141) which is the only Slovenian autochthonous goat breed. Dataset included records from 54 farms. Data for does born after 2002 have been obtained from the database of the National selection program for small ruminants, collected by the ICAR standards. The contribution of farm to phenotypic variance was estimated. Data was analyzed by MIXED procedure in SAS/STAT. The results showed significant effect of breed, farm and year of culling on all traits studied, except the effect of breed on completed lactations in lifetime and number of liveborn kids. The lifetime milk yield was higher in Slovenian Alpine goat compared to Slovenian Saanen goat by 413.26±172.52 kg. The difference in lifetime protein yield between Slovenian Alpine goat and Slovenian Saanen goat amounted to 11.76±5.21 kg. Dreznica goat did not differ in lifetime milk production and protein yield compared to both intensive goat breeds. Dreznica goat yielded about 25.50±5.21 kg more fat in lifetime compared to Slovenian Saanen goat. However, compared to Slovenian Alpine goat the difference was not significant. Comparison of Slovenian Saanen goat and Slovenian Alpine goat revealed higher lifetime fat yield of Slovenian Alpine goat by 13.28±5.21 kg. The results suggested reasonably good performance and adaptation of the autochthonous breed Dreznica goat in local agro climatic conditions.

Key-words: Slovenian Saanen goat, Slovenian Alpine Goat, Dreznica goat, lifetime production, milk yield

INTRODUCTION

Over the last few years, small ruminants breeding in Slovenia have become more important due to the extensive farming on grassland and pastures. The number of goats has been on the rise since the start of the nineties and up to 2009 when the total number was the largest - 29.896 goats (Statistical Office RS, 2014). Currently, there are about 22,000 goats kept on approximately 3,000 farms in total.

Three local dairy goat breeds are mainly used in Slovenia: the locally adapted Slovenian Alpine goat (the number of the purebred does is around 4,000) which is the most numerous dairy breed in the country and the locally adapted Slovenian Saanen goat with the number of the purebred does around 2,500 (Register of breeds with zootechnical assessment, 2014). Both dairy breeds are widespread throughout the whole territory of Slovenia and they are considered endangered according to their reproduction capacity, population trend and pure-breeding proportion. The third dairy goat breed is the only Slovenian autochthonous goat breed named Dreznica goat. This breed is the least numerous goat breed in Slovenia. It is composed of about 650 heads and listed as a critical breed due to the concentration of a major part (90%) of the total population in a restricted geographical area, which means within a radius of less than 30 km (Žan Lotric et al., 2013).

The population of Dreznica goat is divided into two types by the purpose of breeding and its original location. Dairy type of Dreznica goat was developed in Bovec area where cheesemaking tradition dates back to the 13th century, whereas meat type of Dreznica goat has developed in Dreznica region. Flocks of goats for meat productions are prevailing. Dreznica goat is a seasonal extensive breed, well adapted to the harsh Alpine

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conditions and it is kept also for landscape management in these regions. All does of the Slovenian local goat breeds are seasonally fertile and kid once per year.

In Slovenia goat milk does not have such economic importance and long tradition as it has in other European (e.g. Mediterranean) countries. However, there are some farms where goat milk producing is important. Although the economics of goat milk production is not on a high level, the demand for goat milk and dairy goat products is constantly rising and is recently greater than the supply. The main source of income in Slovenian local goat breeds, beside milk, is cheese, produced by farmers themselves. There are also two by-products known as ricotta and whey. Most dairy products are sold directly at home, as well as at the high and mountain pastures (in the case of dairy type Dreznica goat breed) where goats graze in the summer. There is unfortunately no information about the lifetime production of milk recorded Slovenian local goat breeds provided in the literature. To the best of our knowledge, very few studies about goat milk of Slovenian local goat breeds are available. Kompan and Kastelic (2009) reported how conventional and organic farming system affected productivity of Slovenian Alpine goat, while Žan Lotrič et al. (2006) reported how different altitude of mountain and highland pasture influences milk fatty acid composition of Slovenian locally adapted goat breeds.

Lifetime performance of a doe is the ultimate indicator of its utility in the flock and it is also important in investigating flock economics (Osman et al., 2010). Economic efficiency is mostly a result of achieved milk production and longevity (Heins et al., 2012; Martens and Bange, 2013). The aim of the study was to compare the lifetime production between three goat breeds in Slovenia and to estimate the contribution of flock to phenotypic variance.

MATERIAL AND METHODS

The production data of Slovenian Alpine breed, Slovenian Saanen breed and Dreznica breed, born between 2002 and 2015 and culled after the 2008, were used for the analysis of lifetime production. The data were obtained from the Slovenian national breeding program for small ruminants recording, collected by the ICAR standards (ICAR, 2014).

The dataset included 1,580 does kept on 54 farms, where the majority of records (1,105) were collected in Slovenian Alpine breed, the most important dairy goat breed in Slovenia. The number of records was considerably smaller in Slovenian Saanen breed (334) and Dreznica breed (141). Extensive farming, highly dependent on grazing, is mostly practiced on all farms that keep autochthonous Dreznica goat whereas most of Slovenian farms breed Slovenian Alpine and Slovenian Saanen goats in a more intensive production system.

The production traits were considered as follows: milk yield, fat yield, protein yield, number of completed lactations and number of liveborn kids. The lifetime production of the total milk, fat, and protein yield, number of liveborn kids in lifetime was calculated as the sum of production from the first to the last kidding.

The average milk yield in lifetime of all three breeds together was 1534.15 kg (σ=1230.15) with 49.51kg (σ=40.19) average fat yield and 46.47kg (σ=37.39) average protein yield (Table 1). Average age at culling was 72.12 months (σ=28.14) while average number of liveborn kids was 6.00 (σ=3.84).

Table 1. Descriptive statistics

Variable	N	$\bar{x}$	σ	min	max
Milk yield (kg)	1580	1534.15	1230.15	47.20	8341.95
Fat yield (kg)	1578	49.51	40.19	0.69	232.92
Protein yield (kg)	1578	46.47	37.39	1.59	225.23
Lactations	1580	4.04	2.24	1.00	10.00
Age at culling (months)	1580	72.12	28.14	15.00	21.00
Liveborn kids	1580	6.00	3.84	0	21.00

N–number of observations; σ–standard deviation; $\bar{x}$ -mean; min–minimum; max–maximum

Statistical analysis

Lifetime production traits were analyzed using the following statistical model (eq. 1):

$$y_{ijkl} = \mu + Y_i + B_j + f_k + e_{ijkl} \quad (1)$$

where y_{ijkl} is analyzed trait, μ is intercept, Y_i is fixed effect of year of culling (i=1,2, …,7) and B_j is fixed effect of breed (j=1,2,3), f_k is farm as random effect (k= 1,2, …,54), and e_{ijkl} is a residual. Analyses were conducted using MIXED procedure in SAS/STAT statistical package (SAS Institute, 2011). The restricted maximum likelihood method was applied. Preliminary results showed sufficient homogenous residual variance structure.

RESULTS AND DISCUSSION

Lifetime production of goats was affected by culling year and farm (Table 2; Figure 1A; Figure 1B). Differences between breeds were obtained in milk, fat and protein yield; however breeds did not differ in number of liveborn kids and number of completed lactations.

The results revealed the significant variance for farm effect (Table 2). The farm contributed similar proportion of phenotypic variation in milk traits: 36.11% in milk yield, 35.03% in fat yield, and 34.16% in protein yield. The contribution of variation caused by farm was smaller in liveborn kids (23.16%) and a number of completed lactations (25.10%).

Table 2. Statistical significance of effects on production traits and estimates of variance components with standard errors

Trait	p-values			Estimates of variances	
	Year	Breed	Farm	σ_f^2	σ_ε^2
Milk yield (kg)	<.0001	0.0454	<.0001	565146±138652	999963±36249
Fat yield (kg)	<.0001	0.0199	<.0001	585.11±149.55	1085.39±39.41
Protein yield (kg)	<.0001	0.0752	<.0001	484.09±121.50	932.83±33.84
Lactations	<.0001	0.1557	0.0001	1.26±0.34	3.76±0.14
Liveborn kids	<.0001	0.5403	0.0001	3.52±0.95	11.67±0.42

σ_f^2 –variance component of farm (in squared units); σ_ε^2-residual variance component (in squared units)

Lifetime milk yield was higher in Slovenian Alpine goat compared to Slovenian Saanen goat by 413.26 ±172.52 kg (Figure 1A); however results showed that Dreznica goat did not differ in lifetime milk production compared to other two breeds (p-value was 0.7715 and 0.3209). Standard errors of least square means for Dreznica goat were larger because of smaller number of animals and variability within the breed. The difference in lifetime protein yield between Slovenian Alpine goat and Slovenian Saanen goat (Figure 1B) amounted to 11.76±5.21 kg (p-value 0.0240). The difference between Dreznica and Slovenian Saanen goat and Slovenian Alpine goat was not significant (p-value was 0.2532 and 0.9772; respectively).

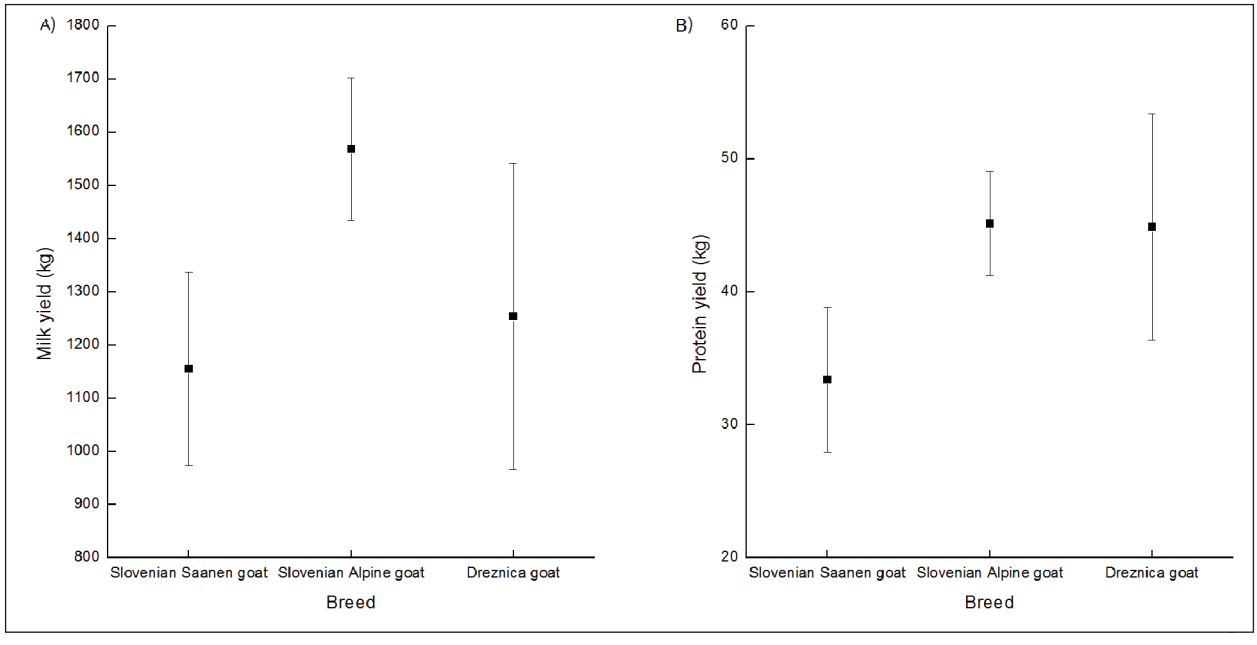

Figure 1. Least square means for lifetime milk yield (A) and protein yield (B) for three goat breeds in Slovenia

Dreznica goat yielded about 25.50±5.21 kg more fat in lifetime compared to Slovenian Saanen goat (p-value 0.0206); however compared to Slovenian Alpine goat the difference was not significant (p-value 0.2330). Comparison of Slovenian Saanen goat and Slovenian Alpine goat revealed higher lifetime fat yield of Slovenian Alpine goat for 13.28±5.21 kg (p-value 0.0188).

Despite the fact that prevalent breeding system in Dreznica goat is extensive with alpine pasture during the grazing season, results showed no difference in lifetime milk production compared to Slovenian Saanen and Slovenian Alpine goat which are characterized by breeding in more intensive production system, generally utilizing advanced technology. Presumably, this could be explained by excellent adaptability of autochthonous Dreznica goat to local environmental conditions and production system. Similar lifetime milk production should be considered for the further steps of conservation of critical endangered the only Slovenian autochthonous goat breed. Furthermore, public awareness of the local and threatened breeds is increasing. Therefore, milk and dairy products of the local goat breeds may well fit into the economics niches, maintaining tradition and cultural values.

The lifetime production of local dairy goat breeds was not very widely studied. Gaddour et al. (2007) studied dairy performance such as, daily milk average, total production by lactation and milking period of local goat, Alpine, Damascus, Maurciana and crossed groups. They found lower dairy production for the local breed in com-

parison to other breeds. Zaitoun et al. (2004) observed that breed and region within geographical site and lactation number significantly affected daily milk production. Some studies were done on dairy local cattle breeds and some conclusions are maybe also applicable on local dairy goats. Avtar (2005) found much higher lifetime performance in crossbreds than in autochthonous cattle breeds. The author continues that crossbred cattle have higher milk productivity and reproductive efficiency, hence are more profitable than local cattle. Similarly, Galukande et al. (2013) found out that lifetime milk yield was higher in cross-breeding cattle compared to local cattle. Krishanender et al. (2014) reported that the overall least squares means estimated for lifetime milk yield per day of herd life, lifetime milk yield per day of total lactation length and lifetime milk yield per day of longevity in Jersey cows were 4.77 or -0.18 kg, 6.46 + or-0.13 kg and 2.92+or-0.12 kg respectively.

CONCLUSION

Milk yield, as well as the milk composition, number of completed lactations and liveborn kids, indicate the differences among the years, farms and breeds. However, breeds did not differ either in number of liveborn kids or number of completed lactations. The lifetime milk yield was higher in Slovenian Alpine goat compared to Slovenian Saanen goat by 413.26±172.52 kg. The difference in lifetime protein yield between Slovenian Alpine goat and Slovenian Saanen goat amounted to 11.76±5.21 kg. Dreznica goat did not differ in lifetime milk production and protein yield compared to both intensive goat breeds. Dreznica goat yielded about 25.50±5.21 kg more fat in lifetime compared to Slovenian Saanen goat. However, compared to Slovenian Alpine goat the difference was not significant. Comparison of Slovenian Saanen goat and Slovenian Alpine goat revealed higher lifetime fat yield of Slovenian Alpine goat for 13.28±5.21 kg.

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DEVELOPMENT OF INTEGRATED CATTLE GENOMICS KNOWLEDGE BASE

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Original scientific paper

SUMMARY

Systems biology approaches being applied to animal breeding represent an opportunity to derive greater benefits from animal production systems. The increasingly detailed investigations in systems biology have led to a large amount of data dispersed over various sources; therefore, a centralized knowledge base is in demand. In this study, we have integrated cattle genomics data of heterogeneous sources and types and developed a bioinformatics tool to study genotype-phenotype associations in cattle: http://integromics-time.com/integromics-database/. The tool enables revealing genomic overlaps within trait-associated loci and identification of potential functional candidates. It might be also used as a tool for planning genotype–phenotype research in cattle.

Key-words: breeding, bioinformatics, cattle, data integration, genomics

INTRODUCTION

A recent development in bovine genome databases has potential to provide advances in finding associations between genotype and phenotype and is beginning to revolutionize gene functions study methods in cattle (Pareek et al., 2011). Comparative integromics combined with systems biology approaches are used (Jiang et al., 2012; Cannistraci et al., 2013) to explain genetic factors and molecular pathways underlying complex phenotypes. In the cattle genomics field regularly updated resources containing genotype-phenotype data are available. For instance, the Animal QTL database (Hu et al., 2013) includes several thousands of QTL for six livestock species, detected with large confidence intervals covering several megabases of the genome containing hundreds of genes. The GeneRIF (Gene Reference Into Function) provides an up-to-date functional annotations of genes. However, the percentage of genes associated with at least one GeneRIF remains quite modest (Lu et al., 2006). Online Mendelian Inheritance in Animals (OMIA) (Nicholas, 2003) is an online database providing up-to-date information on inherited disorders and other familial traits in animal species.

The aim of our study was to: 1) review available sources of genotype-phenotype association data in cattle, 2) develop integrated genotype-phenotype knowledge base for the research in the field of cattle genomics and 3) develop a bioinformatics web application for querying the integrated knowledge base.

MATERIAL AND METHODS

The Cattle genomics integrated knowledge base containing genotype-phenotype association data from three data sources: GeneRIF, Animal QTLdb and OMIA. 1) GeneRIF (http://www.ncbi.nlm.nih.gov/gene/about-generif), downloaded in April 2015, includes short statements (255 characters in length) describing a function of a gene, supported by at least one publication, 2) Animal QTLdb (http://www.animalgenome.org/QTLdb/), release 26, is a database of QTL and single-nucleotide polymorphism/gene association data in livestock animal species, 3) OMIA (http://omia.angis.org.au/) is the catalogue of inherited disorders, other (single-locus) traits, and genes in animal species (Nicholas, 2003).

The data is stored within a relational database management system, MySQL (http://www.mysql.com). The data from the database is retrieved through the web interface and developed using HTML, CSS, JavaScript and Apache web server and servlet written in programming language Java, run on servlet container Apache

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Tomcat. Genomic view, a visualization of distribution of phenotype-related loci along chromosomes, is implemented using Flash GViewer, a freely available flash program developed by the GMOD project (http://gmod.org/wiki/Flash_GViewer). It is inserted into a web page to display chromosomes with genomic locations of individual loci (genes and QTL). To keep the knowledge base up to date, regular updates will be performed by source databases updates.

RESULTS AND DISCUSSION

In the present study, we have reviewed available sources of genotype-phenotype association data in cattle (Table 1) and developed the freely available bioinformatics web application: http://integromics-time.com/integromics-database/. It included genotype-phenotype associations from three data sources, from different research approaches and biological layers, including genomics, proteomics, transcriptomics, and epigenomics. The web interface was used for querying the knowledge base and for visualization of the search results. The user can enter the keyword describing the phenotype of interest. If the knowledge base contains any associations with the phenotype of interest it returns a list of candidate loci and the visualization of loci on the genomic view. The visualization of candidate loci on a genomic view reveals positional overlaps and serves as a tool for analysis of causal genes that underlie complex traits (Figure 1). Numerous regions of the cattle genome have been linked to QTL for body weight and carcass characteristic. For example, there are QTL related with body weight within all cattle chromosomes (Figure 1A). On the bovine chromosome 20 there was an overlap between three QTL associated with body weight and growth hormone receptor (*GHR*) which was found to play a role in body weight determination in three cattle breeds (Qin, Xu, and Gao 2007) (Figure 1B).

Table 1. The number of genotype-phenotype associations, loci, and phenotypes/traits and in cattle in the source databases included in the integrated cattle genomics knowledge base

Source	Number of genotype-phenotype associations	Number of loci	Number of phenotypes/traits
GeneRIF	5178	1817	-
Animal QTLdb	17908	17908	514
OMIA	31	31	31

- The number of phenotypes/traits was not provided

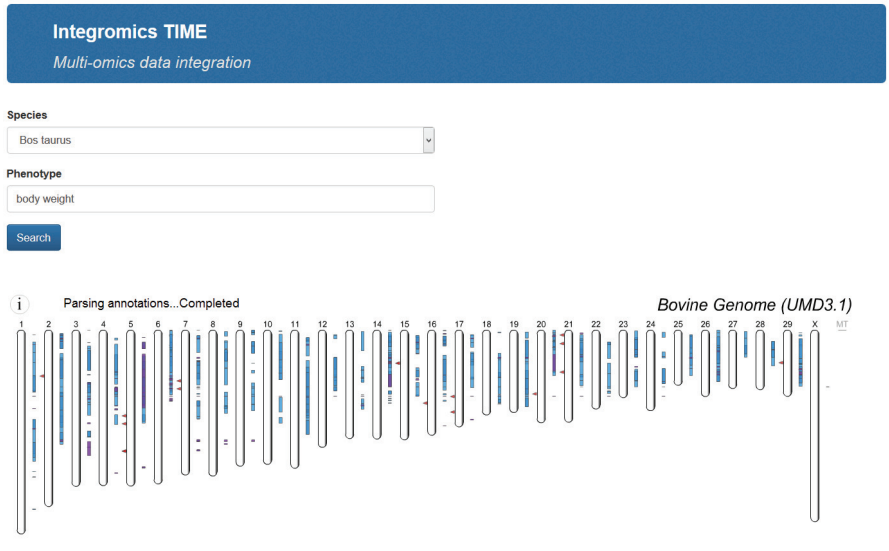

Fig. A

Fig. B

Figure 1. Web interface for querying the integrated cattle genomics knowledge base. A) Search results (genomic view of body weight related loci), B) Bovine chromosome 20 with body weight related loci

More data sources could be included in the cattle genomics integrated knowledge base. For example, the Bovine Metabolome Database (http://www.cowmetdb.ca/cgi-bin/browse.cgi) is a free web database including cattle metabolites information. Additionally, the databases of bovine candidate genes for milk production and mastitis (Ogorevc et al., 2009) and Obesity Gene Atlas in Mammals (Kunej et al., 2013) are centralized databases of candidate genes for traits in cattle. All efforts related to organization and integration of genomic data are a step toward a faster translation of biomarkers from research into practice.

CONCLUSION

In this study, we have integrated cattle genomics data from heterogeneous sources and types and developed a new bioinformatics tool enabling us to mine the integrated knowledge base. The tool was designed to return genotype associations from the knowledge base for a given phenotype term. The results are presented as a list and as a visualization of genomic locations of loci associated with the phonotype of interest. The presentation of genotype-phenotype associations in this manner enables us to reveal genomic overlaps which might reveal functional candidates at trait-associated loci.

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THE EFFECT OF THE SECOND GRAZING PERIOD ON THE FATTY ACID COMPOSITION IN MEAT OF INDIGENOUS CIKA AND SIMMENTAL BULLS

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Original scientific paper

SUMMARY

The aim of the study was to determine fatty acid composition in meat of Cika and Simmental bulls from two different fattening technologies. The herd of 39 young bulls was housed during the winter time and fed the same total mixed ration diet (TMR) based on corn and grass silage with a limited amount of concentrates. In the spring bulls of both breeds were divided into two subgroups. Bulls in the first subgroup (10 Cika, 9 Simmental; S-INT) were fattened indoors with the semi-intensive TMR. Bulls in the second subgroup (10 Cika, 10 Simmental; G+S-INT) were put on all-day grazing in the pasture. After grazing period bulls were housed under the same conditions as the first subgroup. Samples of M. longissimus dorsi were collected from the right carcass side to determine the total fat content and the fatty acid composition. The breed significantly influenced fatty acid composition in meat. The beef of Simmental bulls resulted in higher percentage of PUFA and lower percentage of SFA and MUFA. Higher percentage of n-3 and n-6 PUFA was determined in meat of Simmental bulls but the n-6/n-3 ratio was lower in Cika bulls meat. The fattening technology had less effect on FA composition in meat. The second grazing period produced higher percentage of SFA, beneficially lower values of n-6/n-3 ratio and higher values of long-chain C20-22n-3 PUFA. Higher CLA percentage was determined in beef from S-INT group.

Key-words: beef meat, fatty acids, breed, grazing

INTRODUCTION

In the past, the indigenous Cika cattle was used mainly for the milk production. Nowadays it is considered a low milk productivity breed compared to popular commercial breeds and it is mainly reared in herds with cow-calf system (Simčič et al., 2013a). On the other hand, most widespread breed in Slovenia is a dual purpose Simmental breed. The young growing fattening bulls are usually maintained indoors but a grazing period could be set up in the growing-fattening scheme, e.g., the first grazing season as calves in the suckler herds, the first indoor period as young stock, the second grazing season starting at 300-350 kg and a final finishing period indoors (Dieuguz Cameroni et al., 2006). Regarding natural conditions in Slovenia where cattle diet is based on the forage, the second grazing period for bulls could be easily adapted to the previously mentioned technology. Fats from animal origin have been a subject of many debates because they increase the risk of some diseases when they consumed in excess (Salobir, 2001). Besides concentrated source of energy for the body, fat is also a source of fat soluble vitamins A, D, E and K and essential fatty acids (FA) important for normal growth and play a role in maintaining many body functions. Nutritional guidelines advocate reduction in the intake of total fat, saturated (SFA) and *trans* FA (TFA) and increased intake of n-3 polyunsaturated FA (PUFA), especially long chain PUFA (Shinfield et al., 2008). Fatty acid profile of meat shows a wide variability depending on several factors such as breed, diet, age and sex (Daley et al., 2010). Many studies have confirmed an effect of cattle breed on the FA composition

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in beef (Warren et al., 2008; Brugiapaglia et al., 2014). Manipulating fatty acid profile in ruminant fat by changing their diet is far more difficult than in monogastric animals. That is due to the biohydrogenation process in the rumen. Nevertheless, the results of many studies confirmed that fatty acid composition in ruminant fat can be influenced by different feeding systems (De la Fuente et al., 2009; Humanda et al., 2012). The objective of this study was to investigate the effect of breed and fattening technology on the FA composition in meat, in particular, to examine the difference in the intramuscular fatty acid composition of indigenous Cika and Simmental young bulls.

MATERIAL AND METHODS

Animals

The study was conducted at the Educational and Research Animal Husbandry Centre Logatec (Slovenia) and included 20 Cika and 19 Simmental young bulls bought from farms throughout Slovenia in November 2010. The herd was housed in a feedlot with a closed barn with multiple pens for the winter time (178 days in average) and fed the same total mixed ration (TMR) diet based on maize and grass silage with a limited amount of concentrates. The experimental period started In May 2011 when bulls were divided into two subgroups by their live weight. The first subgroup consisted of 10 Cika and 9 Simmental bulls with initial body weight 445.7 kg and 392.4 kg, respectively. Bulls in the first subgroup (S-INT) were fattened indoors in four pens with a fully slatted floor. They were fed semi-intensive TMR consisted of maize silage (66.0%), grass silage (16.5%), corn grain (9.5%), sunflower meal (7.1%) and mineral-vitamin premix (0.9%) that is commonly used for bulls fattening. The second subgroup (G+S-INT) included 10 Cika and 10 Simmental bulls with initial body weight 339.7 kg and 312.5 kg, respectively. Bulls in the second subgroup were put on all-day grazing in the pasture. After grazing period (131 days in total) bulls were housed in the same conditions as the first subgroup. They were fed with the same semi-intensive diet as bulls in the first subgroup. This period lasted for 233 days on the average. Bulls were slaughtered when they achieved appropriate commercial requirements according by the Slovenian market. The average age of bulls at slaughter for S-INT group was 703 days and for G+S-INT group 740 days. Samples of *M. longissimus dorsi* located between the 7th and 8th rib were collected from the right carcass side to determine total fat content and FA composition. The carcass traits of bulls from this experiment were presented by Simčič et al. (2014).

Total fat and fatty acid composition

Intramuscular fat content was determined using petroleum ether extraction after hydrolysis of sample in 4 M HCl solution by the manufacturer's application notes (Foss, Application note AN 3904). Total fat determination includes acid hydrolysis step, in which fat bound polar components are separated and later extracted. In our case, samples were hydrolysed in 4 M HCl in SoxCap 2047 (Soxtec 2050, Foss system, Höganäs, Sweden). After drying, fat from hydrolysed samples was extracted using petroleum ether in Soxtec 2050 (Foss system, Höganäs, Sweden).

The fatty acid composition of meat samples was analysed using a gas chromatographic method following transesterification of lipids. Fatty acid methyl esters (FAME) were prepared according to Park and Goins (1994) using gas chromatograph (6890 series, Agilent, Santa Clara, CA, USA). FAME were separated using a capillary column (Varian CP 4720, length 100 m, internal diameter 250 μm, film thickness 0.25 μm). Agilent GC ChemStation was used for data acquisition and processing. Separated FAME were identified by retention time comparison and results were calculated using response factors derived from chromatographic standards of known fatty acid composition (Nu Chek Prep). The exactness and reliability of the method used was assessed by the certified reference material NIST SRM 1546 Meat Homogenate. The fatty acid composition was expressed as a percentage by weight (wt%) of the total identified fatty acids.

Statistical analysis

Data were analysed using the GLM procedure in the statistical package SAS/STAT (SAS Institute Inc., 2001). The effect of breed and fattening technology were included in the model. The interaction between these effects was eliminated from the model because it was not significant. The results in the table are presented as Least square means ± SE and they were considered to be significantly different when $P < 0.05$.

RESULTS AND DISCUSSION

Nutrition is the major factor influencing FA composition in beef whereas both nutrition and genetics affect the level of fat (Kamihiro et al., 2015). In this study, the breed significantly influenced total fat content in meat. Cika bulls compared to Simmental bulls had higher total fat content in meat. Regarding total fat content in meat, no difference was observed between fattening technologies (Table 1). This is in contrary with Simčič et al. (2013) who reported significantly higher intramuscular fat content in semi-intensively Cika bulls compared to grazed Cika bulls. This might be due to the fact that all bulls in our experiment were put on the same TMR diet in the finishing period.

Breed significantly affected percentage by weight of total SFA, MUFA and PUFA. Cika meat had higher percentage of SFA (46.94 wt%) and MUFA (40.16 wt%) thus consequently lower percentage of PUFA (12.91 wt%) compared to Simmental meat (44.35 wt%, 35.55 wt%, 20.09 wt%), respectively. This might be due to the fact that meat of Simmental bulls contained

considerably lower proportion of fat and consequently higher proportion of phospoholipids in total lipid content. According to many studies, the wt% of PUFA in meat is correlated with the level of intramuscular fat. Aldai et al. (2007) were found out that the lowest percentage of SFA and MUFA, and the highest percentage of PUFA were observed in meat from the leanest animals. Likewise, the high level of PUFA (23.2 wt%) and the low level of intramuscular fat in the *Longissimus thoracis* muscle in Maremmana bulls was reported by Sargentini et al. (2010). Percentage of major hypercholesterolaemic FA, C14:0 and C16:0 was higher in the meat of Cika bulls, but there was no influence of breed found in the percentage of C18:0 (Table 1). The meat of Simmental bulls contained more n-6 PUFA (10.88 wt%) and n-3 PUFA (1.88 wt%) compared to Cika bulls (17.40 wt%, 2.46 wt%), respectively. The higher percentage of both essential FA, linoleic (C18:2n-6) and α-linolenic acid (C18:3n-3), was established in meat of Simmental bulls. There was no difference between these two breeds found out in the proportion of CLA (C18:2cis-9, trans-11). Ruminant dairy and meat products are the principal sources of cis-9, trans-11 CLA isomer in the human diet. Due to its biological benefits, e.g. reduction of carcinogenesis, atherosclerosis, inflammation, obesity, diabetes, the CLA has received much attention in recent years (Yang et al., 2015). Likewise, Brugiapaglia et al. (2014) found no effect of breed (Piemontese, Limousine, Friesian) on the CLA content in beef. On the other hand, Warren et al. (2008) established the significant effect of breed (Aberdeen Angus cross, Holstein-Friesian) on the CLA proportion in steers, but only when animals were 14 or 19 months old at slaughter. When animals were slaughtered at 24 months no effect of breed was detected. Meat from Simmental bulls contained significantly higher wt% of AA (arachidonic acid), EPA (eicosapentaenoic acid), DPA (docosapentaenoic acid) and DHA (docosahexaenoic acid) compared to Cika bulls meat. These long chain PUFAs are in meat presented in low but important concentrations due to increasing evidence from animal and *in vitro* studies which indicates anticarcinogenic properties of n-3 PUFA, especially EPA and DHA. Concerning n-6/n-3 PUFA ratio, the values in Cika bulls meat (5.82) were lower and thus preferential as in Simmental bulls (7.29). According to DACH (2008) nutrition guidelines the recommended n-6/n-3 ratio is 5:1, but in human diets this ratio is often above 10:1 and connected with many chronic diseases.

Manipulating FA composition in ruminant meat has been extensively studied with purpose to elevate n-3 PUFA and reduce SFA concentrations. SFA tends to dominate in ruminant fat, as a consequence of dietary PUFA being hydrogenated by microbial population in the rumen. Nutritional guidelines recommend a reduction in total fat intake, particularly of SFA (DACH, 2008). In this study fattening technology had no impact on percentage by weight of PUFA, but SFA and MUFA were significantly affected. Higher percentages of SFA were found out in G+S-INT group. That is in agreement with Alfaia et al. (2009) who demonstrated higher percentage of SFA and no difference in PUFA percentage in meat from Alentejano bulls were fed concentrate diet after grazing period compared to bulls fed concentrate diet entire experimental period. The wt% of C14:0 and C16:0 was not affected by fattening technology, but the proportion of C18:0 was significantly higher in G+S-INT group. There was also no effect of fattening technology on wt% of linoleic and α-linolenic acid observed. Regarding CLA, the higher percentage by weight was discovered in S-INT group (0.27 wt%) compared to the G+S-INT group (0.20 wt%). This is in the contrary with many studies which demonstrated a positive effect of grazing on CLA concentration in meat (De la Fuente et al., 2009; Humanda et al., 2012). This is probably due to the fact that all bulls were put on the same TMR diet in the finishing period. Percentages of EPA, DPA and DHA, which are of special interest in the human diet, were influenced by the fattening technology. The higher values of these long chain PUFAs were in meat of bulls from G+S-INT group. The fattening technology significantly affected the percentage by weight of n-3 PUFA and consequently n-6/n-3 PUFA ratio being lower in meat of grazing bulls (5.86) compared to meat from bulls that were fattened indoors (7.25).

Table 1. Total fat content (%) and fatty acid composition (percentage by weight of total identified fatty acids; wt%) in *M. longissimus dorsi* of Cika and Simmental young bulls from different fattening technologies (LSM±SE)

	Breed			Fattening technology		
	Cika	Simmental	P-value	S-INT	G+S-INT	P-value
Total fat	2.03±0.16	1.28±0.16	**	1.81±0.16	1.50±0.16	ns
C14:0	2.42±0.08	1.74±0.08	***	2.12±0.09	2.06±0.08	ns
C16:0	24.40±0.39	21.49±0.40	***	22.50±0.40	23.39±0.38	ns
C18:0	15.64±0.30	15.68±0.31	ns	15.19±0.31	16.14±0.30	*
C18:1	35.63±0.80	31.44±0.82	**	34.67±0.82	32.40±0.80	ns
C18:2n-6	7.95±0.67	12.77±0,68	***	10.17±0.68	10.56±0.67	ns
C18:3n-3	0.77±0.04	0.88±0.04	ns	0.81±0.04	0.85±0.04	ns
C18:2c9,t11(CLA)	0.23±0.01	0.23±0.01	ns	0.27±0.01	0.20±0.01	***
C20:4n-6 (AA)	2.08±0.21	3.51±0.22	***	2.72±0.22	2.87±0.21	ns
C20:5n-3 (EPA)	0.34±0.04	0.40±0.04	*	0.31±0.04	0.52±0.04	**
C22:5n-3 (DPA)	0.62±0.05	0.88±0.06	**	0.65±0.06	0.85±0.05	*
C22:6n-3 (DHA)	0.08±0.00	0.12±0.01	**	0.08±0.01	0.12±0.01	*
SFA	46.94±0.42	44.35±0.44	***	44.71±0.44	46.58±0.42	**
MUFA	40.16±0.89	35.55±0.92	**	39.29±0.92	36.42±0.89	*
PUFA	12.91±1.03	20.09±1.06	***	16.00±1.06	17.00±1.03	ns
n-6 PUFA	10.80±0.91	17.40±0.94	***	13.81±0.94	14.38±0.91	ns
n-3 PUFA	1.88±0.14	2.46±0.14	**	1.92±0.14	2.42±0.14	*
n-6/n-3	5.82±0.21	7.29±0.21	***	7.25±0.21	5.86±0.21	***

*P<0.05; **P<0.001; ***P<0.0001; ns P>0.05; LSM - Least square means; SE - standard error; CLA-C18:2*cis*-9,*trans*-11; AA-arachidonic acid, EPA-eicosapentaenoic acid, DPA-docosapentaenoic acid, DHA-docosahexaenoic acid, SFA-saturated FA, MUFA-monounsaturated FA; PUFA-polyunsaturated FA

CONCLUSION

Data reported here demonstrate considerable differences in beef FA profiles between breeds, in this Cika and Simmental case. The beef from Simmental bulls resulted in higher percentage of PUFA and lower percentage of SFA and MUFA. Higher percentage of n-3 and n-6 PUFA was determined in meat of Simmental bulls but the n-6/n-3 ratio was lower in meat of Cika bulls. On the other hand, the second grazing period had less effect on the fatty acid composition. That is probably due to the fact that bulls were fattened under the same conditions in the last period before the slaughter. Nevertheless, the second grazing period produced higher values for SFA, beneficially lower values for n-6/n-3 ratio and higher values for long-chain C20-22n-3 PUFA.

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ENVIROMENTAL FACTORS AFFECTING RACING TIME OF TROTTER HORSES IN SERBIA

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Original scientific paper

SUMMARY

Speed, the most important trait in trotter horses, forms the basis for examining their racing ability, and is calculated according to the time it takes to run a certain distance. The phenotypic manifestation of a horse's speed is controlled by numerous genes and larger or smaller impacts of environmental factors. To improve trotter horse selection to be more successful and faster in genetic progress it is very important to determine the impacts of such gene-related and environmental factors. The aim of this study was to investigate the effect of year and month of birth, sex, year and season of race, age, racetrack, distance and type of start on trotter horse racing times. Data from the Association for Trotting Sport of Serbia (UKSS) for the registered horses and races in the period from 1998 to 2010 were used. The database is comprised of data for 1263 horses over a total of 14398 races. After calculating descriptive statistics of racing times, the effect of fixed factors using the general linear model (GLM) was examined. The average racing time achieved was 84.21s, and ranged from 73.8 to 132.2s. All of the tested factors had a statistically significant effect on the observed racing times. Thus, each of these factors should be included in future models for genetic prediction of the suitability of animals use as parents of further generations of racing trotters. This should increase the rate of genetic progress and competitiveness of the animals at both national and international levels.

Key-words: trotter, time in race, environmental factors, genetic improvement

INTRODUCTION

Trotting races are the test of the speed, endurance and regularity of trot of trotter horses. Speed, though, is the most important trait in trotters and forms the basis for examining their racing ability. Speed is calculated on the basis of the measured time it takes to run a certain distance. The main goal in breeding trotters is to produce good (successful, durable, healthy) race horses, able to start young in their sports (racing) careers, because once proven in sport, they are generally made available for reproduction. Trotter breeding values are determined by time records achieved in races, from which speed is indicated as well as the number of race starts, being an indicator of horse endurance. These traits are quantitative, meaning that their phenotypic manifestation is controlled by numerous genes and is more or less affected by the environment. It is, therefore, very important to ensure optimal environmental conditions, and conditions in which the genotype of the animals can achieve maximum expression. In addition, when defining a model for genetic estimation, it is important to consider all the environmental factors that can affect the final result of the animals in the race (Ojala et al., 1987).

The results of trotting horse races are influenced by many environmental factors. Čačić and Šimundža (2012) based on the research of numerous authors reported the following factors which can be used for the analysis of accumulated (annual) race results: age, age at first start, month and year of birth. The authors note that the inclusion of individual race records in a model allows the definition of additional factors such as the racetrack, distances, type of start and condition of the track. In order to produce a more reliable model though, the effects of the environment should be included for

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individual results or the results of each race. Specific environmental conditions for each recorded race result can be directly introduced into a statistical model, and it is not necessary to pre-adjust data for this purpose (Rohe et al., 2001; Bugislaus et al, 2006; Langlois and Blouin, 2007). According to Štrbac and Trivunović (2013), sex, racetrack, season, age and distance have a statistically significant effect ($P<0.01$) on horse racing time. Similar results have been shown by Rohe et al. (2001) and Bugislaus et al. (2006). The aim of this study was to investigate the influence of the year and month of birth, sex, year and season of the race, age, racetrack, distance and method of start on racing times of trotters.

MATERIAL AND METHODS

As working material, data from the Association for Trotting Sport of Serbia (UKSS), for registered horses and races in the period from 1998 to 2010 was used. The database is comprised of data for 1263 horses over a total of 14398 races. For consideration of average racing time and variability of racing time, descriptive statistics was performed. For this purpose, standard statistical parameters were calculated: mean, standard error of the mean, minimum, maximum, standard deviation and coefficient of variation. The general linear model (GLM) was used to determine the influence of the year and month of birth, sex, year and season of the race, age, racetrack, distance and method of start on racing times of trotters according to the following two models:

$$Y_{ijklmnoprs} = \mu + Yb_i + Mb_j + S_k + Yr_l + Se_m + A_n + Rt_o + D_p + St_r + e_{ijklmnoprs} \quad (1)$$

$$Y_{ijklmnop} = \mu + YbMb_i + P_j + YrSe_k + A_l + Rt_m + D_n + St_o + e_{ijklmnop} \quad (2)$$

Where:

Y – observed trait

μ – general mean

Yb – year of birth (n=13)

Mb – month of birth (n=11)

YbMb – interaction between year and month of birth (n=114)

S – sex (n=3)

Yr – year of race (n=12)

Se – seasons of race (n=3)

YrSe – interaction between year and month of race (n=14)

A – age (n=11)

Rt – racetrack (n=50)

D – distances (n=3)

St – type of start (n=3)

e – random error

In both models, the factors were defined as fixed effects, and notably, model 2 included interactions between year and month of birth and year and season of racing.

Statistical analysis was performed using software Statistics 12.

RESULTS AND DISCUSSION

The average phenotypic value and variability of racing time of trotter horses in Serbia is shown in Table 1. The average racing time achieved was 84.2 s, and ranged from 73.8 to 132.2 s. According to the European Trotting Statistics, the average time achieved on European racetracks ranged from 77.5 to 71.7 s during the period 1985-2009.

Table 1. Phenotypic parameters of racing time

Trait	N	$\bar{x}$	$S_{\bar{x}}$	Min	Max	SD	CV
Time in race, s/km	14398	84.2	0.038	73.8	132.2	4.6	5.5

$\bar{x}$ – average; $S_{\bar{x}}$ – standard error; Min – minimum; Max – maximum; SD – standard deviation; CV – coefficient of variation

Based on the results in Table 1, we can conclude that, on the average, our horses achieve racing times significantly slower than those of horses from other European countries. This may be due to the fact that selection in Serbia is currently carried out solely on the basis of phenotypic parameters, while genetic evaluation as selection criteria has not yet been implemented. Also, population size, management and quality of race tracks are not on at an adequate level for top results in trotting sports.

Racing time is a simple phenotypic measure that is collected routinely for all horses participating in a trotting race (Ricard et al. 2000). Although its inclusion as a criterion for selection is not always necessary, it is recommended, bearing in mind that it shows the ability of the animals to quickly trot (Thiruvenkadan et al., 2009). In addition, Rohe et al. (2001) suggested that this is the most important trait for selection of sports performance because it has the highest heritability of all traits that have been studied and has a high genetic correlation with a horse's ability to achieve a place in the races.

In Tables 2 and 3, the effects of environmental factors on the racing times achieved are shown.

Table 2. Impact of environmental factors on trotter horse racing time (model 1)

Factors	d.f.	F – value	R^2
Year of birth	12	15.6**	0.505
Month of birth	10	13.9**	
Sex	2	78.2**	
Year of race	13	17.4**	
Season of race	2	28.4**	
Age	10	59.7**	
Racetrack	49	86.7**	
Distances	2	189.1**	
Method of starts	3	492.1**	

** $p<0.01$; d.f. – degree of freedom; R^2 – coefficient of determination

Table 3. Impact of environmental factors on trotter horse racing time (model 2)

Factors	d.f.	F – value	R^2
Year and month of birth	113	8.5**	0.528
Sex	2	76.9**	
Year and season of race	13	8.7**	
Age	10	60.2**	
Racetrack	49	82.4**	
Distances	2	197.5**	
Method of starts	3	474.1**	

** $p<0.01$; d.f. – degree of freedom; R^2 – coefficient of determination

All of the examined factors had a statistically significant effect on trotter horse racing times. Thus, each of these factors should be included in models for genetic evaluation of the animals. Year of the race and birth year of the animals have an impact on horses' sports performance, due to improvement of the training over time, and possible changes in the quality of conditions at racetracks (Thiruvenkadan et al., 2009). The effect of month of birth was also noted by Saastamoinen and Ojala (1991), especially at an early age, which may indicate that there are significant differences in physical growth between animals born at the beginning and end of the year. The influence of sex on horses' racing times has also been proven in other studies (Ojala and Hellman, 1987; Štrbac and Trivunović, 2013). In most cases, males performed better in races than females. Ojala and Hellman (1986) reported that males were superior to females and that stallions were faster on the average by 1.05 to 2.29 seconds. Štrbac and Trivunović (2013) reported that stallions achieved the best racing times, followed by mares, and that geldings produced the worst results. The influence of age in trotter horse races was established by Ojala and Van Vleck (1981). Saastamoinen and Ojala (1991) pointed out that starting trotters at an early age enables profitable production through earlier and more accurate selection. The effect of year-season-racetrack is also included in some models as an indirect measure of the conditions on the track at the time of racing (Ojala et al., 1987a). Racing time largely depends on the race itself - the distance. Čačić and Šimundža (2012) pointed out that the best racing time is achieved over shorter distances (1900m or less). Type of starts also affects the racing time (Ojala et al., 1987; Thiruvenkadan et al., 2009; Thuneberg-Selonen et al., 1999), and Čačić and Šimundža (2012) suggest that much better time occur in races with flying starts.

The R^2 value (see Tables 2 and 3) was higher in model 2, which included interactions between year and month of birth and year and season of race. Overall, the R^2 values showed that about 50% of the variability in racing times was determined by the applied models. The values of R^2 would certainly be higher if the models had also included effects of sire and dam, as around 30% of the variability in racing times can be caused by genetic differences measurable through the horses' bloodlines.

CONCLUSION

This study has shown that trotter horses bred in Serbia currently achieve poorer results (slower racing times) compared to similar horses in other European countries. The selection of trotters in Serbia is based only on phenotypic parameters. Such selection is less efficient than selection using genetic analysis, as changes in the phenotype can occur as a result of interactions of genotype with environmental factors. To further progress the trotter horse selection process, it will be necessary to utilise genetic analysis that includes the impact of environmental factors. Results of this study show that the impact of the year and month of birth, sex, year and season of the race, age, racetrack, distance and method of start should all be included in models for genetic prediction of trotter horse ability. This system of genetic evaluation should result in genetic improvement increase, and increase in the competitiveness of Serbian-bred trotters at the national and international levels.

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THE EFFECT OF DIETARY FIBRE CONTENT ON SKATOLE AND INDOLE PRODUCTION IN FAECES OF IMMUNOCASTRATED MALE PIGS

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Original scientific paper

SUMMARY

The effect of dietary fibre content on intestinal production of indolic compounds was studied in immunocastrated pigs (IC). In addition, entire males (EM) and IC were compared on control diet (with low fibre content). For the study 32 crossbred pigs were assigned, within a litter to 4 treatment groups; 24 pigs were immunocastrated (at the age of 77 and 112 days) and 8 pigs were kept as entire males (EM). IC were split into three groups (IC_H, IC_M and IC_L) fed three diets differing in crude fibre (3.4, 6.0 and 8.0 g/kg dry matter, respectively) and net energy (NE) (10.0, 9.3, 8.5 MJ NE/kg/DM, respectively). EM were fed high NE i.e. low fibre diet. The experiment started when pigs were 84 days old and finished at the age of 172 days, when pigs were sent to slaughter. Skatole and indole concentrations were determined in the samples of intestinal content taken from caecum (CE), ascending (AC) and descending colon (DC). The concentration of indole was the highest in CE and proximal part of the colon, while skatole concentration increased in the distal parts of the large intestine. Concentrations of indolic compounds did not differ between EM and IC that were fed the same diet. Lowering dietary NE by inclusion of high fibre ingredients reduced the production of indole in the intestinal content of IC pigs, whereas the production of skatole was not affected.

Key-words: indole, skatole, dietary fibres, pig; entire males; immunocastrates

INTRODUCTION

Skatole (3-methyl-indole) is a product of bacterial degradation of tryptophan in the hind-gut of pigs, which is absorbed, and if not metabolised, deposited in the adipose tissue (Claus et al., 1994). Skatole and androstenone (testicular steroid) are held responsible for 'boar taint,'a sex-specific odour of meat from entire males (EM) (Lundström et al., 2009). It has been demonstrated that androstenone hindered skatole metabolism in the isolated pig hepatocytes (Doran et al., 2002) explaining higher skatole concentration in male pigs than in castrates. However, the levels of fat skatole can be also critically high in castrated males (Škrlep et al., 2012). Data in the literature shows that dietary factors are very important for the skatole production and that deposited skatole levels in the fat can be reduced by dietary means (Wesoly and Weiler, 2012), such as ingredients with high dietary fibres. However, the effect is not consistently shown (Aluwé et al., 2009) and it seems to depend on the level of inclusion and other dietary factors. Skatole level in adipose tissue is proportional to its production in the hind gut (Claus et al., 1993). The effect of sex on skatole production in the hind gut was not given much of attention, but higher levels of its concentration in fat of EM than castrates were mainly related to anabolic (Claus et al., 1994) or androstenone effect (Doran et al., 2002). In order to investigate the effect of increased dietary fibre content on intestinal production of indolic compounds, a feeding trial with different levels of NE in the diet achieved by different fibre content was conducted using IC pigs. Additionally, in regard to skatole production, IC pigs were also compared to EM.

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MATERIAL AND METHODS

Study is a part of bigger experiment performed in the frame of doctoral thesis of Nina Batorek Lukač made at INRA, Saint-Gilles, France in accordance with French laws on animal experimentation. Thirty-two crossbred pigs were used, 24 pigs were immunocastrated (IC) at the age of 77 and 112 days and 8 pigs were kept as entire males (EM). Pigs were assigned within litter to four treatment groups and individually housed during the experiment from 77 to 172 days of age. IC received three different diets (based on wheat, corn and barley), differing in net energy (NE) content (Table 1). Diet NE was reduced by inclusion of high fibre sources (wheat bran, soybean hulls, dried beet pulp) and by reduced quantities of added oil (Labussière et al., 2014). A high NE/low fibre diet (HNE) was given to EM (n=8) and IC from group IC_H (n=8), pigs in group IC_M (n=8) received medium NE/fibre diet (MNE) and pigs of group IC_L (n=8) received a low NE/high fibre diet (LNE, Table 1).

Table 1. Chemical composition and nutritional value of the experimental diets

	LNE	MNE	HNE
Analysed chemical composition, g/kg of DM			
Ash	60	56	54
Crude protein (N × 6.25)	180	175	185
Starch	416	470	518
Ether extract	33	34	42
Crude fibre	80	60	34
GE, MJ/kg of DM	18.17	18.17	18.31
Nutritional value[1]			
DE, MJ/kg of feed	12.70	13.28	13.87
NE, MJ/kg of feed	9.29	9.83	10.83

LNE – low net energy/high fibre diet; MNE – medium net energy/medium fibre diet; HNE – high net energy/low fibre diet; GE – gross energy; DE – digestible energy; NE – net energy; DM – dry matter

Feed and water were provided *ad libitum*. The experiment started when pigs were 84 days old and finished at the age of 172 days, when pigs were sent to slaughter. Samples of intestinal content from caecum (CE), ascending (CA) and descending colon (DC) were taken at evisceration and immediately frozen in liquid nitrogen. Skatole and indole concentrations in the intestinal samples were determined by HPLC to the method of Denhard et al. (1991). Analysis of variance with fixed effects of treatment group and intestinal section was performed (GLM procedure of SAS Inc., Cary, USA). If significant effect ($P<0.05$) was noted, least square means between treatment groups were compared using Tukey's test.

RESULTS AND DISCUSSION

In the present study, the highest indole production was observed in CE and proximal part of the colon (CA), while skatole concentration was higher in the distal parts of the large intestine (Figure 1) implying that the main sites of indole and skatole production differ within the intestines, and agrees with previously reported data (Claus et al., 1994; Knarreborg et al., 2002). It can be also observed that indole production decreased gradually with increasing inclusion of dietary fibre (i.e. decreasing dietary NE content), whereas skatole concentration of the intestinal content was not affected by higher dietary fibre content (Figure 2). Knarreborg et al. (2002) also studied the effect of low and high non-starch polysaccharides (NSP) diet (i.e. low and high fibre diet) and reported an effect on both, skatole and indole i.e. absorption from intestines into bloodstream was lower in high NSP diet. The differences in results between their and our study might be explained with ingredients used. Namely the main source of NSP in their diet was sugar beet pulp whereas in the present one it was wheat bran and soybean hulls (see discussion later on). Secondly, in their study EM were used while in the present one, IC pigs were used. However the latter does not seem to be a major factor, and it is important to highlight, that in the present study the concentrations of indole and skatole were comparable between EM and IC fed the same dietary regime (fed high NE, low fibre diet) implying that intestinal skatole production is similar in castrated and uncastrated pigs and that the differences between these two sex categories are mainly related to differences in skatole liver metabolism (Doran et al., 2002).

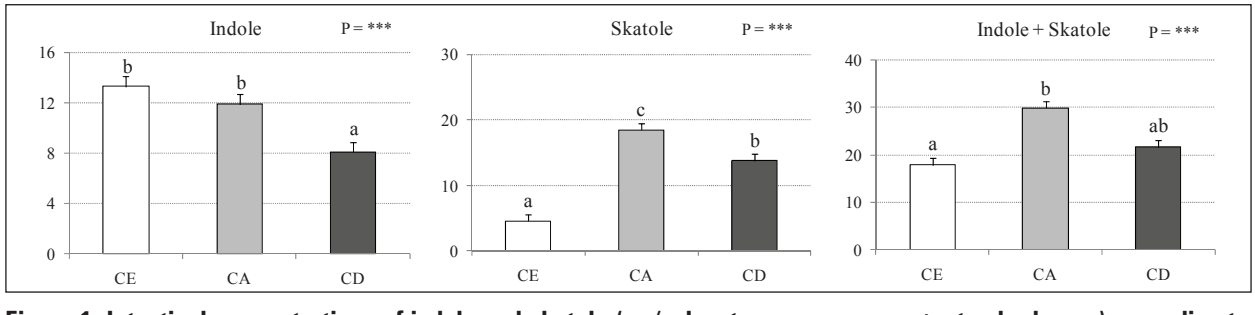

Figure 1. Intestinal concentrations of indole and skatole (μg/g, least squares means ± standard error) according to sampling locations (CE – caecum; CA – ascending colon; CE – descending colon)

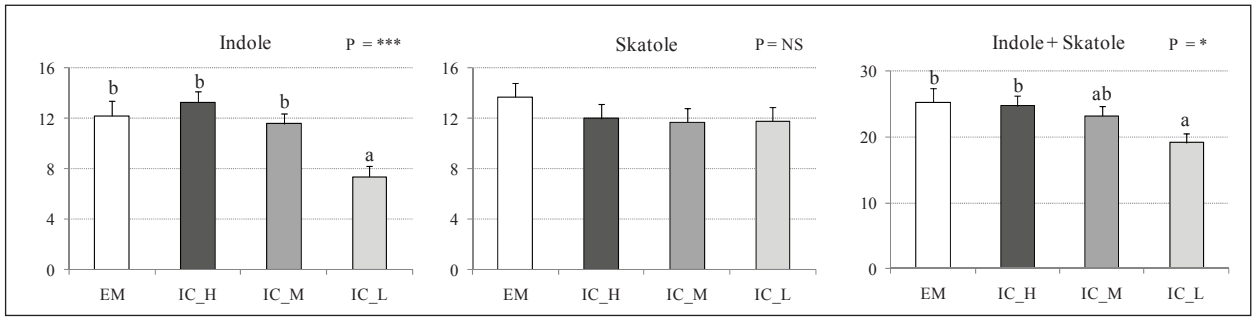

Figure 2. Overall intestinal concentrations (including caecum, ascending colon, descending colon) of indole and skatole (μg/g, least squares means ± standard error) according to treatment groups (EM – entire males, fed high energy/low fibre diet; IC_H – immunocastrates (IC) fed high energy/low fibre diet; IC_M – IC fed medium energy/ medium fibre diet; IC_L – IC fed low energy/high fibre diet)

The amount of skatole stored in adipose tissue depends on the rate of its production, intestinal transit time, intestinal absorption and hepatic metabolism rate (Zamaratskaia and Squires, 2008). Generally, the production of skatole and its resorption into peripheral bloodstream is highly correlated (r=0.79, P>0.001; Claus et al., 1993) whereas skatole deposition depends strongly upon blood plasma concentrations. Therefore reducing skatole production in the intestine can be considered as an effective way for reducing skatole deposition in the adipose tissue. Skatole production can be manipulated by alterations in feed composition e.g. fermentable carbohydrates, like inulin and raw potato starch being effective in reducing skatole levels in gut and fat tissues (Kjos et al., 2010; Vhile et al., 2011). Concerning dietary fibres, as reviewed by Jensen (2006), the exact mechanism of how fibre-rich diets affect skatole deposition in back fat is not clear. The level of skatole production depends on the sufficient availability of carbohydrates (for fermentation) to supply gut bacteria with energy, reducing the skatole production (Jensen et al., 1995). Additionally, dietary fibres can reduce skatole absorption by increasing faecal wet weight and decreasing intestinal transit time (Zamaratskaia and Squires, 2009). Studies interested in the effect of NSP on skatole deposition (Knarreborg et al., 2002; Hansen et al., 2008: Aluwé et al., 2009) show variable response and imply that dietary influences should be considered from a broader perspective of diet composition/ingredients. It is possible that pH of intestinal content (which we didn't measure) varied according to the diet because dietary carbohydrates alter faecal pH and composition (Canh et al., 1998). Jensen et al. (1995) showed the importance of intestinal content pH for production of indolic compounds; with high pH favouring the production of indole and low pH favouring the production of skatole. The amount and source of fermentable carbohydrates in pig diets influence volatile fatty acid concentration and pH of faeces (Canh et al., 1998) which are key factors influencing pathways of microbial production of indolic compound (Jensen et al., 1995). In regard to indole (and sum of indole and skatole) production it is worth noting that LNE (high fibre) diet significantly reduced its production denoting a positive effect in respect of boar taint control (Figure 2). Although indole levels in fat tissue are generally low and seem unproblematic *per se*, its contribution to boar taint is important due to synergy with other compounds (Annor-Frempong et al., 1997). In general, the reduction of indole concentration in faeces in pigs fed LNE/high fibre diet can be considered as positive.

CONCLUSION

Production of indolic compounds varies along the intestinal tract; the concentration of indole was the highest in caecum and proximal part of the colon, while skatole concentration increased in the distal parts of the large intestines. Production of indolic compounds was comparable in entire and immunocastrated male pigs fed the same diet (high NE). Lowering dietary NE by inclusion of high fibre ingredients reduced the production of indole in the intestinal content of immunocastrated pigs, whereas the production of skatole was not affected. Nevertheless, in view of boar taint control, it can be considered that high fibre diet has a positive effect.

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ASSESSING THE POSSIBLE INTERACTION BETWEEN *CARDUUS MARIANUS* AND DIETARY DEOXYNIVALENOL ON CAECAL MICROBIOTA AND FERMENTATION OF GROWING RABBITS

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Original scientific paper

SUMMARY

Contamination of feed with mycotoxins is a common problem encountered in animal farming. Mycotoxin exposure can affect adversely the health of animals. In rabbits caecal fermentation is an essential digestive process being indication of physiological alterations. Deoxynivalenol (DON) is one of the most frequent contaminants of grains which affect the growth of monogastric animals. Data about dietary DON and its effect in rabbits are scarce. Medicinal plants are often used as feed additives to enhance the performance of the animals. Carduus marianus (milk thistle) is known for its hepatoprotective and antioxidant effects (among others) but no data are available about the effect on rabbit caecum. Considering the aforementioned, the aim of this study was to assess the possible interactive effect of Carduus marianus and DON on the caecum of growing rabbits. 75 Pannon White rabbits were reared for six weeks from 35 (after weaning) till 77 days of age. Rabbits received the following diets: control (C), control with DON (CT), control supplemented with C. marianus in 0,5% (H1), control supplemented with C. marianus in 0,5% and DON (H1T), control supplemented with C. marianus in 1% (H2) and control supplemented with C. marianus in 1% and DON (H2T). On slaughter, caecum was collected for the analysis of total volatile fatty acids (VFA) and the microbiota of the caecum, pH of the caecum was also recorded. There was no significant difference in total VFA concentration or individual VFA. Number of aerobic bacteria significantly differed among toxin and non-toxin groups. DON affected adversely the number of aerobic bacteria. An interactive effect of DON and Carduus marianus on E.coli number was observed. There was no effect on total or individual VFA amounts.

Key-words: rabbit caecum, milk thistle, VFA, aerobic bacteria

INTRODUCTION

Mycotoxin contamination of animal feed can pose a serious problem for farm animals, such as pigs, poultry and rabbits, since it frequently affects adversely their health status. In rabbit physiology, caecal fermentation is a very important digestive process that begins around the weaning period (when solid feed is introduced in the diet). The short chain fatty acids produced by fermentation are beneficial as additional source of energy, and also because of their antibacterial effect (Bellier and Gidenne, 1996). The physiological composition of caecal microbiota is crucial for the preservation of the homeostasis (Bagóné Vántus et al., 2014).

Deoxynivalenol (DON) is a mycotoxin mainly produced by *Fusarium graminearum* and *F. culmorum* which

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are common contaminants of cereal grains (Bonnet et al., 2012). Pestka and Smolinski (2005) stated that monogastric animals' growth and weight gain was suppressed upon chronic or sub-chronic exposure of DON with swine being the most sensitive among farm animals. Data for the effect of dietary DON in rabbits are scarce (Khera et al., 1986; Hewitt et al., 2012). Mycotoxins are taken up mainly orally by consumption of contaminated feed. As such, it can be proposed that they may interact with the gut microbiota as it has been shown in the case of ochratoxin A (Guo et al., 2014).

Medicinal plants are often used to improve the health status of animals, especially after the antibiotics have been banned in European Union (European Union Commission, 2005). *Carduus marianus* (syn. *Silybum marianum*) is a member of Asteraceae (Compositae) family which containing several species used in herbal medicine e.g. *Echinacea* (Gao et al., 2010). One of the most popular colloquial names of *C. marianus* is milk thistle (Dunnick and Nyska, 2012). *C. marianus* is known for its hepatoprotective (Flora et al., 1998) as well as for its antioxidant effect (Henning et al., 2014). Some studies suggest a potential positive effect in inhibition of chemically induced diabetes (Shakeel and Yar, 2014) or anti-atherosclerotic effect (Białecka, 1997). As far as we are concerned there are no studies about the effect of *C. marianus* on caecal microbiota of rabbits.

Considering the aforementioned the aim of this study was to assess the possible interactive effect of *C. marianus* and DON on the caecal microbiota and fermentation of growing rabbits.

MATERIAL AND METHODS

Animals and diets

Seventy five Pannon White rabbits were reared in mesh-wired cages from 35 till 77. The rabbits had free access to water (pacifiers) and feed (*ad libitum*). The temperature was 16-18ºC and the photoperiod was set to 16h of light and 8 hours of dark. The weight and feed intake were recorded on a weekly basis whereas the morbidity and mortality were daily monitored.

The animals received three different diets during the first period (3 weeks). The control (C) diet was a nonsupplemented basal diet formulated in such a way to meet the nutritional needs of weaned rabbits, control diet supplemented with the herb (*C. marianus*) in a concentration of 5 g/kg (H1) and control diet supplemented with the herb in a concentration of 10 g/kg (H2; Table 1). During the second period (3 weeks) the rabbits received six different diets after the subdivision of the three initial groups. The diets were formulated in a similar way of the first period but this time supplemented with the toxin (DON) as well (CT, H1 and H2T; Table 1). The experimental protocol was authorized by the Food Chain Safety and Animal Health Directorate of the Somogy County Agricultural Office, under permission number SOI/31/1679-11/2014.

Table 1. Chemical composition of the experimental diets

Chemical composition g/kg of DM	C	H1	H2	CT	H1T	H2T
Dry matter (g/kg of feed; DM)	91.2	90.4	91.0	90.8	90.9	93.9
Crude protein (CP)	18.5	18.8	18.1	18.6	18.3	18.4
Crude fat (EE)	2.8	2.9	2.8	2.9	2.8	2.7
Crude fibre	16.7	16.4	16.4	16.3	17.2	16.3
Ash (ASH)	8.2	8.6	8.3	8.2	8.3	8.3
Starch	18.8	18.6	19.8	18.5	18.0	18.6
Natural detergent fibre (NDF)	35.5	36.0	34.7	35.1	35.2	34.9
Acid detergent fibre (ADF)	22.3	22.4	21.6	22.1	22.0	21.9
Acid detergent lignin (ADL)	6.1	6.3	5.7	6.1	6.2	6.2
Acid insoluble ash (AIA)	1.2	1.4	1.1	1.0	1.6	1.0

Mycotoxin production and plant purchase

Toxin was produced using *Fusarium graminearum* strain number IFA 77 (from "Das Interuniversitäre Department für Agrarbiotechnologie", Tulln, Austria) fungal culture (7 days old), grown on Potato Dextrose Agar (PDA; Chemika-Biochemica, Basil, Switzerland). The inoculated cultures were stored and incubated at 28°C for 2 weeks. When the incubation time was complete the fungus-infected cereal was dried at room temperature and ground.

LC-MS analysis was performed by a Shimadzu Prominence UFLC separation system equipped with a LC-MS-2020 single quadrupole (ultra-fast) liquid chromatograph mass spectrometer (Shimadzu, Kyoto, Japan) with electrospray source.

The homogenized fungal cultures contained DON at concentration of 7140 mg/kg. Fungal cultures were mixed into the feed of the experimental animals, based on the dose presented in Table 2.

Table 2. Concentration of the toxin in the experimental feeds in the different groups

Groups	DON (mg/kg)
CT	10.1
H1T	11.5
H2T	10.6

The mycotoxin production as well as the determination of its concentration, were performed at the Department of Physiology, Biochemistry and Animal Health of Kaposvár University. *Carduus marianus* was purchased from Parceval (Pty) Ltd Pharmaceuticals, South Africa in powder form (seeds).

Determination of composition of caecal microbiota and volatile fatty acids (vfa) concentrations

At 78 days of age 6 animals per group were slaughtered. The digestive tract was removed immediately

and the caecum was separated. The caecal contents' pH values were determined using a pH meter (OP-110, Radelkis, Hungary). One gram of caecal chyme was used immediately after sampling for microbiological culture, and anaerobic conditions were maintained by the use of carbon dioxide. The rest of the caecal content was weighed, freezed and stored at -80ºC for further volatile fatty acids (VFA) analysis.

The analyses for both procedures (microbiological culture and analysis of the volatile fatty acids) were performed as previously described respectively in Kovács et al. (2011).

Statistical analysis

Statistical analysis of the data was performed with SPSS (version 19) statistical software package using one way analysis of variance (ANOVA), LSD was used for the Post Hoc test. T-test was also performed. In all the cases the significance level was $p<0.05$.

RESULTS AND DISCUSSION

Production parameters were not affected adversely by the consumption of the toxin (data not shown).

There was no significant difference in the total VFA (mmol/kg) or the particular VFA (acetic, propionic and butyric; Table 4).

The composition of caecal microbiota showed no significant differences regarding anaerobic bacteria and bacteroides (Table 3). Coliforms' numbers were very low in all the samples (colonies<100; data not shown). On the other hand, there were significant differences in the number of aerobic bacteria. More specifically, groups C and H1 had significantly lower number of aerobic bacteria compared to all toxin groups (i.e. CT, H1T and H2T; Table 3) suggesting an effect of DON on aerobic bacteria regardless the supplementation with the herb. This is confirmed by the T test performed using as factor the consumption of mycotoxin solely (C, H1, H2 and CT, H1T, H2T respectively). *Escherichia coli* (*E. coli*) number significantly differed between H2 and all the toxin groups which can suggest a possible positive effect of the herb in 1% concentration (data not shown).

The pH of the caecal content didn't differ significantly among the groups (Table 4). There was a correlation of VFA and pH, the correlation coefficient was -0.593 and the significance $p=0.01$.

Table 3. Compositon of caecal microbiota (CFU log10/g, mean±SD, p<0.05)

Microbiota	C	CT	H1	H1T	H2	H2T
Aerobic	4.9 ± 0.3[a]	5.8 ± 0.6[b]	5.3 ± 0.6[a.b]	5.7 ± 0.6[b]	5.2 ± 0.2[a.b]	5.7 ± 0.6[b]
Anaerobic	8.9 ± 0.4	9.1 ± 0.4	8.8 ± 0.3	9.0 ± 0.6	8.7 ± 0.5	9.0 ± 0.5
Bacteroides	8.3 ± 0.5	8.5 ± 0.2	8.5 ± 0.3	8.4 ± 0.5	8.3 ± 0.5	8.6 ± 0.4

Table 4. Volatile fatty acids of the caecal chyme (% of total fatty acid content) and caecal pH (mean±SD)

Volatile fatty acids	C	CT	H1	H1T	H2	H2T
Total VFA mmol/kg	46.6 ± 7.7	48.9 ± 7.7	45.8 ± 11.9	39.8 ± 18.8	46.6 ± 16.0	43.4 ± 12.6
Acetic acid (%)	78.6 ± 1.5	78.7 ± 3.7	78.1 ± 2.9	77.2 ± 2.6	79.8 ±2.4	78.6 ± 2.8
Propionic acid (%)	7.2 ± 0.8	7.0 ± 1.2	6.2 ± 0.6	8.0 ± 1.1	7.1 ± 1.8	7.0 ± 1.1
Butyric acid (%)	14.3 ± 1.5[a,b]	14.3 ± 3.7	14.3 ± 1.1	14.7 ± 2.1	13.2 ± 1.2	14.4 ± 2.1
pH	6.04 ± 0.15	6.05 ± 0.39	6.06 ± 0.14	6.08 ± 0.22	5.88 ± 0.15	6.01 ± 0.23

CONCLUSION

This was a preliminary study on the possible interactive effect of *C. marianus* and DON on caecal microbiota and fermentation. DON increased the number of aerobic bacteria in the caecum of rabbits which is an undesirable situation due to the anaerobic conditions (in physiological state) of caecum. The effect of DON was slightly influenced by the 1% herbal supplemantion regarding *E. coli*. More studies should be conducted with more animals to ensure if there is truly interactive effect. There was no interaction of DON and *C. marianus* on total VFA amount.

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EFFECT OF β-ALANINE AND L-HISTIDINE ON CONCENTRATION OF CARNOSINE IN MUSCLE TISSUE AND OXIDATIVE STABILITY OF CHICKEN MEAT

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Original scientific paper

SUMMARY

This paper presents the results of two separate experiments, each involving 75 chickens of Cobb 500 provenience, divided into three experimental groups. During the last three weeks of fattening, chickens were fed finisher diets supplemented with amino acids β-alanine (0%, 0.5% and 1%) and L-histidine (0%, 0.3% and 0.5%) in different portions. After chickens have been slaughtered, 10 samples of breast tissue were taken from each group for carnosine content determination in muscle tissue and lipid oxidation expressed as TBARS. Analysis of THE results referring to carnosine concentration in breast muscle proved that supplementation of 0.5% L-histidine affected the carnosine concentration increase in breast muscles from 941.58 µg/g of tissue (H1) to 1186.06 µg/g of tissue (H3), while supplementation of 1% β-alanine influenced the increase in carnosine concentration from 756.15 µg/g of tissue (A1) to 911.01 µg/g of tissue (A3). Supplementation of amino acids did not have effects on TBARS values, but oxidation values decreased along with the supplementation of higher amounts of amino acids to diets, which was particularly expressed in samples stored for 60 days at -20°C. The experimental group H3 (0.5% L-histidine) exhibited 30.54% lower value of lipid oxidation than the control one H1 (0% L-histidine), while the group with 1% β-alanine (A3) had lipid oxidation value by 17.65% lower than the control group A1 (0% β-alanine).

Key-words: β-alanine, L-histidine, carnosine, TBARS, chicken, oxidative stability

INTRODUCTION

Carnosine is a dipeptide produced by synthesis of amino acids β-alanine and L-histidine with help of carnosine synthetase enzyme in brain and skeletal muscle cells (Hipkiss, 1998). Concentration of carnosine in skeletal muscles depends on animal species and animal age, and it is also affected by feeding treatment, types of muscles (white muscle of chickens contains higher concentration of carnosine than dark muscle) (Maikhunthod, 2003). Carnosine is present in high concentrations in breast muscle of chickens (Kralik et al., 2010). Carnosine has antioxidative activity probably resulting from its ability to bond metal ions and to eliminate several types of free radicals (Kralik et al., 2012). As a dipeptide, it exhibits stronger antioxidative activity than some amino acids as its compounds (Maikhunthod, 2003). Concentration of carnosine in animal, as well as in human tissue, can be modified by supplementation of the amino acids being compounds of carnosine or their combination in feed (Dunnet and Harris, 1999; Nagasawa et al., 2001; Tomonaga et al., 2005; Haug et al., 2008; Boldyrev et al., 2013). The research objective was to determine effects of different concentrations of β-alanine and L-histidine supplemented in chicken feed on the concentration of carnosine and oxidation of lipids in breast muscle tissue of Cobb 500 chickens.

MATERIAL AND METHODS

Chickens were divided in three experimental groups in two experiments (75 chickens per group) and fattened for 42 days. During fattening, chickens were fed com-

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plete starter diets containing 24.39% crude protein and 13.93 MJ ME/kg (1st experiment), i.e. 22.92% crude protein and 13.41 MJ ME/kg (2nd experiment). From 22nd to 42nd day chickens in the 1st experiment were fed finisher diets containing 20.07% crude protein and 13.15 MJ ME/kg (H1–control group), 20.22% crude protein and 13.20 MJ ME/kg (H2 group) and 19.80% crude protein and 13.09 MJ ME/kg (H3 group), which were supplemented with L-histidine in various amounts (H1 0%, H2 0.3% and H3 0.5%). In the 2nd experiment, chickens were fed finisher diets containing 18.79% crude protein and 12.10 MJ ME/kg in the A1 group (control), 18.93% crude protein and 12.90 MJ ME/kg in the A2 and 19.46% crude protein and 13.03 MJ ME/kg in the A3 group, which were supplemented with β-alanine (A1 0%, A2 0.5% and A3 1.0%). Chickens were fed *ad libitum*. Composition of diets is overviewed in the Table 1.

Table 1. Composition of the chicken diets

Ingredient, %	Diet day 1-21	Diets day 22-42					
		1st experiment			2nd experiment		
		H1	H2	H3	A1	A2	A3
Corn	51.50	61.20	60.90	60.70	62.70	62.20	61.70
Alfalfa	2.50	3.00	3.00	3.00	3.00	3.00	3.00
Protein gold	2.00	-	-	-	-	-	-
Soybean toasted	9.00	-	-	-	5.00	5.00	5.00
Soybean cake (46%)	29.50	27.80	27.80	27.80	24.00	24.00	24.00
Sunflower oil	0.50	3.00	3.00	3.00	0.30	0.30	0.30
Kuškovit 5% BK+ Kokcisan + phytase	5.00	-	-	-	-	-	-
Kuškovit 5% BK+ phytase	-	5.00	5.00	5.00	5.00	5.00	5.00
L-histidine	-	-	**0.30**	**0.50**	-	**-**	**-**
β-alanine	-	-	**-**	**-**	-	**0.50**	**1.00**
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00

H1 0% L-histidine, H2 0.3% L-histidine, H3 0.5% L-histidine, A1 0% β-alanine, A2 0.5% β-alanine, A3 1% β-alanine

Composition per 1 kg of premix „Kuškovit“: vit. A 300,000 IUs; vit. D3 40,000 IUs; vit. E 600 mg; vit. K3 40 mg; vit. B1 20 mg; vit. B2 120 mg; vit. B6 40 mg; vit. B12 300 mcg; vit. C 300 mg; niacin 800 mg; pantothenic acid 240 mg; folic acid 10 mg; biotin 2.00 mg; choline chloride 10,000 mg; iodine 12 mg; iron 500 mg; copper 75 mg; manganese 1600 mg; zinc 1000 mg; cobalt 3 mg; selenium 3 mg; antioxidant 2000 mg; calcium min. 38 g; sodium min. 23 g; methionine 55,000 mg; lysine 24,000 mg

The study was conducted in accordance with the Regulations of the Republic of Croatia (Regulations on the animals protection during transport and related operations NN 12/11, Regulations on the Protection of animals at slaughter and killing NN 39/08, Regulations on the protection of animals at the time of killing NN 83/11). After 42 days of fattening and 12-hour fasting, chickens were slaughtered and carcasses were processed as „ready for grill“. Breast muscle tissues were sampled for determination of carnosine content and the lipid oxidation values (fresh tissue and tissue kept for 60 days at -20°C). In order to perform analysis of carnosine content in muscle tissue, there were 10 chickens from each group randomly selected from the mixed sample. Samples of tissues were prepared by the method described by Aristoy and Toldra (2004), and concentration of carnosine was determined by the HPLC device (Varian Prostar, USA) equipped with fluorescent detector and Zorbax column ODS, 4.6 x 250 mm (Agilent, USA). The sample was derivatized before injecting with OPA reagent by the method described by Interpichet and Maikhunthod (2005). Oxidation of lipids in breast muscle tissue was determined by the methods of Vyncke (1970) and Lemon (1975). The research results were analyzed by statistical software Microsoft Office Excel (2007). Significance of differences between groups was determined by analysis of variance (ANOVA). The calculated F value was compared with the theoretical F value at a significance level (5%, $P<0.05$). Significance of differences between mean values was determined by the t-test.

RESULTS AND DISCUSSION

Concentration of carnosine in breast muscles

The Table 2 presents the values referring to concentrations of carnosine in breast muscle tissue of Cobb 500 chickens (mixed sample) fed diets supplemented with L-histidine and β-alanine. The data indicate that supplementation of L-histidine in feed influenced the increase of carnosine concentration in chicken breast muscle tissue. If compared to the control group, supplementation of 0.3% L-histidine in diet affected the increase of carnosine concentration in breast tissue by 8.88% whereas supplementation of 0.5% L-histidine affected the increase of carnosine concentration by 25.96%. Statistical analysis was used to determine significant differences in the content of carnosine between the groups. Supplementation of 1% ß-alanine in diets during the last three weeks of fattening resulted in 20.48% higher concentrations of carnosine in breast muscles of A3 group if compared to the control. Supplementation of 0.5% ß-alanine did not influence the increase of carnosine concentrations. The group A3 fed diets with 1% ß-alanine had statistically higher value of carnosine content in breast muscle than the groups A1 and A2.

Table 2. Concentrations of carnosine (μg/g of tissue) in chicken breast muscle tissue (mixed sample) with dietary supplementation of L-histidine and β-alanine

	Groups			P-value
	H1	H2	H3	
1st experiment	941.58 ± 59.72[c]	1025.22 ± 101.18[b]	1186.06±73.75[a]	***0.001***
	A1	A2	A3	
2nd experiment	756.15 ± 118.56[b]	753.29 ± 85.35[b]	911.01 ± 118.81[a]	***0.003***

H1 0% L-histidine, H2 0.3% L-histidine, H3 0.5% L-histidine, A1 0% β-alanine, A2 0.5% β-alanine, A3 1% β-alanine; Values within a column with different superscript letters a, b, c were significantly different ($P<0.05$)

Haug et al. (2008) performed the experiment of enriching chicken meat with carnosine by supplementing different amounts of L-histidine (1 g/kg, 2 g/kg, 3 g/kg) in chicken feed. Supplementation of L-histidine in 1g/kg of feed already affected the increase of carnosine concentration in breast muscle from 9.96 μmol/g to 16.15 μmol/g, which represented an increase of 62% if compared to the control group of chickens that were not fed histidine. Kai et al. (2015) found out that the chicken meat can be enriched with carnosine if histidine is added in feed in the amount of 200% of NRC norms during 10 days of feeding. Nagasawa et al. (2001) carried out the research to determine whether histidine in feed could affect the content of carnosine in muscle tissue of rats. Supplementation of 2% histidine in feed had positive influence on synthesis of carnosine in muscle tissue and significantly influenced the increase of carnosine concentration in muscle (from 6.48 mmol/g muscle in control group to 9.30 mmol/g muscle in the group fed 2% histidine). Amend et al. (1979) determined that histidine was indispensable in feeding of adult roosters and that the portion of 0.11% in feed was sufficient to keep appropriate live weights and normal concentrations of hemoglobin and to prevent decrease in concentration of histidine dipeptides and free histidine in muscles and in brain tissue. It is assumed that, since then, the needs for histidine increased due to development of new hybrids, shortening of fattening periods, etc. However, other researches proved that the speed of carnosine synthesis in muscles was more affected by β-alanine than by histidine, i.e. β-alanine was the limiting factor in synthesis (Dunnet and Harris, 1999; Harris et al., 2006). Consequently, recent researches are focused on the supplementation of β-alanine in animal feed and water. Tomonaga et al. (2005) performed the experiment within which chickens were given 22 mmol/kg β-alanine orally from 2nd to 6th day of age. Twelve hours after the last dose of β-alanine, chickens were sacrificed and the right breast muscle was taken for analysis of carnosine content. Chicken group that was given β-alanine exhibited significant increase in carnosine concentration from 5459 nmol/g tissue to 8774 nmol/g tissue, which represented an increase of 60.7%. Tomonaga et al. (2012) found out that the addition of β-alanine in water increases the concentration of carnosine in chicken brain and *Musculus pectoralis superficialis*. Dunnet and Harris (1999) investigated the effect of continuous usage of β-alanine in feed on the concentration of carnosine in muscle fibres of *gluteus medius* in horses. Over a period of 30 day, horses were given three times a day β-alanine in the amount of 100 mg/kg of body weight and histidine in the amount of 12.5 mg/kg of body weight. Histidine was also supplemented to feed in order to provide for the appropriate amount of amino acids for biosynthesis of carnosine. Analysis results proved that in muscle fibres that were successfully isolated, concentration of carnosine was higher at the end of the experiment than at the beginning, i.e. availability of β-alanine affected significantly the speed of carnosine synthesis. Chicken meat is sensitive to oxidative changes, which negatively influence taste, smell and meat preservation. Therefore, due to its antioxidative traits, carnosine is especially interesting as a factor in achieving longer meat preservation time.

Indicators of lipid oxidation in meat

Table 3 presents the TBARS values of lipid oxidation from the experiments 1 and 2, shown as mg MDA/kg tissue, measured on fresh breast tissue and on breast tissue stored for 60 days at -20°C. Groups of chickens fed diets with L-histidine exhibited quite equal TBARS values of fresh samples, so there were no significant differences determined between the groups. The sample stored for 60 days at -20°C also did not exhibit statistically significant differences. However, there was a trend of decrease of TBARS values determined in the experimental groups H2 (0.338 mg MDA/kg_{tissue}) and H3 (0.282 mg MDA/kg_{tissue}), if compared to the control group H1 (0.406 mg MDA/kg_{tissue}). In the group with 0.5% L-histidine (H3), TBARS value was lower by 30.54% than in the control group. The group H3 exhibited the highest concentration of carnosine, so it was assumed that the favorable trend of the TBARS value decrease was a consequence of carnosine antioxidative activity. Statistically significant difference was determined only within the group H1, where the storing of samples at -20°C for 60 days resulted in significant increase of TBARS values. The values referring to fat oxidation in breast muscle tissue of chickens fed diets supplemented with β-alanine proved balanced values of TBARS in fresh samples. However, samples of breast tissue stored for 60 days at -20°C exhibited higher values of TBARS in the group A1 (0.425 mg MDA/kg_{tissue}),

while the groups A_2 and A_3 had slightly lower values (0.352 mg MDA/kg_{tissue} and 0.350 mg MDA/kg_{tissue}, respectively). Still, the statistical data analysis did not prove significant differences between the groups.

Table 3. Products of lipid oxidation expressed as TBARS values (mg MDA/kg_{tissue}) in chicken breast muscle tissue samples (fresh and stored for 60 days at -20°C) with dietary supplementation of L-histidine and β-alanine

Storage time	1st experiment			*P-value*
	H1	H2	H3	
Fresh	0.275±0.05[b]	0.298±0.07	0.256±0.02	0.216
60 days	0.406±0.15[a]	0.338±0.11	0.282±0.04	0.056
P-value	***0.015***	0.355	0.084	-
	2nd experiment			
	A1	A2	A3	
Fresh	0.359±0.05	0.341±0.06	0.399±0.04	0.078
60 days	0.425±0.08	0.352±0.07	0.350±0.09	0.103
P-value	0.063	0.745	0.144	-

H1 0% L-histidine, H2 0.3% L-histidine, H3 0.5% L-histidine, A1 0% β-alanine, A2 0.5% β-alanine, A3 1% β-alanine

Although it was determined that duration of storing muscle tissue did not affect TBARS values, more intensive oxidation was found out in group A_1 with the increase in TBARS by 15.52%, while the increase in fat oxidation in group A_2 was small, being only 3.13%. It is important to point out that during the storage time, fat oxidation was lowered in the group A_3 by 14%. Considering the fact that the muscle tissue in the group A_3 had the highest concentration of carnosine, it was expected that antioxidative activity of carnosine would have positive effect on meat preservation. Kralik et al. (2010; 2012) also pointed out that carnosine demonstrated an antioxidant effect in the cell which is amplified by the addition of vitamin E. This fact was also confirmed by Hu et al. (2009), as well as by Djenane et al. (2004).

CONCLUSION

On the basis of the obtained research results, it is concluded that supplementation of particular amino acids, L-histidine and β-alanine, affected the increase of carnosine content in breast muscle tissue of Cobb 500 chickens. Higher average values of carnosine were observed in the study where the chickens received L-histidine in the feed, but in both studies an increase in the content of carnosine in the tissues was observed by increasing the use of amino acids in feed. Dietary supplementation of 0.5% L-histidine (H3) affected the increase of carnosine content by 25.96% if compared to the control group (H1). Dietary supplementation of 1% β-alanine (A3) resulted in 20.48% higher content of carnosine in muscles than in the control group (A1). Although the results referring to lipid oxidation values did not prove significant differences between the groups, it was determined that dietary supplementation of amino acids in higher portions affected the lowering of TBARS values, being particularly expressed in samples kept for 60 days at -20°C. Therefore, it can be concluded that carnosine as a natural dipeptide was a desirable antioxidant used for meat and meat products preservation for a longer period of time.

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EFFECT OF STARTING BODY FAT CONTENT OF LEGHORN-TYPE LAYING HENS ON THE CHANGES IN THEIR BODY FAT CONTENT, EGG PRODUCTION AND EGG COMPOSITION DURING THE FIRST EGG LAYING PERIOD

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Original scientific paper

SUMMARY

The experiment was carried out with 60 TETRA BLANCA laying hens chosen from 250 TETRA BLANCA pullets based on their body fat content, predicted at 16 weeks of age by means of computer tomography (CT). Pullets with the highest (n=20), lowest (n=20) and average (n=20) body fat content were chosen for the investigation. Changes in the body fat content of the experimental animals were followed by means of computer tomography in vivo, scanning the hens at every fourth week of the experiment, between 20 and 72 weeks of age. Eggs produced by these birds, were collected at 32, 52 and 72 weeks of age and, after breaking them, their yolk, albumen and egg shell ratio was determined and their dry matter, crude protein and crude fat content was chemically analyzed. Based on the results it was established that the body fat content of the hens started the egg production with high body fat content being higher than that of the hens started the egg production with average or low body fat content during the whole experimental period. The differences in the body fat content of the two extreme groups were statistically proven between 24 and 48 weeks of age (P<0.05). The total egg production of the hens with high starting body fat content was lower by 11 and 12 eggs per hen on the average than that of the hens with low and average starting body fat content, respectively. The composition of the produced eggs was mainly not affected significantly (P>0.05) by the starting body fat content of the hens.

Key-words: hen, body fat, egg production, egg composition, computer tomography

INTRODUCTION

It is well known from former experiments that the success in the hen house is dependent upon the success in the pullet house. However, during the pullet's rearing period we are mainly focused on managing pullet body weight and body weight uniformity. However, we should also realize that the cumulative nutrition program can have a significant effect on pullet's body composition.

Nowadays the pullet feeding programs can develop pullets of similar body weight, but with markedly different body compositions and subsequent reproductive patterns. Therefore, the optimal body conformation at photostimulation seems to be more important for reproductive success than just obtaining the recommended body weight targets (Powell, 2004).

In the study of Gregory and Robins (1998) it was established that the body condition of laying hens could be very different at the end of the laying period. It was also pointed out in this experiment that the empty body weight increased with increasing body condition score and on the average the birds with a body condition score of 3 were over 50% heavier than the birds scoring 0. About 77% of the difference in empty body weight between the condition score 3 and 0 birds resulted from differences in muscle and fat weight. Differences in absolute fatness accounted for most of that difference,

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and this was evident when the results were expressed as proportions of fat in the empty body.

The determination of the optimal body fat content at the beginning of the laying period seems to be very important, because both the low and high body fat content could have a negative effect on the production of the laying hens (Robinson et al., 1991; Hocking et al., 2002). Therefore, the aim of this study was to examine the effect of starting body fat content of Leghorn-type laying hens on the changes in their body fat content, egg production and egg composition during the first egg laying period.

MATERIAL AND METHODS

The experiment was conducted with 60 TETRA BLANCA laying hens chosen from 250 TETRA BLANCA pullets based on their body fat content, predicted at 16 weeks of age by computer tomography (CT). Pullets with the highest, lowest and average body fat content were chosen for the investigation (n=20 in each group). The live weight of the pullets chosen was similar in all of the experimental groups (1091±33, 1103±54 and 1116±43 g in the group of pullets with low, average and high body fat content, respectively; P>0.05).

The experimental animals were kept in cages in a closed building and were fed commercial diet *ad libitum* during the whole experimental period (Table 1). Drinking water was also continuously available from self-drinkers.

Changes in the body fat content of the experimental animals were followed by computer tomography *in vivo*, scanning the hens at every fourth week of the experiment, between 20 and 72 weeks of age. During the CT scanning procedures birds were fixed with belts in a special plexi-glass container, without using any anaesthetics. Three animals were scanned simultaneously. Due to the special arrangement of the hens, they were separable on the CT images, therefore their body fat content was determined individually.

Table 1. Composition of the diets used in the experiment

Component	Content
Dry matter (g/kg)	903.4
ME Poultry (MJ)	11.56
Crude protein (g/kg)	177.8
Crude fat (g/kg)	43.0
Crude fibre (g/kg)	43.1
Crude ash (g/kg)	47.6
Nitrogen-free extractives (g/kg)	591.9
Sodium (g/kg)	1.7
Lysine (g/kg)	8.7
Methionine (g/kg)	3.9
Methionine + cystine (g/kg)	7.0
Calcium (g/kg)	37.6
Phosphorous (g/kg)	7.0

The CT measurements consisted of overlapping 10 mm thick slices covering the whole body using a Siemens Somatom Emotion 6 multislice CT scanner at the Institute of Diagnostic Imaging and Radiation Oncology of the Kaposvár University. The following scanning parameters were set in: 130 kV – 80 mAs, spiral data collection (pitch 1), FoV 500 mm. From the images obtained so-called fat indices were calculated for the *in vivo* determination of the body fat content in the hens. The calculation was performed by determining the ratio of number of pixels with X-ray density values of fat to the total number of pixels with density values of muscle, water and fat, i.e. the range between -200 to +200 on the Hounsfield-scale.

Eggs, produced by these experimental birds were collected at 32, 52 and 72 weeks of age and, after breaking them, their yolk, albumen and egg shell ratio was determined and their dry matter, crude protein and crude fat was chemically analyzed by the regulations of the following standards (dry matter: MSZ ISO 1442, crude protein: MSZ EN ISO 5983-1:2005 [Determination of nitrogen content and calculation of crude protein content by the Kjeldahl method], crude fat: 152/2009/EK. III/H [Lipid extraction with petroleum ether]).

For the statistical evaluation of the differences in the body fat content, egg production and egg composition between the experimental groups, the One-Way Analysis of Variance was used. For testing the significance of the between-group differences the LSD Post Hoc test was used. The statistical analyses were conducted by the SPSS statistical software package, version 10.0 (SPSS for Windows, 1999).

RESULTS AND DISCUSSION

Examining changes in the body fat content of the hens it was established that it was higher in the hens started the egg production with high body fat content than that of the hens started the egg production with average or low body fat content during the whole experimental period (Figure 1).

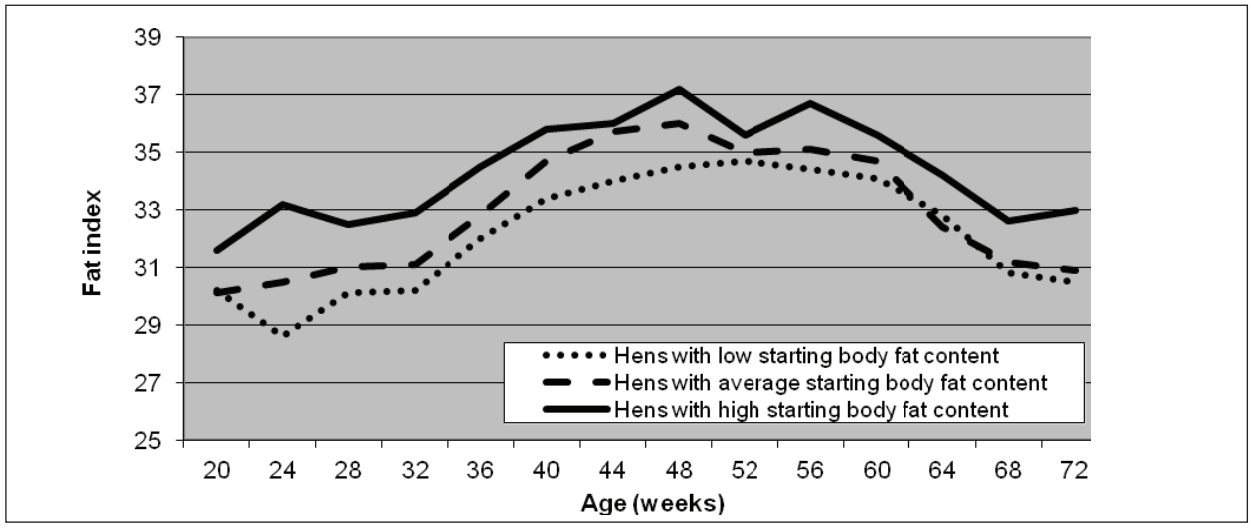

Figure 1. Changes in the body fat content of TETRA BLANCA laying hens started the egg laying period with low, average or high body fat content

The body fat content showed an increasing tendency till 48-52 weeks of age and decreasing thereafter in all of the experimental groups. The differences in the body fat content of the two extreme groups were statistically proven between 24 and 48 weeks of age ($P<0.05$).

The total egg production of the hens started the egg laying period with high body fat content was less by 11 and 12 eggs per hen on average than that of the hens started the egg production with average or low body fat content (281±25, 292±20 and 293±10, respectively). Although these differences were not statistically proven in this experiment ($P>0.05$), they are not worthy for the egg producers in general.

The composition of the produced eggs was mainly not affected by the starting body fat content of the hens significantly ($P>0.05$; Table 2).

Table 2. Composition of the eggs produced by TETRA BLANCA laying hens started the egg production with different body fat content

Traits (%)	Age (weeks)	Starting body fat content		
		Low	Average	High
Yolk ratio	32	29.4±4.6	28.0±2.4	29.2±3.6
	52	29.1±2.4	30.1±1.7	30.6±2.7
	72	28.8±4.3	27.6±3.8	28.1±2.7
Albumen ratio	32	57.8±4.5	59.2±2.6	58.4±3.9
	52	57.6±2.6	57.2±2.5	57.1±2.7
	72	55.4±3.9	54.9±3.1	55.0±3.4
Egg shell ratio	32	13.2±1.3	12.7±1.7	13.1±1.1
	52	12.3±0.7	12.3±1.3	12.1±0.8
	72	13.3±1.4	14.1±1.4	14.3±1.6
Eggs' dry matter content	32	24.4±0.7	24.3±1.1	24.3±0.9
	52	24.8±1.5	24.9±0.8	24.7±0.8
	72	24.7±1.3	24.8±0.9	25.0±1.0
Eggs' crude protein content	32	12.7±0.3	12.7±0.3	12.6±0.2
	52	12.5^{b}±0.6	12.4^{ab}±0.3	12.1^{a}±0.2
	72	12.6±0.3	12.7±0.6	12.8±0.4
Eggs' crude fat content	32	9.6±0.5	9.4±0.9	9.5±0.8
	52	10.0±1.1	10.1±0.7	10.2±0.8
	72	10.1±1.2	10.3±0.8	10.3±1.0

[a, b] Different letters in the same row indicate significant differences ($P<0.05$)

The increase in the body fat content of the hens in the first phase of the egg laying period was similar to our former results, but its decrease in the second part of the egg laying period was not observed in our former experiment (Szentirmai et al., 2013).

The non-significant effect of the starting body fat content of the TETRA BLANCA hens on the composition of their produced eggs was similar to our former results, where the production of TETRA SL laying hens was examined (Szentirmai et al., 2014).

CONCLUSION

Based on the results it was concluded that the starting body fat content of the TETRA BLANCA hens has significant effect on the changes in their body fat content, but it has no significant effect on the composition of their eggs produced in the first egg laying period. The total egg production of the hens was not affected significantly by the starting body fat content of the hens, but the lower production of the hens starting the egg laying period with high body fat content seems to be remarkable.

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COMPARISON OF COMMERCIAL DNA KITS AND TRADITIONAL DNA EXTRACTION PROCEDURE IN PCR DETECTION OF PORK IN DRY/FERMENTED SAUSAGES

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Original scientific paper

SUMMARY

In the present study four commercially available DNA extraction kits (Wizard® Genomic DNA Purification Kit, High Pure PCR Template Kit, DNeasy mericon Food and GeneJET PCR Purification Kit), as well as standard phenol/chloroform isolation technique have been evaluated regarding their concentration, purity and suitability for amplification of porcine DNA in dry/fermented sausages. The isolates were assessed for quantity and quality using spectrophotometer (IMPLEN GmbH, Germany). To verify template usability and quality of isolated DNA, the polymerase chain reaction (PCR) targeting at porcine cytochrome b by species specific primers was used. The comparison of extraction methods revealed satisfactory efficiency and purity of all extraction kits, while with standard phenol/chloroform isolation method high concentrations of DNA with low $A_{260/280}$ were obtained. However, all the investigated techniques proved to be suitable for identification of porcine DNA in dry/fermented sausage. Thus, the standard phenol/chloroform DNA extraction method, as the cost-effective one, can be recommended as a good alternative to more expensive isolation kits when investigating the presence of pork DNA in dry/fermented meat products.

Key-words: dry/fermented sausage, DNA, extraction method, pig

INTRODUCTION

Meat products, especially traditional ones are often being adulated due to the high prices they achieve on the market. However, adding even the smallest amounts of meat into the product other than one stated on declaration is illegal and misleading the consumer. Furthermore, it presents serious health, economical and religious problem. For that reason, identification of meat species in traditional meat products is of great importance. The methods for identification of animal origin are based on electrophoresis, isolectric focusing, chromatography, DNA hybridisation, polymerase chain reaction (PCR), enzyme-linked immunosorbend assay (Ong et al., 2007) and recently used aqueous ionic liquid buffer system (Fujita et al., 2006; Ressmann et al., 2015).

Among these methods PCR based techniques proved to be adequate, as they are fast, reliable and inexpensive. However, as every method, they also have their limitations, especially in cases where much of DNA has been degraded due to changes in pH or temperature (cooking, sterilisation, smoking etc.) foods are often subjected to during the production process (Pascoal et al, 2005; Aslan et al., 2009). This is particularly true for dry/fermented products, such as sausages, where DNA has been subjected to substantial degradation due to salting, smoking and other technological operations involved in making such product. It should be emphasised that DNA quality, purity and quantity has considerable effect in the species identification, and because of that methods for extracting DNA should be carefully selected (Sagi et al., 2009).

There are few methods available for extracting DNA from animal products. First is technique called phenol/chloroform extraction method commonly used for extraction DNA from various samples, but seldom from animal products. It is based on extraction of

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DNA by adding an equal volume of phenol-chloroform to aqueous solution of lysed cells, mixing the phases and allowing them to separate by centrifugation. The extracted DNA is precipitated with alcohol (Ausubel et al., 2000). Although rather laborious, this method is commonly used for DNA extraction because of its rather small price per sample. In recent years, a number of kits for extraction of DNA from different sources have been commercially available. In terms of researching the genetics of the animals, most of them are used for extraction of DNA from mammal blood or tissue. When extracting DNA from those kind of samples high quality DNA with very good yield can be obtained. Price per sample is however higher than in phenol/chloroform extraction method.

In this paper, yield, purity and suitability of two DNA extraction methods (spin column-based and conventional phenol/chloroform extraction procedure), as well as efficiency of four commercially available DNA extraction kits in PCR detection of pork in dry/fermented sausages have been evaluated.

MATERIAL AND METHODS

The investigation was carried out on 10 samples of Croatian traditional dry/fermented sausage called "kulenova seka" produced from pork, salt, garlic, red paprika and pepper, filled in natural casing and exposed to smoking, drying and ripening for approximately 40 days.

DNA was extracted from the sausage using the following commercially available DNA extraction kits: Wizard® Genomic DNA Purification Kit (Promega, USA), High Pure PCR Template Kit (Roche, Germany), DNeasy mericon Food Kit (Qiagen, Germany) and GeneJET PCR Purification Kit (Thermo Fisher Scientific Brand). DNA was isolated according to the manufacturer's protocol. In brief, all kits are based on use of 40 mg – 2 g of the raw material digested with lysis buffer and proteinase K. After the lysis, the lysate was centrifuged through silica membrane and binded to the columns. After series of washing steps, the pure DNA was finally extracted through dilution.

Also, the DNA was isolated using a standard extraction protocol with phenol-chloroform-isoamil (25:24:21) alcohol (Ausubel et al., 2000). In both methods used, the sausages were homogenized with knife mill using liquid nitrogene, mortar and pestle. Concentration and quality of the obtained DNA was determined by measuring the absorbance at 260 nm. DNA quality (purity) was measured by calculating the ratio of absorbance at 260-280 nm. UV/VIS spectrophotometer Nanophotometer® (IMPLEN GmbH, Germany) was used for spectroscopic analyses. The isolated DNA samples were analysed by PCR evaluation of suitability for amplification of porcine DNA. For the detection of pork DNA, a set of species-specific oligonucleotide primers previously reported by Doosti et al. (2014), which yield a 149 bp PCR fragment at porcine cytochrome b were used. The PCR reaction was set up using SapphireAmp® Fast PCR Master Mix (Takara Bio, Inc., Japan) in a 25.0 μL reaction volume containing 12.5 μL of mastermix, 9.5 μL of ultra-pure water, 1.0 μL of each primer and DNA. The PCR was performed in 30 cycles of denaturation at 98°C for 10 sec, annealing at 59°C and 72°C of elongation in a thermal cycler (Eppendorf Mastercycler Gradient). The obtained PCR products were visualised on a 1.5% agarose gel stained with Olerup SSP® GelRedTM Dropper (Olerup SSP AB, Sweden).

RESULTS AND DISCUSSION

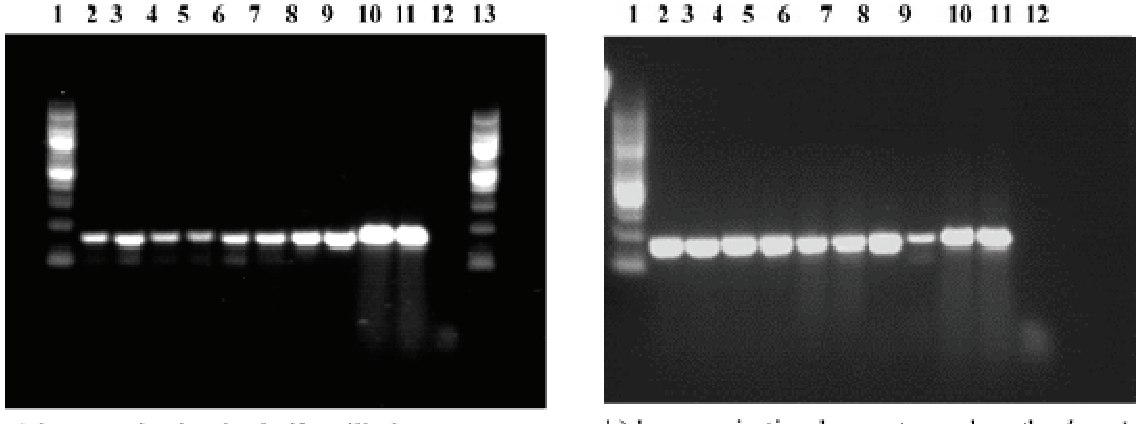
Table 1 presents concentrations and quality of the obtained DNA using of four commercially available DNA extraction kits and standard phenol/chloroform extraction protocol.

Table 1. Concentration and purity of the obtained DNA

Procedure/extraction kit	Homogenisation method	Concentration (ng/μl)	Purity ($A_{260/280}$)
Wizard® Genomic DNA Purification Kit	Knife mill	19.5	1.773
	Mortar and pestle	37.5	1.564
High Pure PCR Template Kit	Knife mill	32.0	1.641
	Mortar and pestle	37.3	1.500
DNeasy mericon Food Kit	Knife mill	126.0	1.780
	Mortar and pestle	164.0	1.777
GeneJET PCR Purification Kit	Knife mill	20.0	1.083
	Mortar and pestle	6.0	0.900
Phenol/chloroform extraction	Knife mill	2123.5	1.149
	Mortar and pestle	1304.0	1.137

As it was expected, the highest concentration of DNA was obtained by conventional phenol extraction method using knife mill for sample homogenisation. However, the purity of DNA was rather unsatisfactory ($A_{260/280}$=1.149 for homogenisation using knife mill and 1.137 for homogenisation using mortar and pestle, respectively) implying a certain amount of phenol in the DNA sample. Among commercially available extraction kits, the highest concentration and DNA purity was obtained using DNeasy mericon Food Kit (both with knife mill and mortar and pestle homogenization method). This was also predictable, as this kit is intended for highly processed foods, where high degradation of DNA can be expected. Amongst the extraction kits originally used for isolation of DNA from muscle tissue or the whole blood, the highest concentration of DNA was obtained with High Pure PCR Template Kit, but the most satisfactory $A_{260/280}$ ratio was obtained with Wizard® Genomic DNA Purification Kit where the sample was homogenized with knife mill. This is opposite to results of Di Pinto et al. (2007), who found a low DNA extrac-

tion efficiency of horse meat using Promega Wizard Magnetic DNA Purification for Food kit. In the work of Nesvadbová et al. (2010) aiming at choosing the most effective commercially available DNA extraction kit for chicken identification in different kind of food and feed, the later authors found that the highest DNA yields were obtained by NucleoSpin Food, Wizard Genomic DNA Purification Kit and JetQuick Tissue DNA Spin and the best DNA quality by using NucleoSpin Food, Wizard Genomic DNA Purification Kit and Invisorb Spin Food Kit I (ratio A_{260}/A_{280} close to 1.8). It was concluded that the results are highly depended on different food or feed using and different isolation system.



a) homogenization by knife mill: *lane 1- 100 bp DNA ladder; lane 2 and 3 – Wizard® Genomic DNA Purification Kit; lane 4 and 5 - High Pure PCR Template Kit; lane 6 and 7 - DNeasy mericon Food Kit; lane 8 and 9 - GeneJET PCR Purification Kit; lane 10 and 11 – standard extraction method; lane 12 – blank; line 13 – 100 bp DNA ladder*

b) homogenization by mortar and pestle: *lane 1- 100 bp DNA ladder; lane 2 and 3 – Wizard® Genomic DNA Purification Kit; lane 4 and 5 - High Pure PCR Template Kit; lane 6 and 7 - DNeasy mericon Food Kit; lane 8 and 9 - GeneJET PCR Purification Kit; lane 10 and 11 – standard extraction method; lane 12 – blank;*

Figure 1. The result of primer-specific identification of pig DNA by using commercial kits and standard isolation method (*a*-using knife mill for homogenization; *b*-using mortar and pestle for homogenization)

Figures 1 a) and b) show the obtained PCR fragment using porcine specific primers. It can be noticed that PCR amplification revealed 149 bp PCR product using all commercially available kits and standard phenol/chloroform DNA extraction procedure, regardless the homogenisation method.

CONCLUSION

The results of the present study indicate good efficiency of the all investigated DNA extraction techniques for determination of pig DNA in dry/fermented sausages. Although standard phenol/chloroform DNA isolation method did not show good $A_{260/280}$ ratio indicating phenol contamination, this contamination can be overcome by diethyl ether extraction or reprecipitation of the gDNA. As this method proved to be reliable and most importantly cost-effective, it can be recommended as a good alternative to more expensive commercial kits when extracting DNA from dry/fermented sausages for detection of pig DNA.

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PRELIMINARY STRUCTURAL OPTIMIZATION OF SOME FUMONISIN METABOLITES BY DENSITY FUNCTIONAL THEORY CALCULATION

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Original scientific paper

SUMMARY

Maize (Zea mays L.) is often contaminated with Fusarium verticillioides. This harmful fungus produces fumonisins as secondary metabolites. These fumonisins can appear both free and hidden form in planta. The hidden form is usually bound covalently to cereal starch. From the hidden fumonisins, during enzymatic degradation, glycosides are formed, and the fumonisin is further decomposed during a de-esterification step. In this short communication some preliminary DFT calculated structural results which could be useful in the future to help to understand the van der Waals force controlled molecular interactions between these kinds of mycotoxin molecules and enzymes are demonstrated.

Key-words: Fumonisin, fumonisin metabolites, DFT, electron density surfaces, structural optimization

INTRODUCTION

The term "mycotoxin" is usually reserved for the secondary metabolites produced by *fungi* that readily colonize crops. The sphingosine-like fumonisins are produced by several species of *Fusarium* molds, such as *Fusarium verticillioides*. The most commonly contaminated crop is maize (*Zea mays*). Fumonisins are common mycotoxins in maize, although these toxins can occur in a few other crops as well (Placinta et al., 1999). The primary health concerns associated with fumonisins are carcinogenic properties and acute toxic effects (Voss et al., 2007). The disruption of sphingolipid metabolism by inhibition of ceramide synthase has been proposed to be responsible for the carcinogenicity and toxicity (Wang et al, 1991). Four groups of fumonisins (FA, FB, FC and FP) were classified based on different structure of the carbon backbone and the location of the of nitrogen functional group (Musser and Plattner, 1997). Fumonisin (FB_1) is the most common and economically important form, followed by B_2 and B_3, where the index numbers refer to the different location of the hydroxyl groups on the carbon chain. Therefore, the metabolic investigations are focused on FB_1 *in vivo* (Fodor et al., 2008) and *in vitro* (Cirlini et al. 2015; Falavigna et al. 2012).

Mycotoxins undetectable by conventional, extraction-based analytical methods are termed as masked or hidden mycotoxins (Berthiller et al., 2013). Extractable mycotoxins can be easily detected but bound and/or hidden mycotoxins cannot be directly analysed. They have to be liberated from the matrix by chemical or enzymatic pre-treatment prior to chemical analysis. Dall'Asta et al. (2010) reported that with an *in vitro* digestion model method - after an enzymatic pre-treatment -, significantly more (30-40%) fumonisin was detected as compared to the measured moiety with conventional extraction method. In case of naturally contaminated (field derived) crop samples the hidden proportion of fumonisin is identical (35.6±22.3%, Dall' Asta et al., 2010). In case of inoculated crop cultures produced at laboratory-scale this value is 38.6±18.5% (Szabó-Fodor et al., 2015). Chemically modified mycotoxins are currently the largest group of modified mycotoxins and can be classified as "thermally formed" and "non-thermally formed" (Rychlik et al., 2014). Thermal degradation products have been described for several mycotoxins. A prominent example is fumonisin FB_1 which can react

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in a Maillard-type reaction with reducing sugars leading to *N*-(1-deoxy-D-fructos- 1-yl) fumonisin B_1 (NDF). The formation of hydrolyzed fumonisins (HFB_1), on one hand, are biologically modified and formed by the intestinal microbiota (Fodor et al., 2008) and, on the other, are formed under alkaline conditions during food processing (Humpf and Voss, 2004).

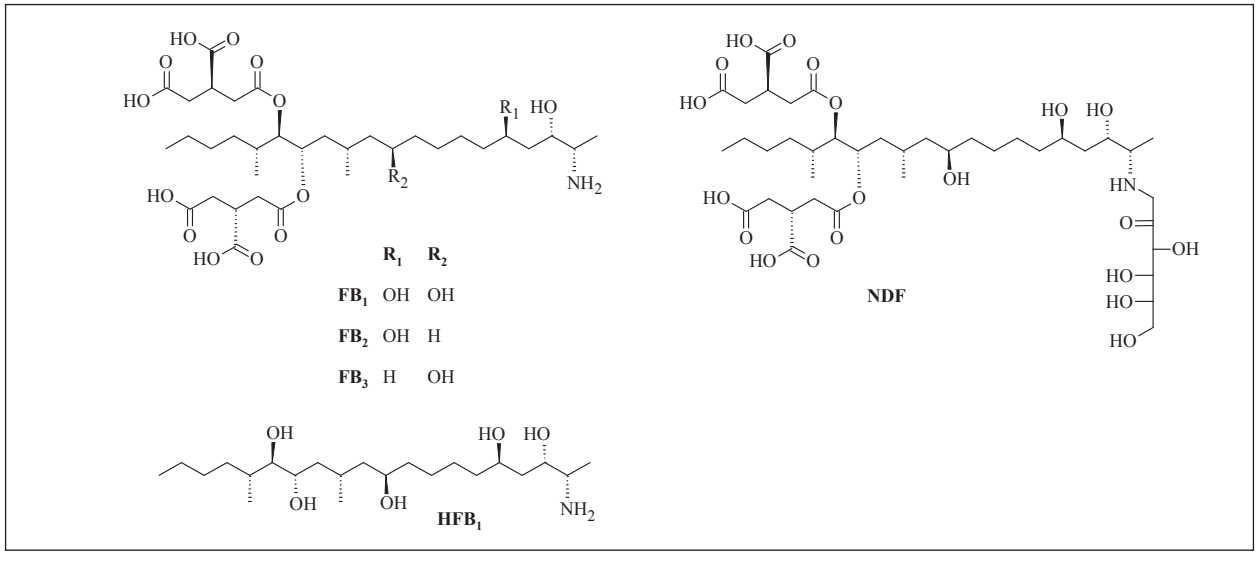

Figure 1. Structures of mentioned fumonisins

FB_1 is an inhibitor of sphinganine *N*-acyltransferase and increases the ratio of sphinganine/ sphingosine (Sa/So). Thus, elevation in the Sa/So ratio in different tissues (e.g. serum, urine, liver, kidney) is the exclusive biomarker of fumonisin exposure in exposed animals or human. However, best to our knowledge, no additional computation study was made to clarify the type of connection between sphinganine *N*-acyltransferase and fumonisin's monogastic metabolites. Density Functional Theory (DFT) calculation is an efficient method to determine the hydrophilic and hydrophobic site of molecules and it provides feasible input structures for the profound enzyme-substrate docking studies, in order to support the toxicological investigations.

From structural point of view, fumonisins provide a special nature: the long hydrophobic carbon chain bears electronegative (hydroxyl, amino- and carboxyl) functional groups. Due to free rotation around the carbon chain's single bonds, there could be several conformations – with different spatial location of functional groups –, being convenient for the above mentioned enzymes as "key", according to the lock and key enzyme model.

Several toxin molecules were investigated by theoretical DFT studies (e.g. Song et al., 2011; Rahmani brothers, 2014) including also mycotoxins (e.g. Türker and Gümüş, 2009). The first step of these computional studies is always the structure optimization followed by enzyme docking studies (e.g. Kumar and Garg, 2014). Docking studies needs huge computing power, usually executed in a computer cluster, which was not available in this case. The aim of this work was to highlight the lack amidst fumonisin metabolites and computational chemistry, as well as to provide some preliminary results for further, enzyme docking studies.

MATERIAL AND METHODS

All calculations were performed by Gaussian 03W. The structures were built in Advanced Chemistry Development, Inc. (ACD/Labs-ACD/3D) as .mol files and then converted to GaussView 3.09. The geometry of fumonisin metabolites were first pre-optimized with semi-empirical PM3 method and these structures were re-optimized in terms of DFT Gaussian 03 calculations for structural parameters using B3LYP (Becke's three-parameter functional with exact HF exchange and Lee-Yang-Parr exchange-correlation)/6-311G basis set. The FMO analysis (HOMO - highest occupied molecular orbital, LUMO - lowest unoccupied molecular orbital), are acronyms for and, involving hybridizations of selected bonds are also calculated at B3LYP methods and 6-311G level of the theory. Although most of all biologically relevant DFT studies set water as model solvent to investigate the solvation effect, in this case solvent interactions were neglected because of the long computation time; therefore all model calculations were executed in vacuum. GaussView 3.09's cube generator was used to calculate and visualize the electrostatic potential maps from the DFT results.

RESULTS AND DISCUSSION

Although the calculations are performed in vacuum – which is obviously not the medium of a living cell –, some ascertainment should be noted. The optimized structure of FB_1 is more spheroidal than expected which

is be explained with the zwitterion-like force between the Lewis base amine and acidic carboxyl group. It is in agreement with the more linear structure of NDF, where amine form *N*-glycosidic bond which is unable to show the above mentioned intermolecular interaction. HFB_1 became also a semicircle instead of a line, but with a smaller curve than FB_1, representing the ground state torsional angle between bonds (Figure 2.). Computed energy gap values (Δε) are shown in Table 1. The results are in agreement with the awaited stability (the first and second hydrolyzation step of NDF): $HFB_1 > FB_1 >$ NDF.

Table 1. B3LYP/6-311G calculated LUMO, HOMO and Δε (energies, are in eV) ($\Delta\varepsilon = \varepsilon_{LUMO} - \varepsilon_{HOMO}$)

Energy – (eV)	FB_1	HFB_1	NDF
LUMO	-4.08	-2.48	-5.01
HOMO	-9.82	-9.88	-8.98
Δε	5.74	7.40	3.97

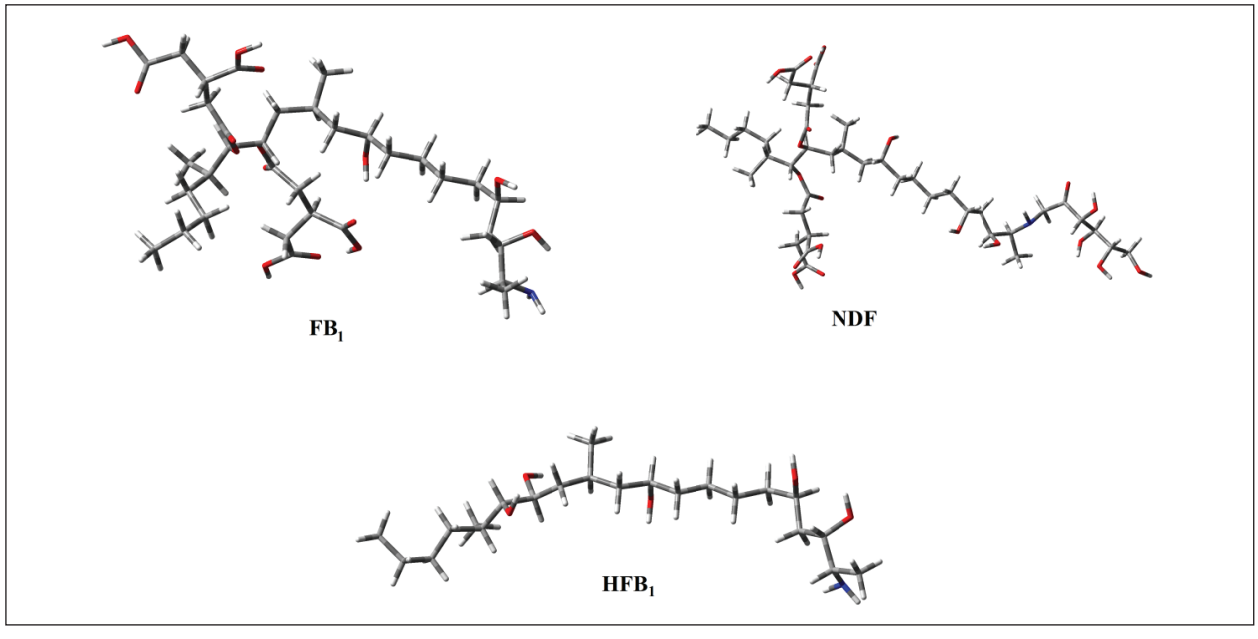

Figure 2. Optimized geometry of the investigated fumonisin molecules at 37°C *in vacuo* – tube model

CONCLUSION

The structures of FB_1, NDF and HFB_1 have been optimized by the Gaussian 03W at B3LYP/6-311G level. With the help of the results – and within the framework of the lock and key model –, researchers can better picture how the active site of the sphinganine *N*-acyltransferase approaches and joins the investigated fumonisin metabolites inhibiting the function of the enzyme in question.

By all means, these calculations should be handled as preliminary results, since solvation effects haven't been taken into consideration. Moreover, pH has momentous influence on the structure, especially in the case of FB_1. It should be calculated at isoelectric point as a zwitterion, with $-NH_3^+$ and $-COO^-$ functional groups. Due to the limited computational sources, we have to decide in the future to use smaller basis set instead of B3LYP/6311G and recomputing with solvent effect or to use a computer cluster to assess exact calculations.

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THE EFFECT OF TRANSITION FROM EUROP 5-POINT SCALE TO 15-POINT SCALE BEEF CARCASS CLASSIFICATION ON CARCASS DISTRIBUTION OF YOUNG SLAUGHTERED BULLS IN SLOVENIA

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Preliminary communication

SUMMARY

In 2007, the EUROP 15-point scale of carcass conformation and fatness classification system was introduced in Slovenia and replaced existing 5-point scale. Data (carcass weight, carcass conformation and fatness) from Slovenian commercial slaughterhouses were collected from January 2005 to December 2013. In total, data from 374,122 animals were used. The analysis was conducted for the category of young bulls from 12 to less than 24 months of age. In the first year after the transition, the classifiers preferentially used $_0$ classes in classification of carcass conformation and carcass fatness as well. In period 2008 - 2009 the classifiers adapted the new scale and started to use + and – subclasses more frequently. The distribution of conformation and fatness subclasses was brought near normal distribution.

Key-words: EUROP-carcass classification, distribution, young bulls, Slovenia

INTRODUCTION

Meat production and especially beef production is an important part of agricultural production in Slovenia. Around 18% of total value of purchased agricultural products in 2012 represented slaughtered calves and cattle (SURS, 2013). The main aim of the carcass classification and grading is to describe the carcass using standard terms to facilitate trading (Polkinghorne and Thompson, 2010). Carcass conformation and fatness are the traits used in EUROP classification system and thus the most important traits affecting the achieved price and the income of the producers. In the EU countries the five main classes with suitable subdivisions in subclasses were accepted as adequate to describe the very variable cattle population (Fisher, 2007). The Slovenian regulation first introduced EUROP carcass classification in 1994 and foresaw 5-point scale of conformation and fatness classification (Rules…, 1994). In 2005, the regulation was changed so that in 2007 the 15-point scale was introduced with further discriminate carcass prices due to differences in conformation and fatness. Furthermore, carcass subclasses were introduced (Rules…, 2005). 15-point scale should encourage all the participants to use traits, like weight, conformation and fatness score in genetic evaluation of cattle according to ICAR recommendation (ICAR, 2014). On the other hand, beef carcass classification is subjective and the individual classifiers had to adapt to those change. Measures were undertaken to encourage them to use also subclasses. The main objective of the our work was to find out how this transition affected classification results of slaughtered young bulls, representing the most important category of slaughtered cattle in the Slovenian slaughterhouses.

MATERIAL AND METHODS

Data from young bulls from 12 to less than 24 months of age were collected in commercial slaughterhouses in Slovenia from January 2005 to December 2013. The carcass weight was defined within 45 min after the slaughter. The conformation and fatness were estimated by independent classifiers according to the EUROP classification system with subclasses. Conformation classes expressed with letters were transformed to the numbers (E+ = 15, E_0 =14,…, P- = 1)

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and fatness classes as follows 1- = 1, 1_0 = 2, 1+ = 3,…, 5+ = 15. Data of total 374,122 animals were processed into statistical analysis. UNIVARIATE procedure in SAS statistical package (SAS, 2001) was used to test the normal distribution for each year.

RESULTS AND DISCUSSION

The number of graded carcasses from young bulls increased from 2005 to 2007 and decreased after that (Table 1). Carcass weight significantly increased from 345 to 354 kg in the first three years and then to 359 kg in the 2013. Together with mean carcass weight, variability also increased. Carcass conformation was relatively stable, with slight increasing trend in the last years. Similar results can be noted on the basis of 5-point as well as 15-point scale. On the contrary, carcass fatness slightly decreased in the last years. In Austria in the same period from 2007 to 2013, the average carcass conformation was relatively constant and varied from 3.44 to 3.51 (Daten and Fakten, 2015). Variability of carcass conformation and fatness as well remained constant during all the studied years. The average coefficient of variation was around 25% for carcass conformation and fatness as well. The transition from 5-point scale to 15-point scale had no effect on the average carcass conformation and fatness score.

Table 1. The number of graded carcasses of young bulls and the average carcass weight, conformation and fatness score in different years

Year of slaughter	N	Carcass weight, kg		EUROP conformation, 1-5		EUROP fatness, 1-5		EUROP conformation, 1-15		EUROP fatness, 1-15	
		mean	std	mean	std	mean	std	mean	std	mean	std
2005	40302	345.41	59.27	3.00	0.73	2.66	0.56				
2006	45001	342.65	59.31	2.95	0.73	2.66	0.54				
2007	49037	354.21	60.47	3.01	0.72	2.56	0.58	7.94	2.09	6.72	1.67
2008	46302	353.77	61.98	3.03	0.74	2.48	0.57	7.99	2.14	6.50	1.62
2009	41113	354.63	62.81	3.05	0.74	2.50	0.62	8.04	2.14	6.54	1.78
2010	39939	358.22	64.12	3.06	0.74	2.53	0.61	8.06	2.15	6.63	1.76
2011	42105	356.51	63.15	3.03	0.73	2.50	0.59	8.00	2.13	6.52	1.65
2012	37259	358.62	65.79	3.06	0.73	2.46	0.57	8.10	2.13	6.39	1.60
2013	33064	359.14	68.32	3.12	0.73	2.41	0.58	8.27	2.09	6.25	1.65

The distribution of slaughtered young bulls into different conformation and fatness subclasses is shown in Table 2. In 2007 the proportion of graded carcass into classes P_0, O_0, R_0,. U_0 and E_0 was higher than expected, whereas the proportion in + and – subclasses was lower. This points to the fact that classifiers preferentially used $_0$ classes. As early as the next year 2008 and further in 2009, the classifiers adapted to the new scale and started to use + and – subclasses more frequently. For example, if we look at the most representative subclass R_0, we can see that in the year 2007 there are 31.12% carcasses graded into those subclass, whereas in 2008 26.22% and 2009 only 23.21%. On the other side, the percentage of carcasses graded into R- and R+ increased by 3.59 and 1.33%. The same is true also for carcass fatness. In 2007, 37.55% of carcasses were graded into class 3_0. In the following year, these percentages declined to 25.92 and in 2009 further to 21.78%. On the other side, the percentage of carcasses graded in + in – class increased by 4.46% and 3.25%, respectively.

The alteration of distribution of slaughtered young bulls into different conformation and fatness subclasses through the studied years is clearly visible in Figure 1 and 2. Most of the changes occurred in the first three years after the introduction of 15-point scale for carcass conformation and fatness.

Table 2. The distribution of slaughtered young bulls into different conformation and fatness subclasses in different years (%)

			Year of slaughter						
			2007	2008	2009	2010	2011	2012	2013
EUROP-conformation, 1-15	P-	1	0.11	0.06	0.06	0.08	0.07	0.08	0.05
	P_0	2	1.07	0.77	0.67	0.70	0.71	0.51	0.45
	P+	3	0.50	0.71	0.69	0.76	0.86	0.70	0.85
	O-	4	3.15	3.86	4.36	4.01	4.42	3.97	3.45
	O_0	5	11.18	9.72	8.11	7.75	7.01	6.95	5.86
	O+	6	5.11	6.29	6.85	7.03	7.85	7.38	6.61
	R-	7	13.74	15.94	17.33	17.85	17.13	17.1	15.23
	R_0	8	31.12	26.22	23.21	23.02	25.2	24.53	22.99
	R+	9	10.76	11.27	12.19	11.93	12.58	13.25	16.11
	U-	10	9.57	11.07	13.00	12.86	10.14	10.61	14.24
	U_0	11	11.57	10.58	9.16	9.66	9.95	10.29	9.60
	U+	12	1.44	2.51	3.32	3.14	3.06	3.07	2.78
	E-	13	0.43	0.71	0.81	0.92	0.74	1.06	1.30
	E_0	14	0.25	0.29	0.23	0.27	0.27	0.39	0.46
	E+	15	0.00	0.00	0.01	0.02	0.01	0.11	0.02
EUROP-fatness, 1-15	1-	1	0.06	0.03	0.01	0.03	0.03	0.02	0.07
	1_0	2	1.34	0.82	1.94	1.67	0.84	0.71	0.66
	1+	3	1.06	1.04	1.37	1.26	1.29	1.07	1.32
	2-	4	3.65	5.21	6.89	5.73	6.69	7.16	10.26
	2_0	5	25.17	26.95	21.3	20.51	21.92	25.04	24.51
	2+	6	11.95	17.65	18.58	18.55	19.89	20.82	22.54
	3-	7	11.47	13.98	15.93	17.09	18.22	17.88	16.68
	3_0	8	37.55	25.92	21.78	22.29	21.06	18.41	15.3
	3+	9	5.61	6.34	9.13	9.58	7.52	6.79	6.18
	4-	10	1.11	1.21	1.54	1.59	1.4	1.2	1.25
	4_0	11	0.87	0.66	1.18	1.31	0.87	0.7	0.94
	4+	12	0.12	0.16	0.25	0.3	0.24	0.18	0.24
	5-	13	0.02	0.03	0.06	0.06	0.02	0.02	0.05
	5_0	14	0.02	0.00	0.04	0.02	0.01	0.01	0.00
	5+	15	0.00	0.00	0.00	0.01	0.00	0.00	0.00

In Table 3, the negative values of skewness for conformation in all the years indicate that the curve is always skewed left, so the tail on the left side of the probability density function is fatter. There is no such rule for fatness. The values of kurtosis near zero indicate a mesokurtic curve type. The presented p-values for Kolmogorov-Smirnov D-values were lower than 0.05 for all the studied years and pointed to non-normal distribution.

Table 3. Some basic measurements from normal distribution testing for carcass conformation and fatness of slaughtered young bulls in different years

Year of slaughter	EUROP conformation, 1-15				EUROP fatness, 1-15			
	Skewness	Kurtosis	Kolmogorov-Smirnov D-value	P-value for D	Skewness	Kurtosis	Kolmogorov-Smirnov D-value	P-value for D
2007	-0.2324	0.0237	0.1624	<0.0100	-0.2747	-0.1488	0.2312	<0.0100
2008	-0.1386	-0.1438	0.1335	<0.0100	0.0860	-0.2545	0.1649	<0.0100
2009	-0.1456	-0.1727	0.1198	<0.0100	0.01227	0.0170	0.1335	<0.0100
2010	-0.1385	-0.1150	0.1227	<0.0100	-0.0027	0.0912	0.1336	<0.0100
2011	-0.1261	-0.0454	0.1321	<0.0100	0.1053	-0.0455	0.1300	<0.0100
2012	-0.0566	0.0151	0.1316	<0.0100	0.2340	-0.0233	0.1470	<0.0100
2013	-0.2038	0.1049	0.1240	<0.0100	0.3882	0.1784	0.1548	<0.0100

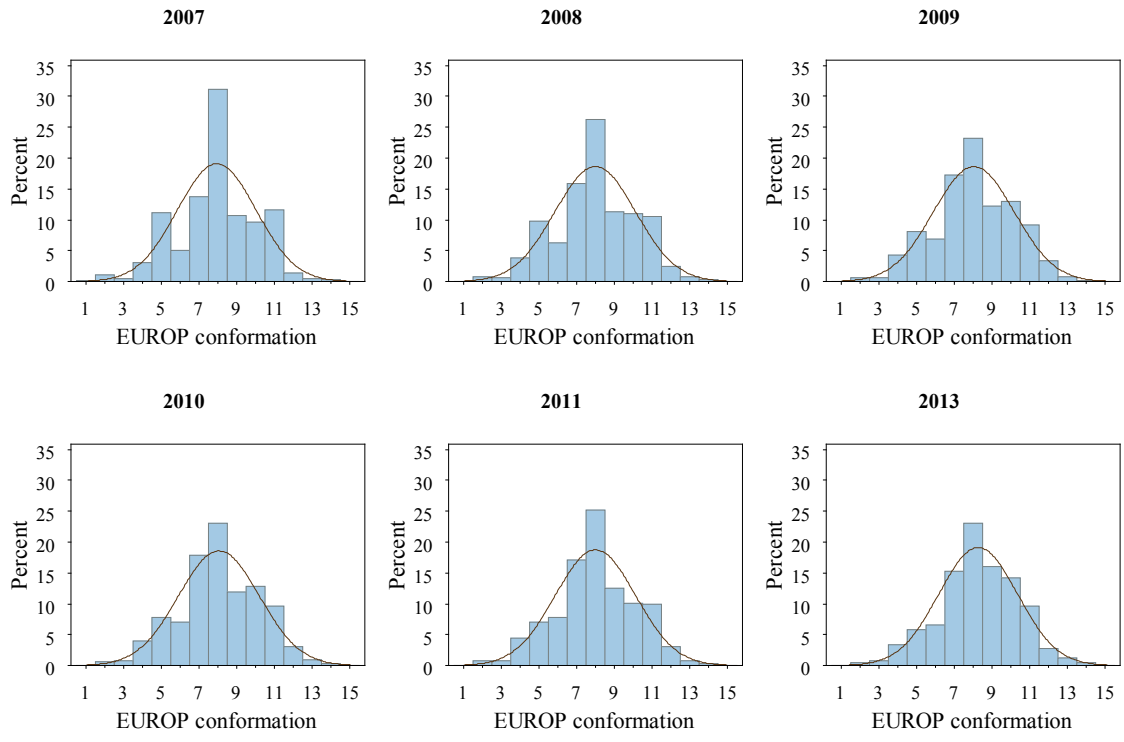

Figure 1. The distribution of slaughtered young bulls into different conformation classes in different years

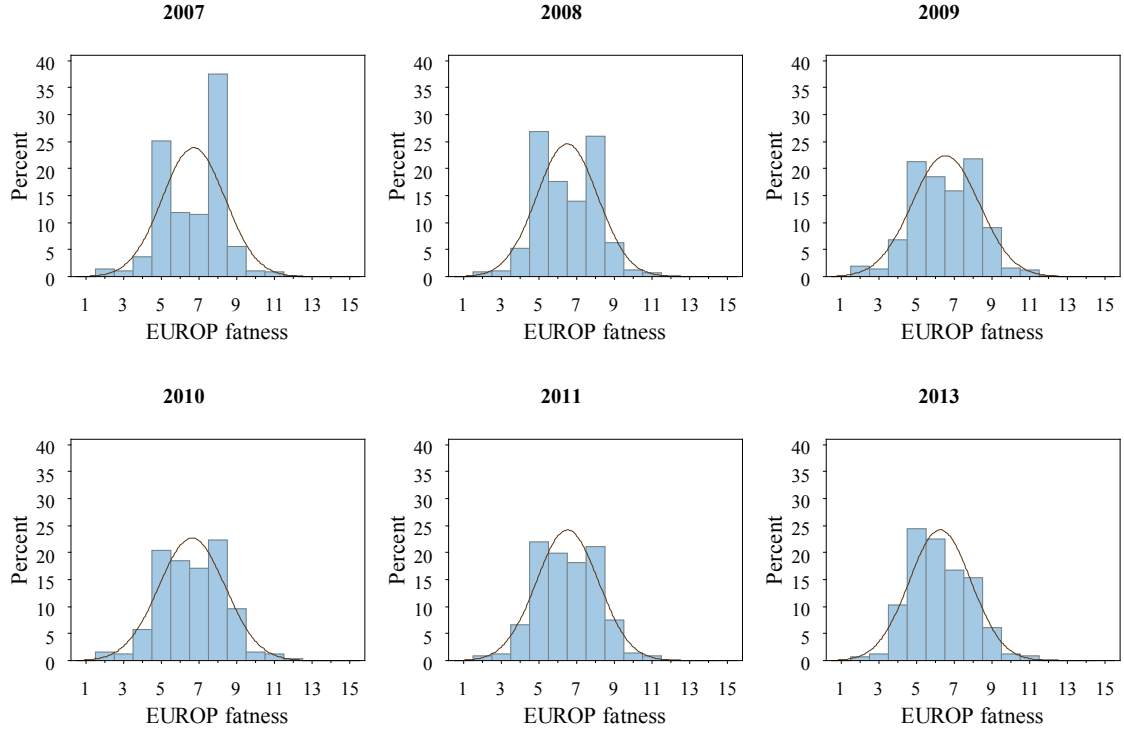

Figure 2. The distribution of slaughtered young bulls into different fatness classes in different years

CONCLUSION

The conducted analysis demonstrated that beef carcass classifiers successfully passed from 5-point to 15-point scale of carcass conformation and fatness classification. The carcass distribution into different conformation and fatness subclasses was brought near normal distribution. Carcass classification into subclasses enables expression of variability inside each class. This provides better quality of raw data for genetic evaluation and a basis for more effective genetic improvement.

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DETECTION OF PIG MEAT, LIVER AND LARD IN BEEF BY CE-SSCP

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Original scientific paper

SUMMARY

Identification of animal species from foodstuffs is important in order to identify frauds to prevent substitutions and admixtures in animal products. In this paper we demonstrate the identification of cattle and pig species by polymerase chain reaction (PCR) capillary electrophoresis - single stranded conformation polymorphism (CE-SSCP) method. The procedure is based on the amplification of the 12S rRNA gene encoded in the mitochondrial DNA (mtDNA). Since mtDNA copy number is highly tissue dependent mixtures of different pig tissues in cattle meat were prepared, at a concentration of 1, 5, 10, 20 w/w% of pig lard, liver and loin. It was determined that regardless the tissue type pig DNA can be detected by CE-SSCP at each contamination level.

Key-words: CE-SSCP, mtDNA, species identification, 12S rRNA

INTRODUCTION

Species identification in beef products has always been important for both the consumers and producers, because of economical, health and religious issues. Adulteration of beef with products from cheaper counterparts is a constant problem of the food industry nowadays. Despite the European Union strict labelling system can be easily evaded with mislabelling. There is a constant need for genetic traceability of food products for fraud detection. Traditional species identification methods are protein-based, including isoelectric focusing (IEF) and immunological methods. IEF separate proteins by their isoelectric point and result in a species specific protein pattern. Drawback of the method is that the obtained results are influenced by temperature and duration of the heat treatment during the technological process. IEF patterns can be too complex and interpretation of the results is difficult when multiple species are present in the sample (Skarpeid et al., 1998). Antibodies, mainly monoclonals can also be used to detect species. Chen et al. (1998) successfully produced monoclonal antibodies against pig thermal-stable muscle proteins with a detection limit of 10% pork in raw and cooked meat as well. However, finding protein antigen for species identification is challenging because fewer species-specific protein marker exist compared to DNA based markers. Production of monoclonal antibodies is also labour intensive and expensive process while the use of polyclonals can be affected by cross-reaction with closely related proteins. On the contrary, DNA-based methods can be characterized with specificity, sensitivity and high reproducibility. DNA is a macromolecule not affected by heat or chemical degradation and less affected by mechanical stress during food processing compared to proteins (Dalvit et al. 2007). DNA can be selectively amplified with PCR while protein amplification method does not exist. Methodologies that target mitochondrial DNA have the following advantages over methodologies targeting genomic DNA: mtDNA is present in much larger copy number compared to gDNA, it improves the possibility to be amplified during PCR and mtDNA has higher mutation rate which induces substantial genetic interspecies variation. The number of mitochondria may vary dramatically in different cell types and physiological conditions. Copy number estimations of the mtDNA in different cells mainly derived from human studies. A normal liver cell contains ~8000, a myocardium ~7000, a skeletal muscle cell ~3700, an adipocyte ~300 while a pancreatic cell contains ~100 copy of mtDNA respectively (Miller et al. 2003;

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Yin et al. 2004; Kaaman et al. 2007). Bellagamba et al. (2001) applied restriction site analysis of PCR products of cytochrome b to discriminate species in meat meal and animal feedstuffs. They used *Bst*N I digestion for species-specific fragment for cattle identification against pig. The principle of the PCR-SSCP technique is that single stranded DNA molecules take on sequence dependent three-dimensional structure after denaturation. Single-stranded molecules under non-denaturing condition differing by as little as a single base substitution can form different conformers and migrate differently in a non-denaturing gel. In our PCR CE-SSCP method, a 12S rRNA mtDNA fragment is amplified using fluorescently labelled primers and separated via capillary electrophoresis. We chose CE-SSCP method due to its simplicity and higher analysis speed because there are no further enzymatic steps involved after PCR compared to RFLP. It can also be carried out on a standard vertical electrophoresis unit. Disadvantage of the method is that reference samples from known origin must be investigated alongside with the unknown sample.

MATERIALS AND METHODS

Different pig tissues were prepared at concentrations of 1, 5, 10, 20 w/w% of pig lard, liver and loin for detection in cattle meat (Table 1). Samples 1-4 were used as controls. Total mass of the samples were 100±1 mg. Homogenization of the samples was performed with an Ultra Turrax T10 rotor-stator (IKA).

Table 1. Food samples and food sample mixtures used in our tests

Sample	Component	Sample	Component
1	100% pork bacon	9	80% cattle spare ribs 20% pork liver
2	100% pork liver	10	90% cattle spare ribs 10% pork liver
3	100% pork loin	11	95% cattle spare ribs 5% pork liver
4	100% cattle spare ribs	12	99% cattle spare ribs 1% pork liver
5	80% cattle spare ribs 20% pork bacon	13	80% cattle spare ribs 20% pork loin
6	90% cattle spare ribs 10% pork bacon	14	90% cattle spare ribs 10% pork loin
7	95% cattle spare ribs 5% pork bacon	15	95% cattle spare ribs 5% pork loin
8	99% cattle spare ribs 1% pork bacon	16	99% cattle spare ribs 1% pork loin

mtDNA was selectively isolated with a method described by Das et al. (2012). In the final step DNA was resuspended in 50μl double distilled water. Concentration and quality of the isolated mtDNA samples were determined using NanoDrop 1000 spectrophotometer (Thermo Fischer Scientific, USA). Nucleotide sequences of 12S rRNA mitochondrial gene of pig (AM158316.1) and cattle (GQ926965.1) were obtained from the NCBI GenBank database. Nucleotide sequences were then aligned using CLUSTAL OMEGA algorithm to check for conservative regions. After that primers forward primer 5′-ACTCTAAGGACTTGGCGGTG-3′ and reverse primer 5′-TTTACTGCTAAATCCTCCTT-3′ were picked with Primer3 software. Following primers targeting RYR1 gene (NC_010448) were designed with Primer3 software too: forward 5′-AGACCTTTCTCTTTGACCTTGAT-3′ and reverse 5′-CCAGACCTGGTGACATAGTTGA-3′. After that polymerase chain reaction (PCR) targeting RYR1 gene was performed to check for gDNA contamination which may interfere with the mtDNA concentration measurement. PCR of the 12S rRNA was performed in 10 μl volume containing 10x Dream Taq buffer (Fermentas, USA), 200 μM dNTP mixture (Fermentas, USA), 4 mM $MgCl_2$ (Promega, USA), 2 pmoles of FAM labelled forward primer (Sigma, Germany), 2 pmoles of VIC labelled reverse primer (Sigma, Germany), 1U Dream Taq polymerase (Fermentas, USA) and 150 ng DNA template. PCR was carried out in a PTC-200 thermal cycler (Bio-Rad). Thermal profile was: 95°C for 1.5 min followed by 35 cycles of denaturation at 95°C for 30 sec, primer annealing at 60°C for 30 sec and extension at 72°C for 30 sec. The final extension step was 5 min at 72°C. Amplified PCR products were analysed in 1.5 m/v% agarose gel (Lonza, France) for 1h at 6V/cm in TAE (Lonza, France) buffer and stained with ethidium-bromide (Applied Biosystems, USA). The samples were prepared for capillary electrophoresis analysis as follows: total volume of 10 μl consisted of 0.5 μl, 2 fold diluted PCR product, 0.5 μl LIZ 500 size standard and 9 μl of HiDi formamide (Life Technologies, USA). Capillary electrophoresis was performed on ABI Prism 310 Genetic Analyzer (Applied Biosystems, USA), equipped with an argon-ion laser, emitting light at 488-514 nm. Samples were electrokinetically -injected at 15 kV for 4 sec to a 47 cm (effective length: 30 cm) long 50 μm diameter (Applied Biosystems) capillary filled with 15 w/t% solution of Pluronic F108 polymer (Sigma, Germany) according to Hwang et al. (2013) containing 0.7x Genetic Analyzer buffer (Applied Biosystems, USA). Electrophoresis was performed at 35°C with 15 kV and 40 min running time. Signal detection was between 525-650 nm. Raw data were collected using the Data Collection software 3.1.0., and processed with GeneMapper® 3.7 (Applied Biosystems) software (Figure 2). To obtain reproducible results, electropherograms were calibrated by fixing the positions of peaks produced by the LIZ 500 size standard (Applied Biosystems, USA).

RESULTS AND DISCUSSION

Figure 1 shows that 12S rRNA PCR products were detected while gDNA RYR1 PCR products were not detected. The absence of gDNA was shown (Figure 1) and subsequently, it could be rough parameter predictor for estimation of mtDNA copy number in a different pig tissue types (Table 2).

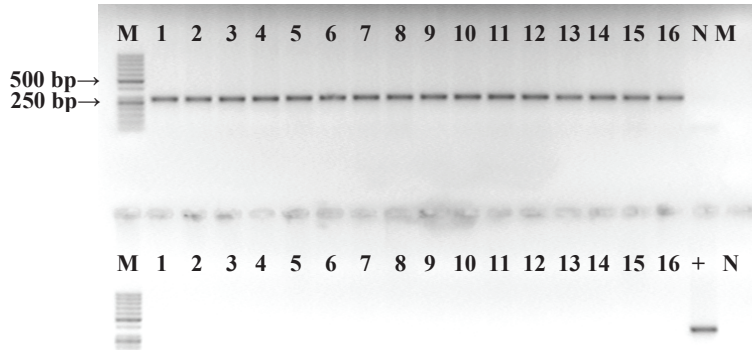

Figure 1. Agarose gel electrophoresis of the labelled PCR products. Upper part shows a single PCR products (283 bp) with labelled primers, lower part shows the PCR products (329 bp) of RYR1 primers. gDNA contamination was not detected. Lanes represents samples 1-16 as defined in the material and methods. +: positive control N: negative control; M: 50 bp ladder (Thermo Fischer Scientific, USA).

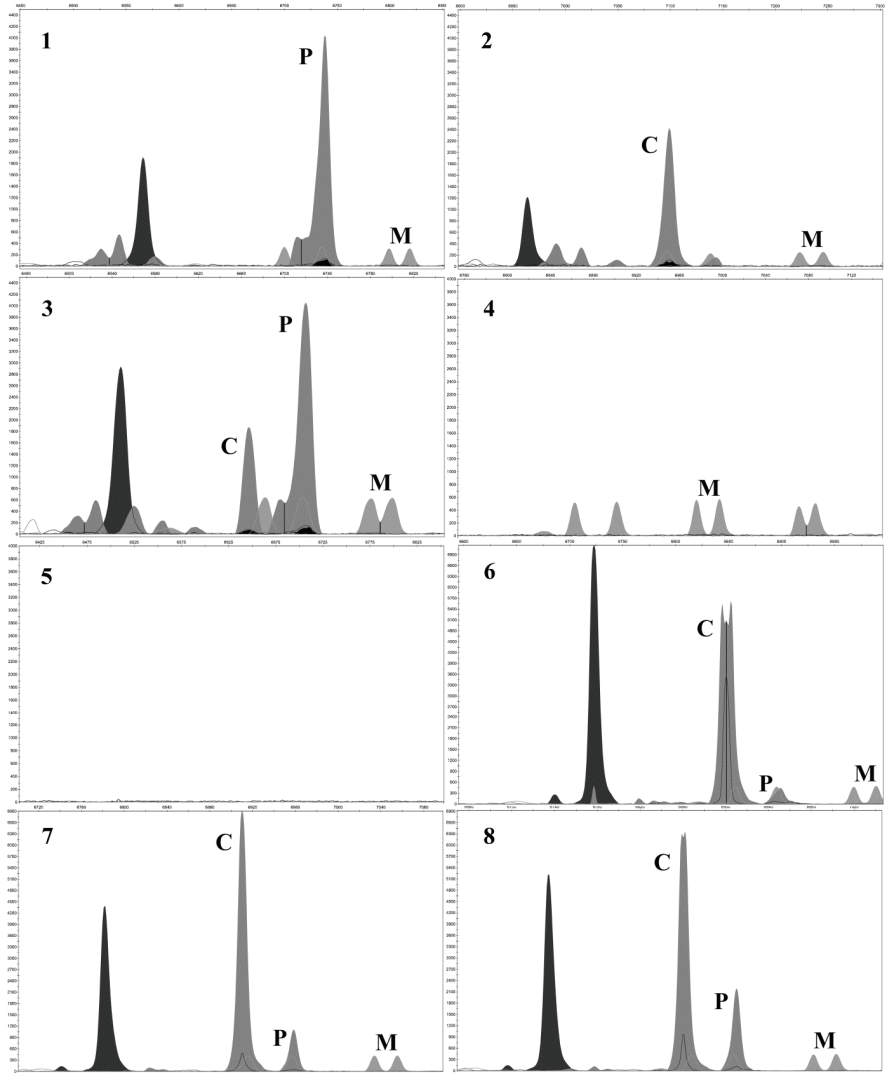

Figure 2. CE-SSCP electropherograms representing species-specific patterns of 12S rRNA of the pig (P) and cattle (C) samples. M: LIZ 500 DNA marker. VIC and 6-FAM labelled strands are shown as light grey and black peaks. Conformation changes were only detectable in the VIC labelled conformers. Vertical axis represents relative fluorescents units (RFU); the horizontal represents data points (1 data point is equal to 220 msec migration time). 1: pig control; 2: cattle control; 3: cattle + pig control; 4: LIZ standard control; 5: negative control; 6: 99% cattle loin + 1% pig lard; 7: 99% cattle loin + 1% pig loin; 8: 99% cattle loin + 1% pig liver

Table 2. Estimation of mtDNA copy number in different tissues, based on our spectrophotometric data

Tissue type	NanoDrop concentration measurement (ng/μl)	Calculated amount (ng) of mtDNA in 1 mg tissue	Rough estimation of mtDNA copy number in 1 mg tissue
Pig liver	2473.58	1236.79	6.87*10^10
Pig loin	506.57	1.41	1.41*10^10
Pig lard	117.91	58.9	3.27*10^9

Calculations in Table 2 are based on the assumption that the average weight of a base pair is 650 g/mol and 16679 base pair is the length of the pig mtDNA (Ursing et al., 1998). According to this estimation, even 1 mg tissue contains abundant amount of template for PCR. Figure 2 shows clear separation of the pig and cattle specific bands in the control runs as well as in the test runs. We determined that this method can detect as low as 1 w/w% pig lard (Figure 2 /6/), loin (Figure 2 /7/) and liver (Figure 2 /8/) mixed with cattle loin. It should be mentioned that our method is not suitable for quantitative estimations of the species in the starting material due the fact that the mtDNA copy number is highly depended on tissue types, which can lead to dissimilar peak intensities (Figure 2).

CONCLUSION

In summary, a 12S rRNA based PCR CE-SSCP method was developed to identify pig and cattle species in the test samples. 1% pig mtDNA was detectable in all cases (cattle meat mixed with pig lard, pig loin and pig liver). Further aim of the experiment is to investigate the applicability of the method with the described primers by involving additional mammalian species and commercially available processed beef products to the analysis.

ACKNOWLEDGEMENT

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THE EFFECT OF OLIVE BY PRODUCTS AND THEIR EXTRACTS ON ANTIOXIDATIVE STATUS OF LAYING HENS AND OXIDATIVE STABILITY OF EGGS ENRICHED WITH N-3 FATTY ACIDS

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Original scientific paper

SUMMARY

The aim of the study was to assess the effects olive leaves, pulp and their extract supplementation on performance, antioxidant status and oxidative stability of eggs. Oxidative stress was induced by the addition of 6% linseed oil in the feed. 94 individually caged laying hens, 40 weeks old, were included in the study. Animals were divided into 6 groups. The feed of each group was composed of a basic feed, supplemented with: group Cont - no supplement, Vit E - 150 IU of α-tocopherol acetate /kg, Olive L - 1% of olive leaves, Olive Ex - extract from olive leaves, the Pulp group - 1% of dried and ground pulp and Pulp Ex - extract from pulp. Based on the results we found out that supplementation of vitamin E, olive leaves, pulp and their extracts had no effect on the performance of hens and showed neither a lymphocyte DNA damage preventive activity nor influence malondialdehyde (MDA) concentration in plasma. The results suggest that α-tocopherol acetate and olive leaves supplementation had significant effect on the MDA content of the stored eggs. Supplements, except vitamin E had neither influence on antioxidant activity (ACL) in eggs nor on n-3 PUFA in fresh and 40 days stored eggs.

Key-words: laying hens, nutrition, olive leaves, olive pulp, egg, oxidative stability

INTRODUCTION

Human dietary recommendations for general population have been focussed on increasing the consumption of polyunsaturated fatty acids (PUFAs), particularly from the n-3 series and reducing the consumption of saturated fatty acids (SFAs). It is also possible to increase the n-3 content of the yolk by enriching hen diets with flax oil. However, PUFAs are more prone to oxidation. These phenomena can be prevented or limited by enriching the eggs with antioxidants such as vitamin E. Feeding trials have shown that vitamin E is an efficient mean for improving the oxidative stability of eggs (Meluzzi et al., 2000). On the other hand natural feed additives in animal diets can also have antioxidant properties (Florou-Paneri et al., 2005). Olive leaves are agricultural residues from the beating of olive trees (*Olea europea L.*) for fruit harvest. They contain many substances, the most important are oleuropein, tyrosol and hydroxytyrosol (Silva et al., 2006). Most of the phenolic compounds are potent antioxidants with anti-inflammatory properties (Benavente–Garcia et al., 2000). Olive pulp is the raw material resulting from extraction of olive oil containing important phenolic compounds. The objective of the present study was to evaluate the effect of n-3 enriched feed supplemented with olive leaves, pulp or their extracts, on laying hen's performance, their antioxidant status and oxidative stability of eggs.

MATERIAL AND METHODS

The study included 94 Isa Brawn laying hens, 40 weeks old individually caged. The animals were randomly assigned to five treatment groups (Table 1). Hens within the control group (Cont) were fed a basal feed containing 6% linseed oil. Other five groups were fed the same diet supplemented with 150 IU/kg feed of α-tocopheryl acetate (Vit E), 10 g/kg of dried and ground olive leaves (Olive L), 1 g/kg of extract prepared from 10 g/kg of olive leaves (Olive Ex), 10 g/kg of dried and ground olive pulp (Pulp), and 1 g/kg of extract prepared from 10 g of olive pulp (Pulp Ex). The trial lasted for 6 weeks.

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Table 1. Composition of the hen diets (per kg)

	Cont	Vit E	Olive L	Olive Ex	Pulp	Pulp Ex
Maize (g)	250.00	250.00	250.00	250.00	250.00	250.00
Wheat (g)	252.53	252.53	252.53	252.53	252.53	252.53
Sunflower meal (g)	80.00	80.00	80.00	80.00	80.00	80.00
Soybean meal (g)	207.82	207.82	207.82	207.82	207.82	207.82
Linseed oil (g)	60.00	60.00	60.00	60.00	60.00	60.00
Wheat starch (g)	10.00	10.00	/	9.00	/	9.00
Vitamin E (IU)	/	150	/	/	/	/
Olive leaves and olive leaves extract (g)	/	/	10.00	1.00	/	/
Olive pulp and olive pulp extract (g)	/	/	/	/	10.00	1.00

The mineral and vitamin calculated for the Isa Brawn hen's requirements

Fresh olive leaves from ecological olive trees and olive pulp were collected for extracts preparation in November. Leaves were dried at 50ºC and then ground to pass 2 mm screen and stored at 4ºC until used. Olive pulp was obtained from local oil mill, lyophilized and ground to pass 2 mm screen and stored as olive leaves. The 70% ethanolic extracts prepared from olive leaves and pulp including ethanol were evaporated. All diets were prepared each week in mash form and stored at 4ºC until use (Table 1).

Table 2. Chemical analysis of feed mixtures and n-3 fatty acids content (g/kg)

	Dry matter	Crude protein	Crude fat	Crude fibre	Crude ash	n-3 PUFA
Average ± std	911 ± 1.4	168 ± 2.2	78.6 ± 5.1	51.1 ± 2.2	144.7 ±7.4	42.2 ± 1.0

Live weight gain was recorded at the end of the experiment. Feed consumption was recorded weekly. The number of eggs and egg weights were recorded daily. At the end of the experiment, blood was collected for the determination of DNA fragmentation of blood lymphocytes, malondialdehyde (MDA) and ACL in plasma. The comet assay was performed by the method of Singh et al. (1988), with slight modifications as described by Rezar et al. (2003). The modified methodology of Wong et al. (1987) was used to measure the concentrations of MDA in blood plasma by HPLC. Plasma antioxidative capacity of lipid-soluble substances (ACL) in egg yolk was determined by the Photochem assay (Analytik Jena, Leipzig, Germany). Data were analysed using the GLM procedure of the SAS/STAT module (SAS 8e, 2000; SAS Institute Inc., Cary, NC). The study protocol was approved by the Animal Ethics Committee of the Veterinary Administration of the Republic of Slovenia.

RESULTS AND DISCUSSION

Poultry in intensive farming systems are frequently exposed to oxidative stress which can result in damage of the body proteins, lipids and DNA and can lead to reduce performance and health (Lykkesfeldt and Svendsen, 2007). The aim of the present study was to evaluate different natural feed additives (olive leaves and pulp) in preventing dietary-induced oxidative stress. Oxidative stress occurs due to inclusion of large amount of high-PUFA vegetable oils in animal feed for creating poultry products with improved nutritional quality (Wood et al., 2004). No differences between the groups were observed in body weight, feed consumption (Table 3), egg production and egg weight. The comparable data in literature are scarce. Botsoglou et al. (2005) did not find differences in egg weight and other egg quality characteristics in the experiment with different aromatic plants supplements. Also Jiang et al. (1994) reported no significant effect on egg weight, when α-tocopherol acetate was supplemented in hen diets at the level of 200 mg/kg.

Table 3. Results of hen's performance

	Cont	Vit E	Olive L	Olive Ex	Pulp	Pulp Ex	p
Body weight (g)	2105.7	2162.1	2062.6	2059.3	2013.7	2092.4	0.61
Feed intake (g/day)	109.4	113.5	108.8	110.8	104.0	114.8	0.07
Egg production (%)	98.4	96.2	95.2	96.2	93.7	98.0	0.09
Egg weight (g)	64.3	64.5	62.9	63.7	62.6	64.5	0.63

High concentrations of PUFA in the diet did not significantly increase the formation of MDA in plasma (Table 4). In contrast, Sahin et al. (2010) found out that the inclusion of resveratrol into diets could enhance antioxidant status in quails, as reflected by dose-dependent decreases in serum MDA. Oxidative damage to DNA is a useful index of oxidative stress. It was proposed that DNA damage is caused by free radical generated as a result of the high intake of PUFA. In our experiment no significant differences between the groups in the rate of lymphocyte DNA damage (head DNA percentage) were observed. Study of Fabiani et al. (2008) showed a potent DNA damage preventive activity of olive oil phenols in humans, but no such studies exist on laying hens.

Table 4. Lymphocytes DNA damage and the marker of oxidative stress and MDA in blood plasma

	Cont	Vit E	Olive L	Olive Ex	Pulp	Pulp Ex	p
Plasma MDA (nmol/ml)	1.66	1.49	1.57	1.39	1.47	1.58	0.54
Head DNA (%)	79.47	76.41	79.59	80.58	76.44	80.56	0.69

The effect of dietary treatments on lipid oxidation of egg yolk, fresh or stored for 40 days is shown in Table 5. The obtained MDA values were very low and confirm generally high oxidative stability of fresh eggs (Cherian et al., 1996). The extend of lipid oxidation differed among the dietary treatments only in 40 days stored eggs and reveals lowest lipid oxidation in groups Vit E and Olive L. This is in agreement with Botsoglou et al. (2005) who found out that natural supplements (rosemary, oregano, saffron) improved oxidative stability of eggs in comparison to unsupplemented control group.

Table 5. MDA concentration (nmol/g egg yolk) in fresh and 40 days stored egg yolk

	Cont	Vit E	Olive L	Olive Ex	Pulp	Pulp Ex	P
Fresh	1.08	0.82	1.54	1.04	1.47	1.06	0.12
40 days stored	1.82^{a}	1.06^{b}	1.15^{b}	1.86^{a}	1.57^{ab}	1.42^{ab}	0.09

^{a,b,c,d} – Least squares means within a row without the same superscript differ significantly ($p<0.05$)

Antioxidative capacity of lipid-soluble substances (ACL) was measured in fresh and 40 days stored egg yolks (Table 6). In comparison with all other groups only vitamin E supplementation (group Vit E) significantly improved ACL in fresh and stored eggs.

Table 6. ACL (nmol/g egg yolk) in fresh and 40 days stored egg yolks

	Cont	Vit E	Olive L	Olive Ex	Pulp	Pulp Ex	p
Fresh	196.2^{a}	660.2^{b}	226.5^{a}	212.7^{a}	215.7^{a}	224.4^{a}	<0.0001
40 days stored	232.7^{a}	846.9^{b}	266.8^{a}	277.0^{a}	288.7^{a}	277.3^{a}	<0.0001

^{a,b,c,d} – Least squares means within a row without the same superscript differ significantly ($p<0.05$)

Table 7 shows that neither α-tocopherol acetate nor any other supplementation exerted any significant effect on the fatty acid composition of fresh eggs. Nevertheless, the results are in agreement with the previous studies reporting no effect of tocopherol supplementation on the fatty acids profile of fresh n-3 enriched eggs (Qi and Sim, 1998). Also, Botsoglou et al. (2012) reported that neither α-tocopherol acetate nor olive leaves supplementation exerted any change in fatty acids composition of eggs.

Table 7. Content of n-3 PUFA in fresh and 40 days stored egg yolk (% of total fatty acids)

	Cont	Vit E	Olive L	Olive Ex	Pulp	Pulp Ex	p
Fresh	12,6	13,8	13,5	14,2	13,8	14,3	0.58
40 days stored	12,6	13,5	13,2	14,2	13,6	14,3	0.39

CONCLUSION

Based on the results we can conclude that supplementation of vitamin E, olive leaves, pulp and their extracts had no effect on the hen performance. The parameters of oxidative stress (DNA damage and MDA concentration in plasma) showed that there was no effect of the supplements on the antioxidant status of the hens. Vitamin E exerted higher antioxidant activity (ACL) of eggs than other feed supplements used in the experiment. The results also show that the oxidative stability of lipids measured as MDA content in egg yolks was improved by vitamin E and olive leaves supplementation in 40 days stored eggs only, and did not change in fresh eggs. There was no effect of any supplement on the n-3 PUFA proportion in egg yolks.

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ECONOMIC SUSTAINABILITY OF THE LOCAL DUAL-PURPOSE CATTLE

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Original scientific paper

SUMMARY

Base economic characteristics (total revenues, total costs, profit and profitability ratio) of the Slovak Pinzgau breed were calculated in this study. Under the actual production and economic conditions of the breed, production system is operated with loss (-457 € per cow and per year) and with negative profitability ratio (-20%). Optimisation of the production parameters on the level defined in the breed standard (5,200 kg milk per cow and year, 92% for conception rate of cows, 404 days of calving interval and 550 g in daily gain of reared heifers) and improved udder health traits (clinical mastitis incidence and somatic cells score) was of positive impact on the total revenues (+34%), on the effective utilisation of costs (+105%) and balanced profit of dairy systems. Next to the positive profitability of the system, higher quality and security of dairy milk products should be mentioned there. Moreover, direct subsidies as an important factor of positive economic result of dairy cattle systems has to be pointed as well. Subsidies should be provided to compensate the real biological limitation of the local breed farmed in marginal areas. However, improvement of the production parameters of the Slovak Pinzgau breed is recommended with the same attention to reach the economic sustainability of dairy production system. To reach economic sustainability of the breed from practical point of view, the farmer activity should be aimed especially to the enhanced herd management.

Key-words: Slovak Pinzgau, economic sustainability, health traits, optimisation

INTRODUCTION

Cattle production plays an important role in agriculture sector especially in countries with substantial forage resources (Doucha et al., 2012). Its economics is determined by many factors, by natural conditions, breed character and its production level, marketing policy, human sources and generally by effective utilisation of inputs (Krupová et al., 2012; Michaličková et al., 2014). The impact of components of farm profit and their relationship should be respected by farmers (Miller et al., 2001).

The Slovak Pinzgau cattle is typically farmed in the mountainous regions of Slovakia. It has been classified as an Animal Genetic Resource since 1994 (Kadlečík et al., 2008). Its breeding aims to produce combined (milk and meat) cattle breed suitable for mountains regions. Production and economic parameters of the breed have been changed substantially in the last seven years period. Milk yield of cows was increased by 278 kg per lactation. However, conception rate of female deteriorated and average calving interval of a cow raised by 10 days (Breeding Service of the Slovak Republic, unpublished data). Increase in market price of outputs (by 0.04 € per kg of milk and by 0.42 € per kg of weaned calf) was not relevant to change in input prices (Michaličková et al., 2014) and finally, the size of dairy cow population was reduced.

The current level of breed production is under the standards defined by Association of Slovak Pinzgau breeders (ASPB, 2014). Therefore the aim of the study was to calculate the main economic characteristics of dairy production system the Slovak Pinzgau breed based on the current production and economical parameters.

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Followed this, optimization of the traits based on breed standard was provided to reach economic sustainability of the breed farmed in marginal areas of Slovakia.

MATERIAL AND METHODS

Economic parameters for the purebred dairy population of Slovak Pinzgau breed farmed in a classical indoor and free housing system with regular access to pasture were calculated. Selling of surplus calves at weaning and surplus pregnant breeding heifers was assumed. The cow herd structure at the stationary state was derived using the Markov chain methodology (described by Wolfová et al., 2007).

The economic efficiency of dairy population was measured as total profit (total revenues minus total costs) per cow entering a reproductive cycle and per year (Wolfová et al., 2007). Profitability ratio of the production system (total profit divided by the total cost expressed in %) including direct subsidies was calculated to measure the effectiveness of expended costs. Revenues came from milk and sold breeding heifers, weaned calves, slaughtered cows and heifers, manure (market price at 3.65 € per t) and subsidies. Revenues from sold milk and animals were function of the production level and the market pricing system. Regarding the revenues, the direct subsidies (average value over the last three years period; MA SR, 2014) were considered. Costs for feeding, housing, veterinary treatment, breeding and fixed costs were calculated for individual cattle category. Daily energy and protein requirement for maintenance, production and activity of the given category were taken into account to define the feeding costs (Wolf et al., 2013).

Actual production, reproduction, management and economic parameters of the breed (averaged over the last three years period) were defined by the own investigations in cooperated farmers (Michaličková et al., 2014), databases of the Breeding service of the Slovak Republic (unpublished data), and on the literature resources (Wolfová et al., 2006; Vasiľ, 2009). For the actual production system one alternative was modelled. The main production parameters of the breed were optimised on the level defined by the breed standard (ASPB, 2014) in this alternative. Base input data of the breed under the actual level and after the optimization are shown in Table 1. The bio-economic model of the program package ECOWEIGHT 6.0.4, Program EWDC version 3.0.4 for cattle (Wolf et al., 2013) was used for all calculations.

Table 1. Basic parameters of the breed under the actual level and after optimization

Parameter (unit)	Input parameters	
	Actual	Optimal
Milk yield (kg/cow and year)	4,473	5,200
Conception rate of cows (%)	88	92
Calving interval (days)	424	404
Daily gain of females in rearing (kg per day)	0.426	0.550
Basic price for milk (€ per kg)	0.310	
Price for nonstandard[1] milk (€ per kg)	0.247	
Revenues from sold breeding heifer (€ per head)	2,028	
Cost per cow (€/day) - fixed[2]	2.315	
Feed	2.51	2.71
veterinary[3]	0.23	0.21

Resource: own calculation; [1]Milk with somatic cells content over 400 thousand per mL; [2]Include costs labour, energy, reparations, insurance, fuel, overhead and depreciation of property; [3]Include costs for clinical mastitis incidence and other veterinary treatment

RESULTS AND DISCUSSION

Base economic parameters of dairy population of Slovak Pinzgau breed are shown in Table 2. When considering the actual conditions, it is operating with loss (-457 € per cow and per year) and with negative profitability ratio (-20%). Economic result calculated for local cattle population is comparable with literature (Komlósi et al., 2010; Hietala et al., 2014) where loss (ranged from -6 to -962 €) per dairy cow per year was published. Regarding the profit in dairy farms, the production level of cows can be defined as the most important factor (Taylor and Field, 1995). In Slovak conditions, it is under the breed standard defined by ASPB (2015), especially for milk yield (up to 5,500 kg of milk per lactation), conception rate of cows (90 to 95%) and average calving interval (up to 400 days). Optimising of the conception rate of heifers and cows by 4 and 6 percentage points (p.p.), respectively has a positive impact on the overall herd structure being in accordance with results published by Taylor and Field (1995). For example, overall proportion of cows at first lactation lowered by 5 p.p. and average lifetime increased from 3.7 to 4.5 years per one cow in this case. Number of calves available to be sold after weaning increased by 2 males and proportion of heifers needed for herd replacement reduced from 32 to only 24 per 100 cows. Surplus heifers (8 heifers per 100 cows) can be sold as breeding animals (in the case of an open herd turnover). In the closed herd turnover, these heifers can remain in the herd and be included in the breeding process. The first option was the case in this study as the stationary state of the herd structure was supposed.

In relation to the rearing of heifers, optimization of growth intensity (from 426 to 550 g per day; see Table 1) has a positive impact on the overall length of rearing period. Age at first calving deteriorated from 1,151 to

958 days and associated costs decreased by 12% to 1,469 € per reared heifer. Similar saving in costs (-15%) was found out for surplus heifers sold at constant live weight (450 kg).

Milk is well known as the main source of revenues in dairy cattle production systems. It ranged from 85% (found in this study for actual production system) to 96% (when surplus calves were sold at young age and no subsidies were included; Hietala et al., 2014). Therefore, the optimisation of milk traits (milk yield per lactation, somatic cell content and clinical mastitis incidence) were expected to be of the higher impact on the system profitability. Increase of the average milk yield by 16% (Table 1) and improvement of the udder health traits (to 2.32 for somatic cell score and clinical mastitis incidence to 0.12 cases per cow-year at risk) enhanced the revenues by 301 € per cow and per year whereas milk accounted for 87% of the total revenues (+3 p.p.). Simultaneously, the veterinary costs were deteriorated by 11% and feeding costs (needed for higher milk production) increased. Finally, increase in the total costs (+3%) were fully compensated by improvement in the total revenues (+34%).

Next to the production level and udder health traits, direct subsidies were also found out as an important parameter of the system profitability. The economic loss of 0.20 per each € of invested costs calculated for actual system would increase nearly two-times (0.34 € per each € of invested costs) when direct subsidies are not considered. For other dairy cattle populations (Komlósi et al., 2010; Hietala et al., 2014) even with the considerably high milk yield of cow (over 8,300 kg per 305 d milking period) the profitability of system strongly depended on agricultural subsidies was indicated. Moreover, low production prices together with the high production costs have resulted in the ineffective utilisation of costs (Hietala et al., 2014). Positive profitability (ranged from 0.4 and 11%) was found out in these studies only when subsidies were taken into account. Proportion of subsidies on the total income for Hungarian and Finnish cattle (ranged from 10 to 21%) was comparable to the Slovak Pinzgau farms (17%, Table 2). In Slovak conditions, a part of direct subsidies was related to milk yield (0.01 € per kg). Therefore, value of payments a slightly increased (by 23 € per cow and per year; Table 2) when optimal value of milk yield was modelled. Similarly, in Finland conditions, considering direct payments per fattened animals (412 € per animal) next to the milk yield (0.1 € per kg) was also taken into account. When comprehensive modelling of the optimal production parameters (given in Table 1) of Slovak Pinzgau breed was provided, positive economic result (21 € per cow and per year) for the production system was found out. In this case, higher level of revenues and effective utilisation of costs was reached in the production system.

To reach the economic sustainability of the breed from practical point of view, the farmer activity should be aimed especially to the enhanced herd management. Activities intended to reproduction parameters of cows (fertile oestrus detection, early pregnancy diagnose) next to the optimal body-condition score over the cows life are recommended. Intensive selection of heifers reared for replacement is expected as the secondary effect of the increased calf production. Higher growth intensity of calves can be reached by effective utilisation of pasture resources and grazing management. Moreover, keeping the appropriate hygienic conditions on farm and early therapy are recommended (Vasiľ, 2009) as an important factor for prevalence and elimination of udder disease in dairy farms.

Table 2. Base economic parameters of dairy population of Slovak Pinzgau breed

Economic variable (€ per cow and year)	Input parameters		Difference
	Actual	Optimal	
Total revenues	1 519.20	2 037.60	+34%
Revenues from milk (€ per cow and year)	1 295.10	1 596.00	+23%
Direct subsidies[1]	313.40	336.10	+7%
Total cost	2 289.70	2 352.20	+3%
Profit or loss	-457.10	21.50	+105%
Profitability[2] (%)	-20.00	0.90	+105%

[1]Sum of direct subsidies paid for milk production, livestock unit, performance testing of cattle and animal genetic resource (MA SR, 2014); value is averaged over the last three years period. Not include subsidies per agricultural land, less favoured areas and other indirect payments; [2]Expressed as proportion of profit (with accounting of direct subsidies) on the total costs

CONCLUSION

Dairy production system of Slovak Pinzgau breed, under current production and economic conditions, is operating with loss and with negative profitability ratio. To reach the sustainability of the local breed its production parameters should be improved. Milk yield, conception rate of females and growth intensity of heifers on the breed standard level is recommended at first. Profitability of dairy production system also depends on subsidies. These should be provided to compensate the real biological limitations of the breed. From practical point of view enhanced herd management should be applied by farmers to reach economic sustainability of the breed.

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QUANTITATIVE ASPECTS OF COAT COLOR IN OLD KLADRUBER BLACK HORSES

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Original scientific paper

SUMMARY

The aim of this study was to evaluate potential factors influencing coat color intensity in black variety of Old Kladruber horse breed and to estimate heritability for this trait. A total number of 145 individuals (105 females, 40 males), aged from 1 to 24 years were included in the analysis. The measurement of coat color was performed by Minolta Spectrophotometer 2500d using CIE (L*a*b*) color system. Measurements were taken at 4 body parts (neck, shoulder, belly and back) on each horse. The GLM procedure (SAS/STAT® software) was used to examine the influence of effects of age, line, sex, body part and housing on recorded coat color characteristics. All the effects except line were found statistically significant ($p<0.001$) related to all three parameters. The estimated heritabilities using REMLF90 (Misztal, 2002) ranged between 0.14–0.37 by the parameter and the body part.

Key-words: Old Kladruber horse, black coat color, variability, heritability, Minolta Spectrophotometer

INTRODUCTION

Old Kladruber horse (OKH) is the only autochtone warmblood breed of Czech origin created on a basis of the old Spanish and Italian blood. This breed has been breeding on Czech territory continuously more than 400 years the General Studbook of the breed has been held since 1757. Nowadays, OKH is included in gene resources of the Czech Republic and the Studbook has been fully closed since 2002. The breed is kept in two coat colors – grey and black one. The breeder's aim is to keep the OKH in carriage (galakarosier) type usable for ceremony and representative purposes, driving event, dressage, baroque and leisure riding. The OKH population consists of 46 stallions and 501 mares, 23 of which are black stallions and 261 black mares. The black variety consists of 5 lines.

The horse coat color is generally taken as a qualitative trait with mendelian inheritance. There are about 10 known genes responsible for coat color in horses (Thiruvenkadan et al., 2008; Rieder, 2009; Adrian, 2013), three basic colors (chestnut, bay, black) are determined by loci AGOUTI (gene ASIP) and EXTENSION (gene MC1R) (Marklund et al., 1996; Rieder et al., 2001). Black coat color is determined by recessive homozygote genotype of AGOUTI locus and at least one dominant allele on EXTENSION locus (***aa E-***) (Sponenberg, 1996).

There are remarkable differences within color phenotypes which are not possible to explain by Mendelian inheritance, for example the differences in level of greying (Curik et al., 2013), the shades of chestnuts or bays (Toth et al., 2006). There are two different types of black coat color – non-fading (jet or raven black) is charcoal black with metallic or blueish shine and fading – black with color without shine, fading to reddish-brown tinge especially when exposed to the sunshine in summer months (Sponenberg, 1996). The genetic determination of these two types of black color is not known. Black OKH population exhibits both color types mentioned, but undesirable „fading black" in high proportion.

The aims of this paper were to get quantitative characteristics of black horse color, to determine factors influencing variability of black color regarding possible effects of line, sex, age, body part, stay on pasture and to estimate heritability for black coat color.

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MATERIAL AND METHODS

There were 145 OKH blacks (105 mares and 40 stallions) at the age of 1 – 24 years included into this study. The statistical evaluation was carried out for 5 groups of age (1-2 years, 3-4 years, 5-10 years, 11-15 years, 16 and more years). The black color parameters were measured using Minolta Spectrophotmeter 2500d. The parameters measured under the system CIE (Commision Internationale de l'Eclairage) consist of: L* - lightness (0=Black, 100=white), a* - redness (+60=red, -60=green), b* - yellowness (+60=yellow, -60=blue). The parameter L* is the most important in case of black color, substantial is also the parameter of axis red-green (a*) detecting possible fading to reddish-brown tinge of hairs. Measurement of parameters mentioned was carried out on every animal on four body parts – neck (place with no mane covering), shoulder, belly and back (thigh). The every value of parameter measured is an average of three consecutive measuring on the same spot. The analysis of effects influencing the intensity of black color was performed by SAS/STAT® software - general linear model (GLM), using the least square method (LSM).

$$\mathbf{Y_{ijklm} = \mu + AGE_i + LINE_j + SEX_k + STABLE_l + PART_m + e_{ijklm}}$$

$\mathbf{Y_{ijklm}}$ – parameter value (L*, a *, b*), $\boldsymbol{\mu}$ – overal mean, $\mathbf{AGE_i}$ – fixed effect of the age group (i = 1, 2, 3, …5), $\mathbf{LINE_j}$ – fixed effect of the line (j = 1– Favory - Generalissimus, 2 - Sacramoso, 3 – Siglavi Pakra, 4 - Solo, 5 - Romke), $\mathbf{SEX_k}$ – fixed effect of the sex (k = 1 - male, 2 - female), $\mathbf{STABLE_l}$ – fixed effect of the housing (l = 1 – stable, 2 – outside), $\mathbf{PART_m}$ – fixed effect of the body part (m = 1 - neck, 2 - shoulder, 3 - belly, 4 - back), $\mathbf{e_{ijklm}}$ – residual error

The variance components and coefficients of heritability were estimated by REML method using the programme REMFL90 (Misztal, 2002). The same fixed effects (except the line) as in GLM analysis were included, the effect of an individual horse and the one of a residual error were considered as random effects. The dependent variables are parameters of black color measured on defined body parts and average value of all four parts on every horse. The pedigree of every horse under the study consists of five ancestor generation.

RESULTS AND DISCUSSION

The observed L*, a* and b* values ranged between 16.24–31.18, 0.72–7.87 and -0.25–13.68, with mean values (± standard deviation) 21.92 ± 2.33, 3.29 ± 1.20 and 3.70 ± 2.01 respectively. All effects except line were found out statistically significant ($p<0.001$) related to all three parameters. The differences between stallions and mares or horses kept in stable vs. horses kept on pasture („tabun") are presented in Table 1. Even though breeding mares are usually kept separately from stallions, there were a number of young horses of both sexes kept under the same conditions (permanent stay on pasture), thus we evaluated sex and stable as the separate effects.

Table 1. Effects of the gender and housing system on L*, a*, b* values

	L*		a*		b*	
	MEAN	SD	MEAN	SD	MEAN	SD
Male	20.48	0.16	2.64	0.09	2.61	0.15
Female	21.06	0.12	3.43	0.07	3.84	0.11
Stable	20.06	0.16	2.81	0.09	2.75	0.15
Outside	21.49	0.13	3.26	0.07	3.71	0.12

The mares showed significantly higher values of all parameters studied, since they are of lighter color with higher fading to reddish-brown tinge in hairs compared to stallions. Also, it was confirmed that black horses on pasture, permanently in summer, show lighter color with reddish-brown tinge. These conclusions are in an agreement with common knowledge on changes of black color influenced by sunlight. Our results are corresponding with conclusions of Stachurska et al. (2004) studying differences in „blue duns" diluted color derived from black.

The differences in parameters under the study in connection with the age are presented in Figure 1. The influence of age on parameter L* was not proved ($p = 0.22$). The values of L* showed substantial differences depending on the age in papers dealing with grey horses (Majzlik et al. 2010, Curik et al., 2013, Hofmanová et al., 2015). The color changes with the age are not remarkable in non-greying horses, but the difference in color especially between foals and mature horses were published (Sponenberg, 1996). Parameters a* and b* showed similar changes. The youngest foals showed the highest values, decreasing with the age to minimum at the age of 11–15 years. Higher values were noticed again in the oldest category may be due to small number of old horses. Higher values of parameters a* and b* (more „red" and „yellow" color) in foals comparing adult horses were presented by Stachurska et al. (2004) in blue duns.

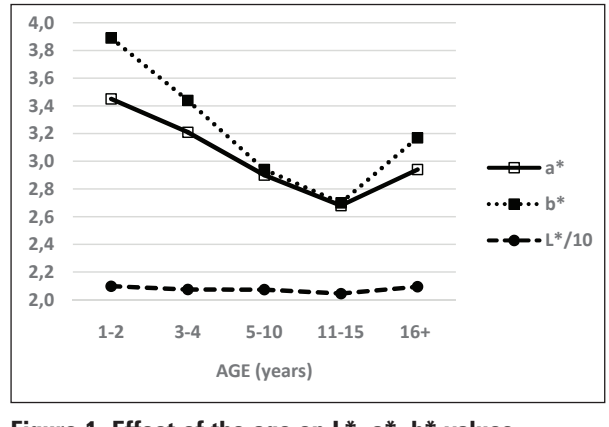

Figure 1. Effect of the age on L*, a*, b* values

The differences between body parts measured on horse reached statistical significance for all parameters under the study. The values measured on the neck and back were lower compared to values measured on the shoulder and the belly (Figure 2). This result is in agreement with practical knowledge – reddish fading in blacks is expressed primarily on belly region. The question is why on this body part, because the back is much more exposed to the sun's rays (there was no equipment covering measured place on the back parts of horses used). Despite the assumption of breeders observation that horses from Romke line (line founder stallion Romke of Friesian breed) are showed darker color, this study did not prove the line influence in any parameter (p=0.06, 0.71 and 0.63 for L*, a*, b* respectively). The explanation is, perhaps, in rotational mating planes in lines used with the result, that every line has some genetic contribution of other black lines.

Figure 2. Effect of the body part on L*, a*, b* values

Estimates of heritability for total color (average value of data measured on all four parts for every horse) and color of four body parts under the study are presented in Table 2. Standard errors of heritability estimate from REMLF90 were not available. We expect them to be high due to the low number of horses. Since the population of Old Kladruber blacks in quite limited, repeated measurements could be an option to assure larger number of observations. The lowest value for total color showed parameter L* (h^2=0.11), whereas the highest value was estimated for parameter a* (h^2=0.18). Heritabilities of body parts measured ranged between 0.14–0.32. The lowest heritabilities showed all parameters of neck color whereas the highest were found out for the back. The parameters a* and b* reached higher values compared to L* which could show the possible multifactorial inheritance of reddish tinge in hairs. Toth et al. (2006) published comparable heritabilities for L*, within-color class heritabilities for parameters a* and b* being negligible in this study. The parameter L* is an important criterion of greying in grey horses, as its value is corresponding to total melanin content in hairs (Toth et al., 2006). Heritability for greying in OKH greys were estimated as h^2=0.52 (Majzlik et al., 2010, Hofmanová et al., 2015) and comparable values for Lipizzaners were estimated by Curik et al. (2013).

Table 2. Genetic and residual variances and heritability estimates

Parameter	L*			a*			b*		
Body part	**V(G)**	**V(R)**	**h^2**	**V(G)**	**V(R)**	**h^2**	**V(G)**	**V(R)**	**h^2**
Neck	0.44	2.59	**0.14**	0.08	0.44	**0.16**	0.20	0.79	**0.20**
Shoulder	0.62	2.68	**0.19**	0.31	0.72	**0.30**	0.88	2.43	0.27
Belly	0.49	2.78	**0.15**	0.22	0.97	**0.18**	0.73	2.84	**0.20**
Back	0.58	1.44	**0.29**	0.23	0.48	**0.32**	0.45	0.78	**0.37**
Average	0.18	1.51	**0.11**	0.11	0.52	**0.18**	0.22	1.34	**0.14**

The differences in genetic determination of fading and non-fading black color are not known yet. The different phenotype is perhaps under the influence of modifying genes having only a minor effect, which could be cumulative. The black color could be theoretically influenced by genotype on EXTENSION locus. We can assume that the most blacks in Kladruby population are homozygous dominant (***EE***) at this locus, but several cases of chestnut foals born in this population proves presence of heterozygous genotype (***Ee***). There is a possibility that presence of recessive allele ***e***, known as a „red factor", could influence the occurrence of fading to reddish-brown tinge in hairs. Rieder et al. (2001) states the influence of EXTENSION locus in bays – bays with lighter shade in hairs were heterozygous (***Ee***), whereas dark bays were homozygous (***EE***).

The presence of polygenic component within black coat color could be solved by genomics in the future. However, the precondition of successful analyses like this is to have available unbiased information on horse color phenotype. This paper and previous papers (Toth et al., 2006; Majzlik et al., 2010; Curik et al., 2013, Hofmanová et al., 2015) dealing with horse color showed that the Minolta Spectrophotometer could be an appropriate equipment for such studies.

CONCLUSION

This paper is the first study dealing with objective evaluation of coat color in black Old Kladruber horse. There are several similar studies in greys or other coat colors in other breeds unlike in blacks with not known such study. The results of this paper confirmed some generally known facts on black color, especially bleaching of hairs by sunlight during the stay of horses on pasture in summer. The influence of the age was proven particularly only – for parameters a* and b*. The results of this study did not show any differences in color between lines. Heritabilities estimated were in range 0.14-0.37 depending on parameter and body part measured.

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THE ACCEPTANCE OF HEALTH RELATED INNOVATIONS IN TRADITIONAL MEAT PRODUCTS BY CROATIAN CONSUMERS

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Preliminary communication

SUMMARY

The aim of this study was to examine Croatian consumers' acceptance of health related innovations in traditional meat products. A face-to-face survey was conducted with a sample of 151 visitors of a specialized fair of traditional food products in Zagreb in 2013. The respondents were asked to indicate their attitudes on a five-point scale, where 1 meant rejection or no impact and 5 meant full acceptance or high impact. Results (mean±SD) showed the highest level of acceptance for innovations related to better control of smoking conditions (3.3± 1.14) and reduction of salt content (3.1±1.15), followed by reduced fat content (3.0±1.14) and controlled fermentation (2.9±1.12). The perceived negative impact of innovations on traditional character of meat products was highest for fat (3.4±0.99) and salt (3.4±1.03) reduction and lowest for controlled fermentation (3.2±1.04) and smoking conditions (3.2±1.05). With regards to respondents' socio-demographic features a nonparametric test statistic (Mann-Whitney U) revealed a higher acceptance of fat reduction and higher willingness to increase a consumption of healthier traditional meat products among females, while age, education level and income had no influence on the investigated parameters. In addition, some health related innovations; e.g. fat reduction and controlled fermentation were generally less acceptable among respondents with a high consumption frequency of traditional meat products. The results of this preliminary study indicated controlled smoking conditions as the best accepted health related innovation by Croatian consumers with the least negative impact on perceived traditional character of product. In general terms, women were more likely to accept some of the investigated innovations and consequently to increase their consumption of innovate products. However, the most regular consumers of traditional meat products were less open towards innovations which may pose a challenge to further improvements in this traditional food sector.

Key-words: traditional meat products, innovations, health, consumers, acceptance

INTRODUCTION

Despite the growing globalisation of today's food markets and the abundance of uniform and cheap industrial food items the traditional food products remain an important part of human culture, identity and heritage, with generally positive perception by consumers due to the characteristics linked to regional identity and sensory quality (Guerrero et al., 2009; Vanhonacker et al., 2013). As a result of this positive public image a demand for traditional foods is increasing in many western countries (Almli et al., 2011). Furthermore, the production of traditional food products, especially those of animal origin, is often closely related to a less intensive traditional production systems which usually rely on local natural and human resources and, as such, play an important role in the maintenance of environment and socio-economic development of rural areas which would, otherwise, be depopulated. In this broader context, both food manufacturers and public authorities are increasingly interested in traditional foods sector, giving it good prospects for future growth. However, the production of traditional foods, which is generally carried out by small and medium low-tech artisanal enterprises, still largely relies on traditional manufacturing practices, often with low competitiveness and poor efficiency (Fito

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and Toldra, 2006). In order to cope better with arising market opportunities the traditional food sector also faces the need to continuously innovate and develop its produce (Kühne et al., 2010; Vanhonacker et al., 2013). In this regard, traditional food producers have been recommended to extend their skills in modern production techniques, management and marketing, as well as in promoting the aspects of their products related to nutritional and health issues (European Communities, 2007). Nevertheless, the success of improvements in traditional food sector is riskier than in other sectors of food industry, because consumers tend to reject innovations affecting the traditional character of the product (Almli et al., 2011), and therefore a good understanding of consumers perceptions, expectations and attitudes towards any innovation is required prior to its implementation (Kühne et al., 2010). Meat products, in general, have been often criticized for being too high in fat (Higgs, 2002) and salt (Žlender, 2009), and thus potentially unhealthy for consumers. Traditional meat products in particular may additionally be associated with lower hygienic and microbial standards (Skandamis and Nychas, 2007) and greater exposure to potentially unhealthy substances from the smoke (Andrés et al., 2007). As consumers today increasingly demand, not only safe and tasteful traditional food products, but also more convenient and more nutritive and healthier types of products, this study aimed to investigate the acceptance of health related innovations in traditional meat products by Croatian consumers.

MATERIAL AND METHODS

In order to collect data a face-to-face survey was conducted in April 2013 at a specialised fair for traditional food products in Zagreb, Croatia. The exhibits in the fair presented various traditional products and customs of Croatian villages, including meat products. Numerous visitors had the opportunity to become familiar with and to buy traditional products during three days of the fair. The fair visitors were selected as a sample group because it could be assumed that these fair visitors are interested in traditional, small farm products and therefore they represent an interesting respondents group for this research. The survey was performed with a randomly selected 151 fair visitors of different sociodemographic characteristics (Table 1).

Table 1. Sociodemographic characteristics of the sample

		% of the respondents
Gender	Male	51.3
	Female	48.7
Age	Less than 25 years	14.6
	25 - 35 years	27.8
	36 – 45 years	20.8
	46 – 55 years	18.8
	56 or more years	18.1
Education	Primary school	6.6
	Secondary education	51.0
	University education	42.4
Perceived family income	Very low	2.0
	Low	12.6
	Average	67.5
	High	15.9
	Very high	0.7

The acceptance of four innovations in traditional meat products (better control of smoking conditions, reduction of salt, reduced fat content and controlled fermentation) that would lead to production of healthier products were measured on a five-point scale, usually used in Croatia, where 1 meant rejection of the innovation and 5 full acceptance of the innovation. Further, respondents were asked to express their attitude on a negative impact of the related innovations on traditional character of the meat products. The attitudes were also measured on a five-point scale with 1 meaning no negative impact and 5 meaning high negative impact. Data were analysed with descriptive statistics (mean values and standard deviations) in order to discuss consumers' acceptance of selected innovations as well as their attitude on a negative impact of different innovations on traditional character of meat products. Mann-Whitney U test was used to test the differences between sociodemographic and consumption frequency groups regarding their willingness to accept innovations as well as their perceived negative impacts of innovations on traditional character of meat products. All analyses were made in SPSS statistical package version 17 (SPSS, 2008).

RESULTS AND DISCUSSION

Buying and consuming behaviour regarding traditional meat products

Two third of respondents of all sociodemographic characteristics make a distinction between traditional and industrial meat products, out of which a great majority (97.1%) prefer traditional products. Most of the respondents (41.1%) buy traditional meat products occasionally and further 29.8% claimed to buy them often or very often. Only a few respondents (2.6%) do

never buy traditional meat products, while 26.5% buy such products rarely or very rarely. Traditional meat products are usually purchased at city markets or at food fairs (about half of the respondents) and less often directly from producers or in the specialised shops (about one fifth of the respondents). Some respondents produce meat products by themselves or they receive them from relatives or friends. Most of the respondents consume traditional meat products few times a week (38.3%) or even a few times a month (36.3%); 19.5% of them eat these products less often, while 6% of the respondents claimed to eat traditional meat products every day.

Acceptance of health related innovations

Nearly half (48%) of the respondents believe that there are no negative health consequences of traditional meat products consumption. The most often mentioned negative impact of traditional meat products consumption on health such as increased cholesterol (21%), followed by food infections (7.3%) and obesity (4.6%). Some respondents also mentioned diabetes and allergies. About 12% of the respondents believed that consumption of traditional meat products can have negative impact on health but they did not mention what kind of impact.

The research results in Table 2 showed above-average level of acceptance of health related innovations in production of traditional meat products, which is in accordance with generally positive acceptance scores for quality innovations in traditional foods reported by Guerrero et al. (2009) and Kühne et al., (2010). The highest willingness to accept innovations was related to better control of smoking conditions (3.3±1.14) and reduction of salt content (3.1±1.15) followed by reduced fat content (3.0±1.14) and controlled fermentation (2.9±1.12). In some earlier consumer studies, e.g. Bruhn et al. (1992) and Kühne et al., (2010), the highest acceptance rates for health innovations in traditional food products were found for fat replace (e.g. in traditional dairy products) or fat reduction, as well as for salt reduction. In the present study, however, the most highly accepted innovation in traditional meat products was more strict control of smoking conditions. In traditional manufacturing in Croatia meat products are typically smoked in the same chamber where the smoke is generated which can lead to greater deposition of potentially unhealthy substances from the smoke, like polycyclic aromatic hydrocarbons (PAH) on the surface of the products (Andrés et al., 2007). In this sense, the results of the present study may indicate that the Croatian consumers, although it is not explicitly stated, are quite aware of the potential health risks associated with consumption of heavily smoked meats.

Table 2. Acceptance of health related innovations in traditional meat products

	Willingness to accept the innovation*	Attitude on a negative impact of the innovation**
Better smoking conditions control	3.3 ± 1.14	3.2 ± 1.05
Reduction of salt	3.1 ± 1.15	3.4 ± 1.03
Reduced fat content	3.0 ± 1.14	3.4 ± 0.99
Controlled fermentation	2.9 ± 1.12	3.2 ± 1.04

*1 – rejection ... 5 – full acceptance; ** 1 - no negative impact … 5 - high negative impact

The perceived negative impact of innovations on traditional character of meat products was the highest for reduction of fat (3.4±0.99) and salt (3.4±1.03) and the lowest for controlled fermentation (3.2±1.04) and smoking conditions (3.2±1.05). This corroborates previous findings of Kühne et al. (2010) that sensory properties of the traditional food products should not be compromised by innovations. More than one third of the respondents (36.7%) claimed that they would increase consumption of traditional meat products if produced with the mentioned innovations that would result in potentially decreased negative influence of traditional meat products on their health. Furthermore, 22.7% of the respondents declared that such innovations would not change their consumption behaviour while a majority of the respondents (40.6%) are not sure about their reaction on such innovations.

Influence of sociodemographic and consumption frequency on acceptance of health related innovations

With regards to respondents' socio-demographic features earlier studies have already shown that females and urban consumers are generally more prone to accept innovations in the traditional foods (e.g. Guerrero et al., 2009; Kühne et al., 2010). The present results (Mann-Whitney U test, not shown) also revealed a higher acceptance of fat reduction and higher willingness to increase a consumption of healthier traditional meat products among females ($p=0.032$), while age, education level and income had no influence on the investigated parameters ($p>0.05$). In addition, some health related innovations; e.g. fat reduction ($p=0.003$) and the use of starter cultures ($p=0.05$) were generally less acceptable among respondents with a high consumption frequency of traditional meat products.

CONCLUSION

The results of this preliminary study indicated controlled smoking conditions as the best accepted health related innovation of traditional meat products by Croatian consumers with the least negative impact

on perceived traditional character of product. In general terms, women were more likely to accept some of the investigated innovations and consequently to increase their consumption of innovate products. However, the most regular consumers of the traditional meat products were less open towards innovations which may pose a challenge to further improvements in this traditional food sector.

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USE OF NEAR INFRARED TECHNOLOGY TO PREDICT FATTY ACID GROUPS IN COMMERCIAL GROUND MEAT PRODUCTS

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Original scientific paper

SUMMARY

Near infrared transmittance (NIT, 850 to 1048 nm) spectroscopy was used to predict groups of fatty acids (FA), namely saturated FA (SFA), monounsaturated FA (MUFA) and polyunsaturated FA (PUFA), in commercial ground meat samples aiming to develope a fast and reliable method for their determination in support of label declaration by the new EC Regulation 1169/2011. Dataset was built using 81 samples of commercial ground meat from different species: beef, pork, chicken and turkey. In some samples, meat was mixtured with different ingredients such as bread, cheese, spices and additives. Samples were first analysed by NIT instrument for spectral information and reference FA values were obtained by gas chromatographic analysis. Prediction models for SFA, MUFA and PUFA expressed on total FA exhibited coefficients of determination of calibration of 0.822, 0.367 and 0.780 on intact samples, and 0.879, 0.726 and 0.908 on minced samples, respectively. Good results were also obtained when FA groups were expressed as g/100g of fresh meat: the coefficient of determination of calibration increased to values larger than 0.915. Moreover, comparing the slightly lower coefficient of determination in cross-validation of intact compared with minced meat suggested that equations developed for minced samples were more accurate than those built for intact products. Results highlighted the effectiveness of NIT spectroscopy to predict the major FA groups in commercial meat products.

Key-words: fatty acid, ground meat, infrared spectroscopy

INTRODUCTION

The nutritional quality of meat is one of the most important aspects for global meat industry and depends on the type of meat, the cut, additives and recipe (Weeranantanaphan et al., 2011; Wyness et al., 2011). Meat and meat products are important sources of a wide range of nutrients such as proteins, fat, vitamins and minerals but their composition varies widely according to the category (Cosgrove et al., 2005; Prynne et al., 2009). Fatty acids (FA) have an important role in meat quality profile thanks to their nutritional value and sensory attributes. Moreover, in the last years many studies have investigated the relationship of red meat consumption with the extension of common cardiovascular diseases, colon cancer, type 2 diabetes and stroke. The main responsible for these diseases seems to be meat FA composition, the high salt content of some preparations and possible carcinogenic compounds formation during cooking (Bingham et al., 2002; Feskens et al., 2013; Kantogianni et al., 2008; McAfee et al., 2010).

In response to human nutritionists and dieticians the European Union has reinforced the attention to labelling law in order to achieve a high level of health protection for consumers. The EC Regulation 1169/2011 has introduced some new mandatory information for labelling such as the specification of main FA groups: saturated FA (SFA), monounsaturated FA (MUFA) and polyunsaturated FA (PUFA). Chemical determination of FA is time consuming, expensive, and it requires long sample preparation. Therefore, the use of fast and reliable tools such as near infrared technology could be

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useful to face these limitations. This paper aimed to investigate the feasibility of using near infrared transmittance (NIT) spectroscopy to predict SFA, MUFA and PUFA in commercial ground meat samples.

MATERIAL AND METHODS

Sample collection

Samples (n=81) were collected randomly in supermarkets and butchers located in Veneto and Trentino-Alto Adige regions (northeast Italy) from October 2014 to February 2015. Products acquired were ground meat, hamburger, meatballs, sausages, and other commercial products with ground meat. Forty-one samples were composed of beef meat as main ingredient, 24 of pork meat, 8 of a mix of beef and pork meat and the remaining 9 samples were made with chicken and turkey. In addition to meat, 46 samples contained spices, bread, cheese, flavourings, preservatives, acidity regulators and other additives based on the formulation of the company recipe.

Near infrared analysis

Meat samples were first analysed without treatments; intact samples were placed into circular glass cup (diameter 140 mm, depth 17.5 mm) at room temperature and NIT spectroscopy was carried out using FoodScan (FOSS, Electric A/S, Hillerød, Denmark) from 850 to 1048 nm (2 nm interval). Then, each sample was cut off, mixed mechanically with the knife mill Retsch Grindomix GM200 (Retsch GmbH & Co, Haan, Germany) and analysed again by FoodScan. Each spectrum was obtained by 24 scans performed at one time for each sample. After spectra collection an amount of minced samples was used for reference analysis.

Chemical analysis

Total lipids were extracted from 4 g of sample by accelerated solvent extraction using a Dionex ASE 350 system (Thermo Scietific, Dreieich, Germany), with petroleum ether extraction solvent. Preparation of ester derivatives of FA for chromatographically analysis were performed with a method adapted from Christie (1993). On 40 mg of the extracted fat 1 mL of sulphuric acid (H_2SO_4) in methanol was added and samples were then placed in an oven at 65°C overnight. At the end of methylation 2 mL of n-heptano and 1 mL of potassium carbonate were added. After centrifugation for 10 minutes at 4000g the supernatant was collected. Separation and quantification of the FA methyl esters were carried out using a gas chromatography Agilent 7820A GC System (Agilent Technologies, Santa Clara, CA) with hydrogen as a carrier gas. Gas chromatograph was equipped with Flame Ionization Detector (FID) and Supelco Omegawax capillary column (30 m×0.25 mm id, 0.25 μm film thickness). The output was quantified using GC ChemStation (Agilent Technologies). Fatty acids were identified upon comparison with the known FA standards (Supelco FAME mixC4–C24 #18919-1AMP; Sigma-Aldrich, Castle Hill, Australia) and expressed as percentage of FA group on the total FA identified. Moreover, FA groups were expressed on the total fresh meat. Fat content, protein content, collagen content and moisture were determined using the FoodScan (FOSS Electric A/S, Hillerød, Denmark).

Statistical analysis

Spectral data were analyzed using WinISI software (Infrasoft International, Port Matilda, PA, USA) and modified partial least squares (MPLS) regressions. Cross-validation was performed splitting the calibration dataset in 5 groups, using one of them to check the results (prediction) and the remaining four to construct the calibration model. Data were treated with different combinations of scattering corrections and different mathematical pre-treatments. The critical "T" outlier value was set at 2.5. The best equation for each FA group was selected on the basis of the highest coefficient of determination in cross-validation (R^2cv). Other fitting statistics used to evaluate the prediction models were the standard error of calibration (SEC), the coefficient of determination of calibration (R^2), the standard error of cross-validation (SEcv), and the residual predictive deviation (RPD) calculated as the ratio of SD of reference data to the SEcv (Sinnaeve et al., 1994)

RESULTS AND DISCUSSION

The fat percentage of intact and mixed commercial ground meat samples obtained by FoodScan is presented in Table 1. Means of SFA, MUFA and PUFA were 43%, 47% and 10% of total FA and 6%, 7% and 1% of fresh meat determined by gold method (Table 2). The variability of each FA group determined on intact samples was similar to the variation of the same FA on minced samples (Table 2).

Comparison of mean FA groups with other studies is difficult because of the variability of the meat type and their recipes. However means of SFA and PUFA were higher and of MUFA lower than Fernández-Cabanás et al. (2011) who obtained values of 40.32% for SFA, 51.96% for MUFA and 7.72% for PUFA in the analysis of 86 sausages. PUFA Mean of turkey hamburgers and meatballs obtained by Ferreira et al. (2000) higher than mean value of PUFA from the present work. However, it is worth noting that in our study chicken and turkey meat samples accounted for only 10% of the data.

Considering FA groups measured on total FA, the R^2 was lower in intact (0.822, 0.367 and 0.780 for SFA, MUFA and PUFA, respectively) than in minced samples (0.879, 0.726 and 0.908, respectively) (Figure 1a). The same pattern was found considering FA expressed on fresh meat, with R^2 values which were always greater than 0.900 for all the FA groups, both in intact and minced samples (Figure 1b). The R^2_{CV} of FA groups

expressed on total FA of intact meat were 0.794 for SFA, 0.315 for MUFA and 0.730 for PUFA, and in minced meat they were 0.863, 0.490 and 0.876, respectively (Table 2). Finally, the R^2_{CV} of FA groups expressed on fresh meat of intact samples were 0.979, 0.957 and 0.896 for SFA, MUFA and PUFA, respectively, and in minced samples they were 0.985, 0.983 and 0.958, respectively.

Table 1. Fat content (%) of intact and minced commercial ground samples obtained by NIT instrument (FoodScan)[a]

	Mean	SD	CV (%)	Min	Max
Intact meat	14.13	5.11	35.73	2.73	23.73
Minced meat	13.95	4.95	35.48	2.45	25.21

[a]Abbreviations: SD, standard deviation; CV, coefficient of variation; Min, minimum; Max, maximum

Table 2. Descriptive and prediction statistics for FA groups of intact and minced commercial ground meat samples [a]

Trait	Mean	SD	CV(%)	Math	T	SEC	R^2	SEcv	R^2cv	RPD
Intact meat, % of total FA										
SFA	42.55	6.59	15.50	SNV 1,4,4,1	7	2.67	0.822	2.86	0.794	2.21
MUFA	46.92	4.51	9.61	MSC 2,10,10,1	1	2.45	0.367	2.59	0.315	1.19
PUFA	10.25	6.86	66.89	NONE 2,5,5,1	10	2.32	0.780	2.58	0.730	1.92
Intact meat, g/100 g of fresh meat										
SFA	5.89	2.4	40.78	SNV+D 1,4,4,1	9	0.26	0.987	0.33	0.979	6.87
MUFA	6.67	2.63	39.36	SNV+D 2,5,5,1	9	0.42	0.973	0.54	0.957	4.75
PUFA	1.46	1.18	80.92	SNV+D 2,5,5,1	10	0.26	0.915	0.28	0.896	3.1
Minced meat, % of total FA										
SFA	42.89	6.63	15.46	MSC1,8,8,1	8	2.20	0.879	2.36	0.863	2.68
MUFA	46.78	4.37	9.34	SNV+D 2,5,5,1	10	1.85	0.726	2.52	0.490	1.4
PUFA	10.04	6.9	68.66	Detrend 1,4,4,1	10	1.52	0.908	1.76	0.876	2.84
Minced meat, g/100 g of fresh meat										
SFA	5.9	2.29	38.86	SNV+D 2,5,5,1	9	0.23	0.990	0.28	0.985	7.99
MUFA	6.59	2.5	37.91	MSC 2,5,5,1	10	0.28	0.988	0.33	0.983	7.46
PUFA	1.42	1.17	82.37	MSC 2,5,5,1	10	0.17	0.964	0.18	0.958	4.88

SFA (saturated fatty acids): sum of $C_{4:0}$, $C_{6:0}$, $C_{7:0}$, $C_{8:0}$, $C_{9:0}$, $C_{10:0}$, $C_{11:0}$, $C_{12:0}$, $C_{13:0}$ (and iso and anteiso), $C_{14:0}$ (and iso and anteiso), $C_{15:0}$ (and iso and anteiso), $C_{16:0}$ (and iso and anteiso), $C_{17:0}$ (and iso and anteiso), $C_{18:0}$ (and iso and anteiso), $C_{19:0}$, $C_{20:0}$, $C_{21:0}$, $C_{22:0}$, $C_{23:0}$, $C_{24:0}$; MUFA (monounsaturated fatty acids): sum of $C_{10:1}$, $C_{12:1}$, $C_{14:1}$ (and isomer), $C_{15:1}$, $C_{16:1n9}$, $C_{16:1n7}$, $C_{16:1}$, $C_{17:1n7}$, $C_{18:1}$ (and isomers), $C_{19:1}$, $C_{22:1n9}$, $C_{24:1n9}$; PUFA (polyunsaturated fatty acids): sum of $C_{18:2n6}$, $C_{18:2}$ (and isomers), $C_{18:3n6}$, $C_{18:3n3}$, $C_{20:2n6}$, $C_{20:3n6}$, $C_{20:3n3}$, $C_{20:4n6}$, $C_{20:5n3}$, $C_{22:2n6}$, $C_{22:5n3}$, $C_{22:6n3}$.

[a]Abbreviations: SD, standard deviation; CV, coefficient of variation; Math, mathematical treatment; T, number of terms used to perform the calibration model; SEC, standard error of calibration; R^2, coefficient of determination of calibration; SE_{CV}, standard error of cross-validation; R^2cv, coefficient of determination of cross-validation; RPD, residual predictive deviation, calculated as ratio of SD of reference data to the SE_{CV}; SNV, standard normal variate; MSC, multiplicative scatter correction; NONE, without treatment of scatter correction; SNV+D: standard normal variate and detrending. The first digit of the mathematical treatment represents the number of the derivative, the second the gap over which the derivative is calculated, the third the number of data points in the first smoothing, and the fourth the number of data points in the second smoothing.

The results evidenced better fitting statistics for prediction models calculated for minced than intact samples; this was expected because sample preparation affects the reliability of near infrared prediction models (Prieto et al., 2009; Guy et al., 2011). The RPD value allows an impartial evaluation of the performance of calibrations between studies examining the same traits with different measurement units and different samples. De Marchi et al. (2012) analysed the FA profile of chicken breast expressed as % of total FA and reported similar RPD values for MUFA and PUFA as well as lower values for SFA compared with our study. Regarding the prediction of FA (expressed on fresh meat) in the ground bovine *Longissimus thoracis* muscle samples, Mourot et al. (2014) obtained slightly worse RPD values than those obtained in the present work. Mourot et al. (2015) investigated the FA profile in four beef cattle breeds (Angus, Blond d'Aquitaine, Charolais, Limousin) and three muscles, *Longissimus thoracis*, *Rectus abdominis* and *Semitendinosus*, and calculated RPD values for SFA, MUFA and PUFA of *Longissimus thoracis* being lower than our results. The same authors also reported that the inclusion of samples from several breeds allowed to capture more variability of FA content, thus leading to an increased accuracy of calibration models.

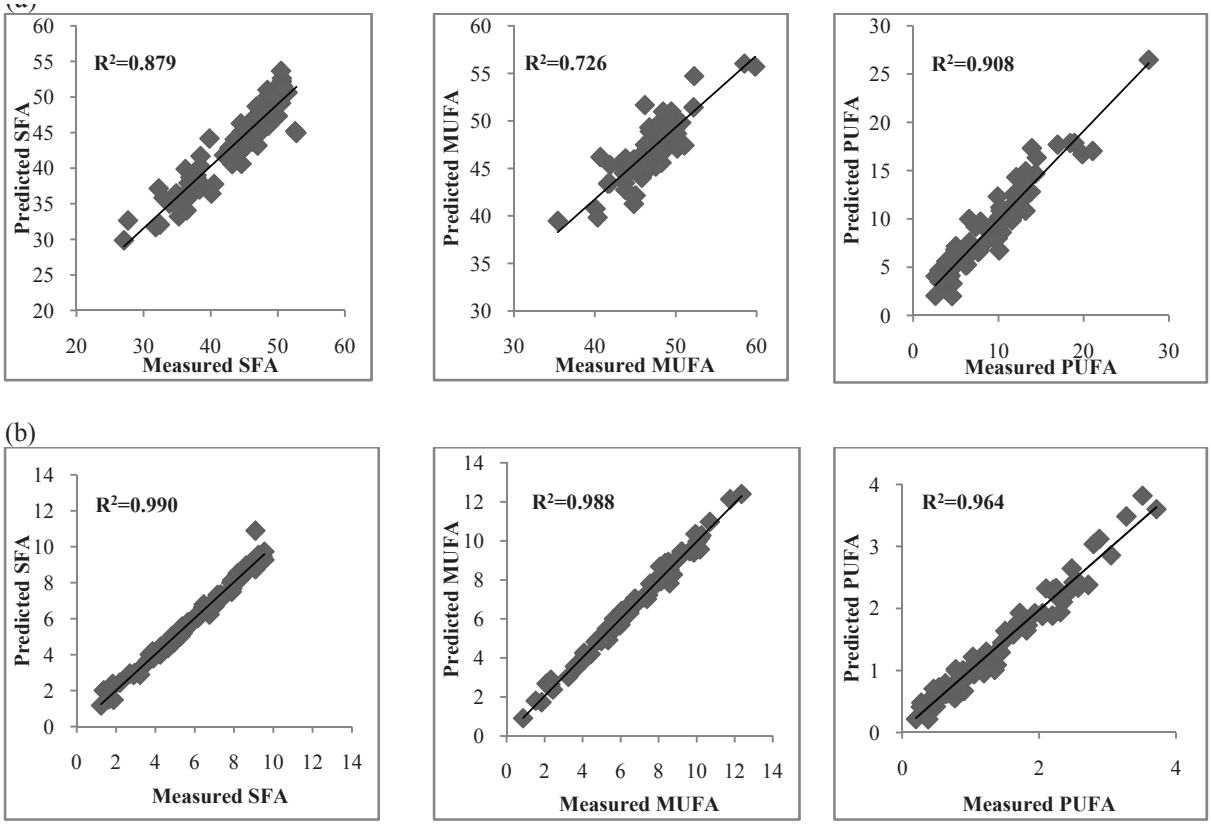

Figure 1. Relationship between reference and predicted SFA, MUFA and PUFA expressed on % of total FA (a) and g/100g of fresh meat (b) of minced meat. The coefficient of determination of calibration is included for each trait

CONCLUSION

The present study underlined the ability of NIT spectroscopy to predict FA groups in commercial ground meat products. Better predictions were obtained for SFA, MUFA and PUFA expressed on fresh meat than on the total FA. This study confirmed the positive effect of mincing the samples on the development of robust prediction models. The calibration models developed in this study can be used by meat industry to address the requirements of EC Regulation 1169/2011.

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MILK COAGULATION PROPERTIES OF CATTLE BREEDS REARED IN ALPINE AREA

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Original scientific paper

SUMMARY

The aim of the present study was to apply mid-infrared spectroscopy prediction models developed for milk coagulation properties (MCP) to a spectral dataset of 123,240 records collected over a 2-year period in the Alpine area, and to investigate sources of variation of the predicted MCP. Mixed linear models included fixed effects of breed, month and year of sampling, days in milk, parity, and the interactions between the main effects. Random effects were herd nested within breed, cow nested within breed, and the residual. All fixed effects were significant (P<0.01) in explaining the variation of MCP. In particular, milk clotting characteristics varied significantly among breeds, and local Alpine Grey breed exhibited the most favourable processing characteristics. Milk coagulation properties varied across lactation and were at their worst after the peak.

Key-words: mid-infrared spectroscopy, milk quality, local breeds

INTRODUCTION

European Union (EU) dairy products market is addressed in maximizing added value of milk, by processing it into different food categories. For instance, EU contributes to half of the total world cheese production. Therefore, an important challenge for the dairy industry is to segregate milk based on its processing quality. Milk coagulation properties (MCP) are indicators of milk processing characteristics and they include rennet coagulation time (RCT, min), curd-firming time (k_{20}, min), and curd firmness (a_{30}, mm). Large-scale monitoring of these traits is difficult due to expensive and time- consuming gold standard methods (e.g., Formagraph). Mid-infrared spectroscopy (MIRS) can overlap this issue and has been proposed as an effective tool to predict innovative milk quality traits at the population level, such as MCP (De Marchi et al., 2013, 2014). There is a paucity of studies that have investigated MCP variation among cattle breeds, including local populations. Therefore, aim of the present study was to investigate sources of variation of MIRS-predicted MCP in major cattle breeds in Bolzano Province, North-Eastern Italian Alps.

MATERIAL AND METHODS

Data collection

A total of 132,380 individual milk samples from 15,173 cows were collected between January 2012 and December 2013 in Bolzano Province, a mountainous area of the Italian Alps. Cow breeds included in the dataset were Holstein-Friesian (HF, 13%), Brown Swiss (BS, 36%), Simmental (SI, 30%), and Alpine Grey (AG, 21%). Cows were from 6 to 450 days-in-milk (DIM), and from 1st to 15th parity. Immediately after collection, samples were added with preservative (Bronysolv; ANA. LI.TIK Austria, Vienna, Austria) and processed in the laboratory of the South Tirol Dairy Association (Bolzano, Italy) according to recommendations by International Committee for Animal Recording. Each milk sample was analyzed using a MilkoScan FT6000 (Foss Electric A/S, Hillerød, Denmark) to determine protein (%), casein (%), fat (%), lactose (%), urea (mg/dL), and pH. Spectral information of milk quality traits, containing 1,060 transmittance data in the region between 900 and 5,000 cm^{-1}, were also retrieved from the South Tirol Dairy

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Association. Somatic cell count (SCC) was assessed by Fossmatic (Foss Electric A/S, Hillerød, Denmark) and transformed to somatic cell score (SCS) through the formula $SCS = [3 + \log_2(SCC/100{,}000)]$.

Laboratory analysis and MIRS prediction models

A calibration dataset of 923 milk samples was used to build MIRS prediction models for RCT, k_{20}, and a_{30}. Reference values of MCP were determined by Formagraph (Foss Electric A/S, Hillerød, Denmark), and MIRS spectra were stored by Milkoscan FT6000 (Foss Electric A/S, Hillerød, Denmark). Full details about the reference method are available in De Marchi et al. (2013). Prediction models were built through partial least squares regression after uninformative variables elimination as recently implemented by Gottardo et al. (2015). Coefficient of determination (root mean square error) in validation was 0.55 (2.86 min), 0.58 (1.00 min), and 0.56 (8.43 mm) for RCT, k_{20}, and a_{30}, respectively.

Phenotypic characterization

Statistical analysis was carried out using SAS software (ver. 9.3, SAS Institute Inc., Cary, NC, USA). Principal component analysis (PROC PRINCOMP) was performed on both the initial validation dataset and the calibration dataset, providing a new matrix of uncorrelated variables called principal components (PC) which explain, in a descending order, a portion of the total variance. This procedure aimed to select spectral observations in the validation dataset similar to those in the calibration dataset. This was achieved by selecting only spectral data in the validation dataset whose first and second PC (PC-1 and PC-2, respectively) were within the same range of PC-1 and PC-2 of spectral data in the calibration dataset. Therefore, 123,240 observations from 15,066 cows were retained for further statistical analysis, whereas the remaining 9,140 records were classified as outliers and discarded. Prediction models were subsequently applied to the edited validation dataset to predict RCT, k_{20}, and a_{30}. Sources of variation of MCP were investigated using PROC MIXED according to the following mixed linear model:

$$Y_{ijklmno} = \mu + B_i + M_j + Y_k + DIM_l + Parity_m + (B \times M)_{ij} + (B \times Y)_{ik} + (B \times DIM)_{il} + (B \times Parity)_{im} + (DIM \times Parity)_{lm} + H_n(B_i) + Cow_o(B_i) + e_{ijklmno},$$

where $Y_{ijklmno}$ is the dependant variable (MIRS-predicted RCT, k_{20}, or a_{30}), m is the overall intercept of the model, B_i is the fixed effect of the ith breed (i = HF, BS, SI, AG), M_j is the fixed effect of the jth month of sampling (j = 1 to 12), Y_k is the fixed effect of the kth year of sampling (k = 2012, 2013), DIM_l is the fixed effect of the lth class of DIM (l = 6 to 30, 31 to 60, 61 to 90, 91 to 120, 121 to 150, 151 to 180, 181 to 210, 211 to 240, 241 to 270, 271 to 300, 301 to 330, 331 to 360, 361 to 390, 391 to 450 days), $Parity_m$ is the fixed effect of the mth parity (m = 1 to 5, with class 5 including cows from parity 5 to 15), $(B \times M)_{ij}$ is the fixed interaction effect between breed and month of sampling, $(B \times Y)_{ik}$ is the fixed interaction effect between breed and year of sampling, $(B \times DIM)_{il}$ is the fixed interaction effect between breed and DIM, $(B \times Parity)_{im}$ is the fixed interaction effect between breed and parity, $(DIM \times Parity)_{lm}$ is the fixed interaction effect between DIM and parity, $H_n(B_i)$ is the random effect of the nth herd nested within the ith breed $\sim N(0,s^2_{H(B)})$, $Cow_o(B_i)$ is the random effect of the oth cow nested within the ith breed $\sim N(0,s^2_{cow(B)})$, and $e_{ijklmno}$ is the random residual $\sim N(0,\sigma^2_e)$. A multiple comparison of means was performed for breed effect using Bonferroni's test ($P<0.05$).

RESULTS AND DISCUSSION

Means and variation

Descriptive statistics of milk quality traits and MCP included in calibration dataset are reported in Table 1. Coefficient of variation of MCP ranged from 24.8% (RCT) to 44.1% (a_{30}). These results are comparable with those (20.0 and 38.7%, respectively) of another large-scale research considering MIRS-predicted MCP on a multi-breed dataset (Penasa et al., 2014). Large data variation is desired when information is used to develop MIRS prediction models that will be subsequently applied for phenotyping at the population level (Visentin et al., 2015).

Table 1. Descriptive statistics[1] of milk samples included in calibration dataset (n=923)

Trait[2]	Mean	SD	Minimum	Maximum	CV, %
Fat, %	4.09	0.72	1.71	9.19	17.6
Protein, %	3.61	0.45	2.34	5.50	12.6
Casein, %	2.82	0.36	1.71	4.38	12.9
SCS	2.80	1.86	-3.64	8.87	66.3
pH	6.65	0.07	5.87	6.92	1.1
RCT, min	18.57	4.61	4.30	29.00	24.8
k_{20}, min	5.20	1.63	2.00	13.15	31.3
a_{30}, mm	28.07	12.37	2.40	57.12	44.1

[1]SD=standard deviation; CV=coefficient of variation; [2]SCS=somatic cell score; RCT=rennet coagulation time; k_{20}=curd-firming time; a_{30}=curd firmness

Table 2. Least squares means (standard error) of milk coagulation properties across breeds

Trait[1]	Holstein-Friesian	Brown Swiss	Alpine Grey	Simmental
RCT, min	22.44 (0.15)[a]	22.15 (0.07)[a]	21.84 (0.09)[b]	21.80 (0.08)[b]
k_{20}, min	7.08 (0.05)[a]	5.75 (0.02)[b]	6.13 (0.03)[c]	6.21 (0.03)[c]
a_{30}, mm	14.82 (0.39)[a]	19.86 (0.19)[b]	19.71 (0.24)[bc]	18.90 (0.21)[c]

[1]RCT=rennet coagulation time; k_{20}=curd-firming time; a_{30}=curd firmness

Sources of variation for milk coagulation properties

Fixed effects included in the mixed linear model for MCP were highly significant ($P<0.01$; data not shown). Cow breed was an important source of variation for milk coagulation characteristics, and least squares means for the breed effect are reported in Table 2. Dual-purpose cows (SI and AG) exhibited more favourable MCP than dairy breeds (HF and BS). These findings are consistent with another large-scale study on Italian dairy cattle breeds conducted by Penasa et al. (2014). Recently, Pretto et al. (2013) demonstrated that a_{30} has a positive association with cheese yield. This is a crucial point of the present study, especially in countries highly specialized in cheese production, such as Italy. Indeed, although characterized by lower milk production compared to cosmopolitan breeds, milk of local AG cows can potentially result in greater cheese yield and this could lead to more profit for the dairy industry. Moreover, this can also contribute to the valorisation of local breeds, that nowadays are giving a service to rural area populations (e.g., maintenance of the territory where they are reared and preservation of the local traditions) without recognition in any milk payment system. These results, anyway, need further investigation.

Least squares means of MCP across lactation are depicted in Figure 1. At the very beginning of lactation, MCP had the most favourable values for cheese processing, with low values of RCT and high values of a_{30}. The opposite trend of RCT and a_{30} is due to the strong and negative correlation between them (-0.87). The strong association between these two traits was previously reported by Cassandro et al. (2008) in Italian Holstein-Friesian dairy cows. Rennet coagulation time increased up to 180 DIM and decreased slightly thereafter, until the end of lactation. Curd firmness, on the other hand, exhibited less desired values immediately after the lactation peak, and then became more favourable for cheese-making after 6 months of lactation. None of the traits, anyway, reached the levels observed at the beginning of lactation. These findings are consistent with Ikonen et al. (2004) and Penasa et al. (2014).

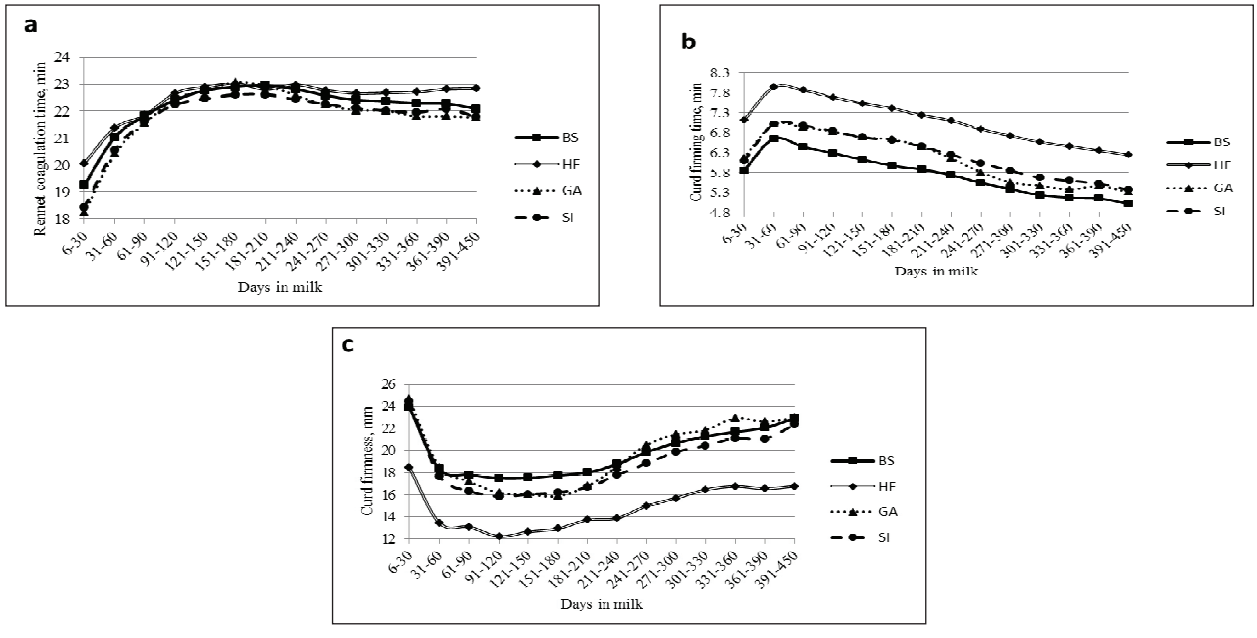

Figure 1. Least squares means of (a) rennet coagulation time, (b) curd-firming time, and (c) curd firmness across lactation.

CONCLUSION

This study provides for the first time a description of predicted MCP of AG cattle breed using individual samples and describing MCP variation in Alpine dairy system. The results demonstrated that MCP variation depended significantly on several environmental factors, including cow breed and DIM. Local AG cow had more favourable clotting characteristics compared to Holstein-Friesian breed, and this can be exploited as a strong point for its valorisation. Further research will estimate genetic parameters of MCP for these breeds.

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