UNIVERSITÀ
DEGLI STUDI
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DIPARTIMENTO DI AGRONOMIA ANIMALI ALIMENTI RISORSE NATURALI E AMBIENTE - DAFNAE

SCUOLA DI DOTTORATO DI RICERCA IN SCIENZE ANIMALI E AGROALIMENTARI

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# FUNCTIONAL MEAT AND MEAT PRODUCTS FROM UNCONVENTIONAL MEAT SPECIES

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*Alla mia famiglia e a Mara…*

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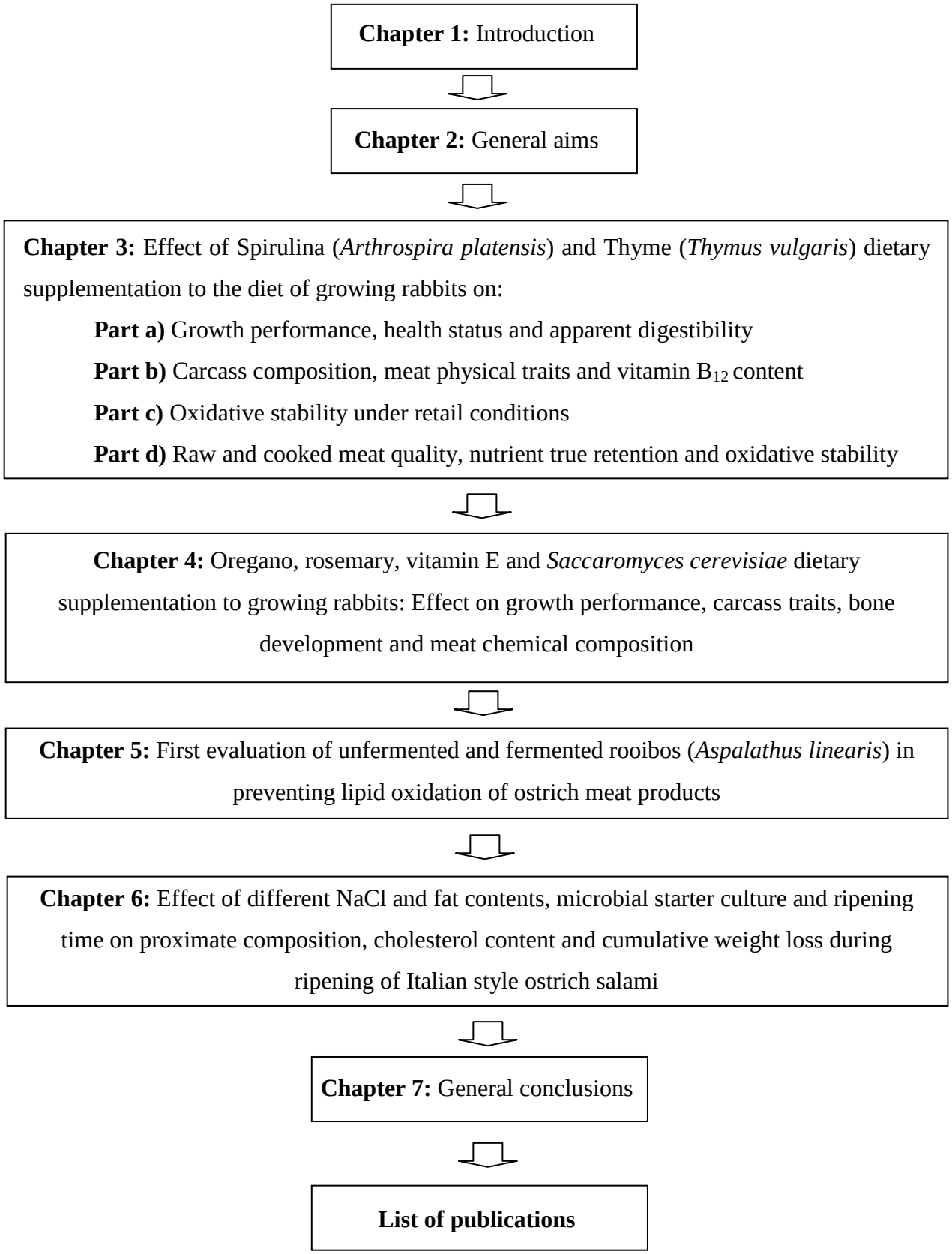
## THE OUTLINE OF THE THESIS

In the first part, some topics to understand and contextualize the research of the present PhD thesis are presented (Chapter 1). Initially, a brief description the role of meat in human evolution and nutrition, as well as the risks and benefits derived from its consumption is provided. Subsequently, one of the most problematic issues concerning meat quality and the possible remedies to prevent it are depicted: lipid oxidation and antioxidants. Then, after describing the history, meaning and role of functional meat and meat products, the two meat species used for the research are presented: the ostrich and the rabbit. In Chapter 2, the general aims of four main contributes are provided: Chapter 3, which includes four different subchapters, and Chapter 4 deal with dietary strategies to improve rabbit meat quality. Chapter 5 and 6 approach two possible complimentary interventions to reduce some compounds or incorporate functional ingredients in ostrich fresh meat as well as processed meat products. The first three subchapters of Chapter 3 (a, b and c) and Chapter 5 have been already published in peer reviewed scientific journals, whereas Chapter 3d, Chapter 4 and 6 have been submitted to peer reviewed scientific papers. The last part of the present thesis (Chapter 7), provides the general conclusions of the research work. Figure 1 graphically shows the contents and organization of the present thesis.

**Figure 1.** The outline of the thesis



# ABSTRACT

The present thesis, which includes four main researches, aimed to study functional meat and meat products obtained from unconventional meat species, namely the rabbit and the ostrich.

The first research tested the single and combined dietary supplementation with 5% Spirulina (*Arthrospira platensis*) and 3% Thyme (*Thymus vulgaris*) to growing rabbits for 3 and 6 weeks, and it is articulated in four different subchapters.

The first subchapter considered the effect on total tract apparent digestibility of nutrients and on the performances of growing rabbits and showed that that single and combined supplementation with Spirulina reduced the nutritive value of the diets. Despite this, no substantial effect on growth performance and health status, was observed. Future studies should take into consideration feed processing technology, pelleting, storage and packaging conditions, as they may reduce or nullify the nutrient and functional compounds' availability. Moreover, Spirulina and/or Thyme effect on health status should be tested under poorer sanitary conditions.

The second subchapter studied the effects on growing rabbit carcass composition, meat and bone rheological traits, and the vitamin $B_{12}$ content of *Longissimus dorsi* (LD) meat. Spirulina was confirmed as a rich source of vitamin $B_{12}$ that was successfully transferred into LD meat, thus demonstrating its value as an effective natural supplement in producing food fortified with this vital element. With this exception, the supplement as well as and the duration of treatment had no effect on the considered traits.

The third subchapter evaluated the effect on rabbit meat during retail display. Thyme improved colour parameters and reduced exudative losses during a simulated retail display, also considering a shorter supplementation period. This would positively impact consumers at the time of purchase as well as farmers demand to limit production costs. Differently, Spirulina had no effect on oxidative stability of rabbit meat, maybe for the poor absorption from the gut as a result of interference on uptake of antioxidants by Spirulina, or maybe because dietary level of Spirulina was not adequate for rabbits requirements.

The fourth subchapter studied the effects on raw and cooked rabbit meat quality, nutrient true retention and protection against oxidative stress conditions. The dietary inclusion of Spirulina improved the FA profile of the *Longissimus dorsi* and hind leg meat by significantly increasing γ-linolenic acid content. Thyme improved the oxidative stability of raw and freeze-dried HL meat but not that of cooked meat.

As for the shelf-life trial, Spirulina was not observed to improve the oxidative stability of rabbit meat subjected to severe oxidative stress.

The second research of the present thesis, aimed to evaluate the effect of the dietary single or combined supplementation with different natural additives (Oregano, Rosemary, vitamin E and *Saccaromyces cerevisiae*) on the performance of growing rabbits, the nutritional composition and oxidative stability of their meat and on their hind leg bone traits. The results of this research demonstrated that an adequate supplementation with natural antioxidants can also have a positive effect on productive performance and meat quality.

The fifth chapter evaluated, for the first time, the unfermented and fermented rooibos (*Aspalathus linearis*) in preventing lipid oxidation in ostrich meat patties and ostrich salami. This plant showed interesting and promising antioxidant potential when applied to meat products, even if further study are necessary to deeply investigate long-term effects.

The sixth chapter studied the effect of two different fat inclusion levels, NaCl contents and two LAB starter cultures on the weight loss, proximate composition and cholesterol content of ostrich salami ripened for 10 and 20 weeks. A lower fat content consistently shortened ripening time, thus being a positive aspect in terms of productivity, and it determined a higher nutrients concentration compared to high fat salami. Reducing the NaCl inclusion, retarded the weight loss of the product of about 1 week, without affecting its proximate composition. Finally, the metabolic activity of tested LAB starter cultures seemed to be influenced by the fat inclusion level, ultimately affecting the healthiness of the products at 10 weeks of ripening.

# RIASSUNTO

La presente tesi si compone di quattro ricerche principali e si propone di studiare carne e prodotti carnei funzionali ottenuti da specie non convenzionali, lo struzzo e il coniglio.

La prima ricerca ha testato l'inclusione singola o combinata, per 3 o 6 settimane, con il 5% di Spirulina (*Arthrospira platensis*) ed il 3% di Timo (*Thymus vulgaris*) nella dieta di conigli in accrescimento. Questo primo capitolo si articola in quattro sottocapitoli.

Il primo ha considerato l'effetto sulla digeribilità apparente delle diete e sulle prestazioni produttive di conigli in accrescimento e ha evidenziato che l'inclusione separata o combinata di Spirulina ha ridotto il valore nutritivo delle diete. Nonostante ciò, non è stato osservato alcun effetto sulle prestazioni produttive e sullo stato di salute degli animali. Studi futuri dovranno considerare la tecnologia di produzione del mangime, la pellettatura e le condizioni di confezionamento e stoccaggio, in quanto potrebbero ridurre o nullificare la disponibilità di componenti nutritivi e funzionali. Inoltre, l'inclusione di Spirulina e/o Timo dovrebbe essere testata in condizioni sanitarie più critiche.

Il secondo sottocapitolo ha valutato l'effetto dell'inclusione di Spirulina e Timo sulla composizione della carcassa, le caratteristiche reologiche di carne ed ossa ed il contenuto di vitamina $B_{12}$ del *Longissimus dorsi* (LD). Spirulina ha confermato di essere una fonte di vitamina $B_{12}$, la quale è stata trasferita con successo nella carne del LD. Spirulina ha quindi dimostrando il suo valore quale additivo naturale per produrre alimenti fortificati con questo elemento. Per quanto riguarda gli altri aspetti considerati nella presente ricerca, gli additivi naturali testati non hanno avuto alcun effetto.

Il terzo sottocapitolo ha studiato la "shelf-life" della carne fresca di coniglio durante una simulazione di esposizione finalizzata alla vendita. Il timo ha migliorato il colore e ridotto le perdite essudative della carne, anche quando è stato somministrato per il periodo più breve. Questo risultato da un lato è in grado di influenzare positivamente il consumatore al momento dell'acquisto, e dall'altro va incontro alle esigenze dell'allevatore di limitare i costi di produzione. Al contrario, Spirulina non ha avuto alcun effetto sulla stabilità ossidativa della carne, forse per uno scarso assorbimento intestinale dovuto all'interferenza degli antiossidanti presenti nella Spirulina stessa, oppure perché il livello di inclusione nella dieta non era adeguato alle esigenze dei conigli.

Il quarto sottocapitolo ha testato l'effetto sulla qualità della carne cruda e cotta, sulla ritenzione reale dei nutrienti e sulla protezione nei confronti di condizioni di stress ossidativo. L'inclusione di Spirulina ha migliorato il profilo acidico del *Longissimus dorsi* e dell'arto posteriore di coniglio, attraverso l'aumento del contenuto dell'acido grasso γ-linolenico. Il Timo ha migliorato la stabilità ossidativa della carne dell'arto posteriore cruda e liofilizzata, ma non quella della carne cotta. Come era stato osservato nel precedente esperimento sulla "shelf-life" della carne di coniglio, Spirulina non ha migliorato la stabilità ossidativa della carne sottoposta a stress ossidativo intenso.

Il secondo capitolo della presente tesi, ha considerato l'effetto dell'inclusione singola o combinata con diversi additivi naturali (Origano, Rosmarino, vitamina E e *Saccaromyces cerevisiae*) sulle prestazioni produttive di conigli in accrescimento, la composizione nutrizionale e la stabilità ossidativa della carne nonchè sulle caratteristiche ossee degli arti. I risultati di questa ricerca hanno dimostrato che un'adeguata inclusione di antiossidanti naturali nella dieta di conigli in accrescimento ha avuto un effetto positivo anche sulle prestazioni produttive e sulla qualità della carne.

Il quinto capitolo, invece, ha studiato per la prima volta l'applicazione di rooibos (*Aspalathus linearis*), fermentato e non, sulla carne e prodotti derivati. In particolare, è stata valutata la sua capacità di prevenire l'ossidazione lipidica in polpette e salami di struzzo. I risultati hanno rivelato un interessante e promettente potenziale antiossidante di questa pianta nei confronti dei prodotti carnei testati. Tuttavia, sono necessari ulteriori studi per esaminarne l'efficacia a lungo termine.

Il sesto ed ultimo capitolo, ha valutato due diversi livelli di grasso e NaCl, e due diversi starter microbici, sulle perdite di peso, composizione centesimale e contenuto di colesterolo di salami di struzzo stagionati per 10 e 20 settimane. Un minore contenuto di grasso ha ridotto considerevolmente il tempo di stagionatura, essendo quindi un aspetto positivo in termini di produttività, e ha determinato una maggiore concentrazione di nutrienti rispetto al salame preparato con il più alto livello di grasso. La riduzione del contenuto di NaCl ha ritardato le perdite di peso dei salami di 1 settimana, senza tuttavia modificare la composizione centesimale del prodotto. Infine, l'attività metabolica degli starter microbici testati è sembrata essere condizionata dal contenuto di grasso del salame e ciò, a 10 settimane di stagionatura, ha influenzato la salubrità del prodotto.

# CHAPTER 1

## General introduction

### *1. Meat: role in human evolution, nutritional composition, risks and benefits derived from its consumption*

According to the European legislation, meat refers to the edible parts removed from the carcass of domestic ungulates such as bovine, porcine, ovine and caprine, but also domestic solipeds, poultry, lagomorphs, farmed game, small and large wild game (European Commission, 2004).

Historically, human evolution has gone hand in hand with meat consumption. Initially humans were opportunistic hunters, but the frequency of the adoption of hunting as a means to obtain food increased and lasted 2 to 3 million years. Subsequently, around 10,000 years ago, human being started to domesticate animals and cultivate plants, thus meat consumption still increased. Then, in the last decades, meat started to be seen as a food containing harmful compounds which could increase the risk to develop diseases (Larsen, 2003). A higher quality of the diet through centuries, including an increased meat consumption, brought important modifications in the human body: morphological changes regarded cranium and dentition, but also gastrointestinal tract. Specifically, as the need of tearing and chewing meat rather than grinding vegetables grew, the size of molar teeth decreased whereas front teeth and jaws became stronger. Moreover, as humans are omnivorous, their gastrointestinal tract is different from that of pure herbivorous as well as that of carnivorous. They have a simple stomach and a long small intestine which reflects its adaptation to a varied diet, with a reduced caecum and colon. Finally, an increase meat and bone marrow consumption partly contributed, together with other factors, to the extraordinary enlargement of brain during human evolution (Navarrete, van Schaik, & Isler, 2011). In the brain, lipids constitute the 60 % of this system, mainly phosphoglycerides and cholesterol (Diau, Hsieh, Sarkadi-Nagy,Wijendran, Nathanielsz, & Brenna, 2005).

Meat is an unequivocal source of high biological value protein with a high Protein Digestibility-Corrected Amino Acids Scores digestibility equal to 0.92, whereas proteins of plant origin can reach a maximum of 0.71 (FAO/WHO, 1991). This score takes into account the quantity and quality of the amino acids which constitute proteins. A total of twenty amino acids are necessary in order to synthesize meat proteins, but only eight of them are essential

and must be supplied by diet as they can't be synthesized by the human body (Table 1). As a consequence, an inadequate consumption of essential amino acids can lead to protein malnutrition. When a certain food supplies enough of seven of the eight essential amino acids, that lacking is called limiting amino acid and this is the case of proteins derived from vegetables such as cereals or legumes (Young & Pellet, 1990). One important amino acid that is not part of proteins but which can be found almost exclusively in animal products, included certain meat types such as duck, poultry, venison, beef, pork, veal, horse, rabbit and lamb is taurine (Spitze, Wong, Rogers, & Fascetti, 2003). This amino acid is fairly produced by humans body from methionine and cysteine which are their precursor, thus it must be provided through diet. Taurine showed several important biological functions which are related to cardiovascular disease prevention, as it is an antioxidant and anti-inflammatory agent (Wójcik et al., 2010).

**Table 1.** Essential amino acids and non-essential amino acids (Wu, 2009)

| Essential amino acids | Non essential amino acids |
|---|---|
| | Alanine |
| | Asparagine |
| Isoleucine | |
| Leucine | Arginine |
| Lysine | Cysteine |
| Methionine | Aspartic acid |
| Tryptophan | Glutamic acid |
| Treonine | Proline |
| Valine | Hystidine |
| Phenilalanine | Tyrosin |
| | Serin |
| | Glycine |

Meat is also an excellent source of B vitamins and minerals such as zinc, selenium and iron, but it contains also useful amounts of magnesium, copper, cobalt, phosphorus, chromium and nickel (Williamson, Foster, Stanner, & Buttriss, 2005).

Vitamin $B_{12}$ (cobalamin) is the largest and most complex of all vitamins. Also its gastrointestinal absorption is complex and determines its bioavailability which is about 50 % in healthy adults. Low dietary intake and malabsorption in the elderly people are thus the main cause of vitamin $B_{12}$ deficiency. Vitamin $B_{12}$ is required by enzymes which participate to the methylation cycle and low intakes of it, but also of vitamin $B_9$ (folate) and vitamin $B_6$ (pyridoxine), have been associated with elevated homocysteine, which is a risk factor for cardiovascular disease and stroke. The major signs of vitamin $B_{12}$ deficiency are megaloblastic anemia and neuropathy (Scott, 1999; Stabler, & Allen, 2004). Meat is known to be the primary source of vitamin $B_{12}$ in the diet and, even if data are limited, bioavailability of cooked ground patties from mutton ranged from 40 to 89 % of the initial vitamin $B_{12}$ content of raw patties (Watanabe, 2007).

Vitamin A is essential for growth and development of cells and tissue and plays a significant role in the respiratory epithelium and the lung), but while meat in general can't be considered a source, this is not true for offal meats (Biesalski, 2005). For example, 100 g of liver were estimated to provide more than 338 % of the dietary recommended value for retinol (Pereira & Vicente, 2013).

Iron is fundamental for many processes at the tissue and cellular levels, thus being crucial for human health. It is a component of hemoglobin, thus being responsible for hemoglobin and myoglobin oxygenation in blood and skeletal muscle, respectively. Moreover, due to the catalytic activity, enzymes containing iron are involved in host-defense responses and cellular energy metabolism. Iron possesses unfilled atomic orbitals which allow it to coordinate electron donors and participate in redox processes (Biesalski, 2005). Despite 90 % of it has an endogenous source (breakdown of red cells) and it is not excreted, iron deficiency is a major nutritional disorder in the world. This is because iron losses can occur in specific situations like damaged skin or digestive tract, as well as it affects specific segments of the population like children and women during child bearing age (Mc Afee, McSorley, Cuskelly, Moss, Wallace, Bonham, & Fearon, 2010). For these reasons, diet has a key role in maintaining iron balance in the body. Iron can be found in two different forms: heme-iron and non-heme iron. The first is only present in animal foods as it comes from hemoglobin and myoglobin, whereas the second one is present mainly in vegetable. Heme-iron is easily absorbed as an intact molecule by enterocytes in the intestinal lumen, whereas non-heme iron has a low bioavailability (2-20 %) and its absorption can be further reduced by the presence of inhibitory compounds that can be normally present in vegetables like phytate, some phenolics, oxalate and non-digestible carbohydrates (Pereira & Vicente, 2013). Meat and meat products

are the primary source of highly bioavailable iron and they can provide up to 18 % of iron daily requirements which makes it fundamental in preventing nutritional deficiencies (WHO/UNICEF/UNU, 2001).

Selenium has an important role in the activity of glutathione peroxidase, which is a crucial enzyme that protects cellules from free radical oxidative damage thus being involved in detoxification processes, and cancer prevention (Pereira, & Vicente, 2013). Dietary requirement for selenium in adults is 55 mcg/100 day and meat is an important source as it can contain 40 to 50 mcg/100 g of fresh meat, with good bioavailability (Fairweather-Tait, Collings, & Hurst, 2010).

As it is associated with the activity of a wide variety of enzymes which are important for the optimal function of the immune and reproductive systems, gene expression, cell division and growth, zinc deficiency raises the risk of infections, oxidative stress and genetic damage (Prasad, 2009). The recommended zinc intake for adults is 7.0 and 9.5 g/day for females and males, respectively, and also a low red meat consumption (≤41 g/day) can guarantee such requirements (McAfee et al., 2010) as meat is classified as a source of zinc.

Meat is also an important source of fats which provide energy and facilitate the absorption of fat-soluble dietary components such as liposoluble vitamins. Fat content differs significantly among different meat species, cuts, age of the animal and diet and ranges around 3-25 g/100 g of edible portion. Meat fats are mostly monounsaturated (MUFA) and saturated (SFA) with oleic (C18:1), palmitic (C16:0) and stearic (C18:0) acids being most ubiquitous (Valsta, Tapanainen, & Männistö, 2005). But meat can also contribute up to 20% to long chain omega 3 PUFA intake, which have been recognized a protective factor against cardiovascular diseases and in favor of general health promotion. Moreover, *n*-3 PUFA content in meat is mainly dependent on the feeding strategy thus it can be enhanced through appropriate strategies like grass or forage-based diets, as well as supplementation with fish oil or flaxseed (Dalle Zotte, Gottardo, Segato, & Andrighetto, 2002; Nuernberg, Dannenberg, Nuernberg, Ender, Voigt, Scollan, Wood, Nute, & Richardson, 2005; Witsuba, Kegley, & Apple, 2006; Kronberg, Barceló-Coblijn, Shin, Lee, & Murphy, 2006; Webb, & O'Neill, 2008). Consequently, as meat is an important source of arachidonic acid (C20:4 *n*-6) which was shown to increase the risk of thrombosis, the presence of omega 3 PUFA in meat could counteract this effect (Christophersen & Haug, 2011). As several studies have associated total fat content, SFA consumption with the risk of developing cardiovascular disease, dietary guidelines have recommended to avoid their consumption (Gidding, Dennison, Birch, Daniels, Gillman, Lichtenstein, et al., 2005). One of the mechanism proposed to explain this

association is that excessive consumption of SFA can promote white adipose tissue expansion and hypertrophy leading to apoptosis. This would promote the release of inflammatory proteins such as cytokines and chemokines inducing inflammation and insulin resistance, which increase the risk of cardiovascular disease and metabolic syndrome (Kennedy, Martinez, Chuang, Lapoint, & Mcintosh, 2009). Moreover high SFA intake has also been associated to certain types of cancer as well as to a raise total and low-density lipoprotein (LDL) cholesterol, with myristic and palmitic acids, but not stearic acid, being main responsible (Radder, & Le Roux, 2005; Valsta et al., 2005).

For this reason most recent dietary guidelines suggest that dietary fat should provide between 15 and 35 % of total calories and that saturated fats should be <10 % of total caloric intake. MUFA and PUFA should account for 12-20 % and 6-12 % of total energy intake, respectively (Aranceta, & Pérez-Rodrigo, 2012). For what concern cholesterol, recent epidemiologic studies and clinical trials suggest that the recommendation to limit its intake to 300 mg/day lacks of convincing evidence (Fernandez, & Calle, 2010). In a study by Li, Sinclair, Mann, Turner, Ball, Kelly, Abedin, & Wilson (1999), it was observed that consumers eating ≥285 g meat/day had higher plasma total cholesterol, as well as LDL cholesterol and triglycerides than vegans, vegetarians, low and moderate consumers of meat. However, the daily intake of 285 g meat is very high and no distinction regarding the type of red meat (unprocessed or processed) was made (Mc Afee, et al., 2010). In fact, an experiment on Irish consumers showed that a moderate red meat consumption (24-72 g/day) provided about 14% of total SFA intakes, a percentage that was similar to non-consumers of red meat (Cosgrove, Flynn, & Kiely, 2005). Moreover, when hypercholesterolemic subjects were fed with low-fat diets, the addition of up to 180 g/day of lean beef didn't compromise the LDL-cholesterol lowering effects of the diet (Beauchesne-Rondeau, Gascon, Bergeron, & Jacques, 2003).

Finally, other aspects should be further considered when studying meat consumption and health risks. Particularly, the effect of processing techniques and cooking methods on health risks derived from meat consumption have been assessed only in few studies, but they represent important variables that could contribute to the bad image of meat (Pereira & Vicente, 2013). Moreover, type of cooking generally produces high losses of B vitamins, as they are thermally instable and water soluble (Lombardi-Boccia. Lanzi, & Aguzzi, 2005). Interestingly, available data of most EU Countries concerning patterns of food and nutrient intake, showed that a higher intake of total fat was associated with a higher energy intake and, often, with all main classes of fatty acids (Valsta et al., 2005). Hence, in order to prevent

food-dependent diseases, the recent tendency of most reports is that to recommend the control of total calorie intake focusing on general dietary patterns and lifestyle rather than on single nutrients (Schönfeldt & Gibson, 2008; Mc Afee,  et al., 2010; Aranceta, & Pérez-Rodrigo, 2012). Thus, the standing advice is to consumer less than 500 g of red meat/week, reduce processed meat consumption to occasional situations and avoid cooking meat at very high temperatures as it leads to the formation of mutagenic compounds such as heterocyclic amines and polycyclic aromatic hydrocarbons (WCRF, 2007).

**Table 2a.** Proximate composition, energy value, main lipid classes and cholesterol contents of several meat species (referred to 100 g of edible portion, USDA 2014)

| Nutrient | Unit | Beef (loin), Lean | Veal (loin), Lean | Pork (loin), Lean | Lamb (loin), Lean | Duck (carcass), meat only | Chicken (carcass), meat only | Turkey (carcass), meat only | Ostrich, composite of cuts | Rabbit, composite of cuts |
|---|---|---|---|---|---|---|---|---|---|---|
| Proximates | | | | | | | | | | |
| Energy | kcal | 139 | 116 | 143 | 143 | 135 | 119.5 | 112 | 118 | 136 |
| Water | g | 72.4 | 74.9 | 72.2 | 72.6 | 73.8 | 75.4 | 75.4 | 75.6 | 72.8 |
| Protein | g | 21.9 | 20.2 | 21.4 | 20.9 | 18.3 | 21.6 | 22.6 | 21.7 | 20.1 |
| Fat | g | 5.74 | 3.34 | 5.66 | 5.94 | 5.95 | 2.98 | 1.93 | 2.80 | 5.55 |
| Lipids | | | | | | | | | | |
| SFA | g | 1.96 | 1.01 | 1.95 | 2.13 | 2.32 | 0.77 | 0.46 | 1.08 | 1.66 |
| MUFA | g | 2.12 | 1.07 | 2.56 | 2.39 | 1.54 | 0.87 | 0.48 | 1.01 | 1.50 |
| PUFA | g | 0.42 | 0.34 | 0.61 | 0.54 | 0.75 | 0.72 | 0.41 | 0.54 | 1.08 |
| Cholesterol | mg | 61 | 80 | 59 | 66 | 77 | 69 | 67 | 74 | 57 |

**Table 2b.** Mineral and vitamins contents of several meat species (referred to 100 g of edible portion, USDA 2014)

| Nutrient | Unit | Beef (loin), Lean | Veal (loin), Lean | Pork (loin), Lean | Lamb (loin), Lean | Duck (carcass), meat only | Chicken (carcass), meat only | Turkey (carcass), meat only | Ostrich, composite of cuts | Rabbit, composite of cuts |
|---|---|---|---|---|---|---|---|---|---|---|
| Minerals | | | | | | | | | | |
| Ca | mg | 13 | 17 | 17 | 12 | 11 | 12 | 11 | 6 | 13 |
| Fe | mg | 2.50 | 0.75 | 0.84 | 1.91 | 2.40 | 0.88 | 0.86 | 3.76 | 1.57 |
| Mg | mg | 12 | 25 | 23 | 27 | 19 | 25 | 27 | 22 | 19 |
| P | mg | 215 | 211 | 211 | 190 | 203 | 175 | 190 | 213 | 213 |
| K | mg | 289 | 324 | 389 | 276 | 271 | 231 | 235 | 310 | 330 |
| Na | mg | 44 | 91 | 52 | 68 | 74 | 77 | 118 | 78 | 41 |
| Zn | mg | 3.26 | 2.49 | 1.84 | 3.19 | 1.90 | 1.49 | 1.84 | 3.74 | 1.57 |
| Vitamins | | | | | | | | | | |
| $B_1$ | mg | 0.05 | 0.07 | 0.99 | 0.13 | 0.36 | 0.07 | 0.05 | 0.20 | 0.10 |
| $B_2$ | mg | 0.32 | 0.26 | 0.27 | 0.23 | 0.45 | 0.14 | 0.19 | 0.29 | 0.15 |
| $B_3$ | mg | 4.76 | 9.08 | 4.92 | 6.51 | 5.30 | 8.43 | 8.10 | 4.71 | 7.27 |
| $B_6$ | mg | 0.62 | 0.56 | 0.53 | 0.17 | 0.34 | 0.44 | 0.65 | 0.51 | 0.50 |
| $B_9$ | μg | 4 | 14 | 5 | 24 | 25 | 7 | 7 | 8 | 8 |
| $B_{12}$ | μg | 3.53 | 1.18 | 0.63 | 2.21 | 0.40 | 0.37 | 1.24 | 4.96 | 7.16 |
| C | mg | 0 | 0 | 0.6 | 0 | 5.8 | 1.6 | 0 | 0 | 0 |
| A | μg | 2 | - | 2 | 0 | 24 | 15 | 9 | 0 | 0 |
| D | μg | 3.0 | - | 0.5 | - | 0.1 | - | 0.2 | - | - |
| E | mg | 0.23 | 0.26 | 0.18 | 0.19 | 0.70 | 0.22 | 0.09 | 0.20 | 0.16 |
| K | μg | 1.5 | - | 0.0 | - | 2.8 | 2.4 | 0.0 | - | - |

*2. Shelf-life of meat: lipid oxidation*

Oxidative stress has been suggested to be involved in many chronic diseases such as cancer, cardiovascular disease and general ageing processes (Mayne, 2003). Lipid oxidation represents one of the most important causes of deterioration of meat and meat products and it affects unsaturated fatty acids, particularly PUFA in membrane phospholipids, and cholesterol, mainly LDL cholesterol. The end-products of this process impair color, aroma, flavor, texture of meat and meat products, hence determining a loss of nutritive value (Gray, Gomaa, & Buckley, 1996). Besides nutritional deterioration, lipid oxidation generates cytotoxic and genotoxic compounds which are deleterious for humans health (Kanner, 2007; Muselík, García-Alonso, Martín-López, Žemlička, & Rivas-Gonzalo, 2007).

Specifically, lipid hydroperoxides, which are primary products of the lipid oxidation, have a higher polarity than normal fatty acids, thus they can disrupt the integral structure and function of the membrane generating detrimental effects to cells and tissues (Min & Ahn, 2005). The aldehyde 4-hydroxynonenal, which is generated during lipid peroxidation, possesses cytotoxic properties for human and animals as it binds to protein, inhibiting their functions (Okada, Wangpoengtrakul, Osawa, Toyokuni, Tanaka, & Uchida, 1999). Lipids are subjected to oxidation when catalytic systems such as light, heat enzymes, metals, metalloproteins and microorganisms are present. With the presence of intermediate reactive species and/or free radicals, these conditions lead to autoxidation, photooxidation, thermal or enzymatic oxidation. However, lipid oxidation is mainly caused by autoxidation that consists of a spontaneous reaction of lipids with oxygen through a chain reaction of free radicals, and it is a self-propagation and self-accelerating phenomena (Shahidi & Zhong, 2010). Lipid oxidation is very slow in the first stage, but then it increases very rapidly after an induction period. The process consists of three phases: Initiation, propagation and termination (Figure 2).

**Figure 2**. Lipid autoxidation (adapted from Shahidi & Zhong, 2010)

1. Initiation:
   (a) $RH + O_2 \longrightarrow R\cdot + \cdot OOH$
2. Propagation:
   (b) $R\cdot + O_2 \longrightarrow ROO\cdot$
   (c) $RH + ROO\cdot \longrightarrow ROOH + R\cdot$
   (d) $ROOH \longrightarrow RO\cdot + \cdot OH$

3. Termination:

(e) $R\cdot + R\cdot$

(f) $R\cdot + RO\cdot$

(g) $R\cdot + ROO\cdot$

(h) $ROO\cdot + ROO\cdot$

} Non radical products (R-R, ROR, ROOR, ROOR + $O_2$)

In the presence of one or more of the above mentioned initiators, a hydrogen atom is abstracted from a methylene group in the hydrocarbon chain of a lipid molecule (RH), especially of unsaturated lipid molecules, and this produce free radicals (R·). The lipid radical tends to be stabilized through the rearrangement of methylene-interrupted double bonds in PUFA, which generates conjugated dienes. Under aerobic conditions, a conjugated diene tends to react with oxygen to form lipid peroxyl radicals (ROO·). Differently, in very low oxygen conditions, conjugated dienes react each other within the membranes or other membrane components like protein and cholesterol (Min & Ahn, 2005). Once peroxyl radicals are formed, they are very unstable and they attack new lipid molecules, thus determining a rapid progression of the reaction. During propagation, peroxyl radicals abstract an hydrogen atom (H·) from another lipid molecule to form lipid hydroperoxides (ROOH), which are the primary products of the oxidation process (Brewer, 2011). Secondary oxidation products, which originated from lipid hydroperoxides, include aldehydes (ex. hexanal, 4-hydroxynonenal, malondialdheyde), ketones, alcohols, hydrocarbons, volatile organic acids and epoxy compounds, depending on the fatty acid substrates and reaction condition, and some of them have undesirable odors (rancidity) which can be detected at very low threshold values (Ladikos & Lougovois, 1990). The decomposition of hydroperoxides, which at low temperatures is dominated by metal ion-mediated (heme and non-heme iron) electron transfer mechanism, generates alkoxyl, peroxyl, hydroxyl and new lipid radicals which then further participate in the chain reaction of free radicals thus initiating further oxidation reactions. The propagation step is then repeated until no more hydrogen is available or when the chain is interrupted (ex. antioxidants). At this stage of the oxidative process (termination), lipid radicals neutralize each other forming stable non-radical products (R-R, ROR, ROOR).

*2.1 Antioxidants*

In order to control lipid oxidation in meat and meat products, the use of antioxidants is the most effective and convenient method. Antioxidants are "substances that, when present at low concentrations compared to those of an oxidizable substrate, significantly delay or prevent oxidation of that substrate" (Halliwell & Gutteridge, 1990). Antioxidants can be divided in:

1. Preventive antioxidants, which protect target lipids from oxidation initiators trough blocking the formation of reactive oxygen species or scavenging species responsible for oxidation initiation
2. Chain-breaking antioxidants, which stop the propagation phase intercepting radical oxidation propagators or indirectly stopping radical chain propagation

As there is a wide range of oxidation initiators, preventive antioxidants can exert their activity through different pathways. The most relevant ones are chelation of transition metals, singlet oxygen quenchers and reactive oxygen species (ROS) detoxification. Chelation of transition metals (ex. copper and iron) decrease the pro-oxidant effect of metal ions by forming a thermodynamically stable complex and reducing their redox potential (Shahidi & Zhong, 2010). Chelators of transition metals are polyphosphates, ethylenediaminetetraacetic acid (EDTA), citric acid, phenolic acids and flavonoids. Singlet oxygen quenchers, like carotenoids, tocopherols and thiols, can inactivate singlet oxygen by absorbing its excess energy and converting it to ground state triplet oxygen. The energy absorbed is then released in the form of heat. Interestingly, some antioxidants with different pathways can interact, thus creating mixed mechanisms. It's the case of ascorbic acid, a water-soluble antioxidant whose effect on lipid stability is mainly due to its synergistic interaction with α-tocopherol, citric acid, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and metal chelators. Ascorbic acid can regenerate tocopherols from their radicals by replenishing hydrogen atom, thus inhibiting depletion of important antioxidants. Finally, ROS detoxification is an oxidation prevention pathway which is mainly carried out by endogenous enzymatic systems like superoxide dismutase, glutathione peroxidase and catalase (Laguerre, Lecomte, & Villeneuve, 2007).

Chain-breaking antioxidants usually stop radical oxidation propagation by donating an hydrogen atom to free radicals, thus inactivating them. Some antioxidant belonging to this group simply reduce the peroxidation rate, whereas others, the most potent chain-breakers, practically induce a lag phase in which the substrate is not oxidized and this phase keeps on going until the antioxidant is not consumed. After that, oxidation process rises again reaching

the same rate as during uninhibited oxidation. Some of the most known chain-breaking antioxidants are tocopherols, tocotrienols, flavonoids and phenolic acids (Laguerre et al., 2007).

In order to obtain positive results it necessary to study the appropriate concentration at which one antioxidant is most effective on a certain substrate. Differently, inappropriate concentrations may lead to an insufficient antioxidant activity or to a pro-oxidant effect. Finally, antioxidant activity can be affected also by environmental factors of the system, like bulk oils *vs* oil-in-water emulsions (Shahidi & Zhong, 2010).

*3. Functional meat and meat products: meaning, market trends and consumers opinion*

The term functional foods was firstly introduced in Japan in 1980, referring to food products that, besides the nutritive function, contained ingredients possessing specific advantageous physiological effects for humans body (Kumar, Kumar, Sharma, Mendiratta, Verma, & Patel, 2013). Up to now, Japan is also the only Country having a specific regulatory approval process for functional products which is called FOod for Specified Health Use (FOSHU) and which compete to the Japanese Ministry of Health and Welfare. Regulations include the establishment of specific health claims for this type of food. The interest in such products rapidly spread also to Europe and USA, as they saw both a great commercial potential for the food industry, as well as the possibility to lower the cost of healthcare of the aging population (Siró, Kápolna, Kápolna, & Lugasi, 2008). However, the nature of functional food greatly differs between Japan and the rest of the World: in Japan functional foods are a distinct class of products (FOSHU) in which function is superior to taste. Differently, in Europe and USA functional food means adding functionality to an existing traditional food product, and such products don't belong to a distinct group (Kotilainen, Rajalahti, Ragasa, & Pehu, 2006). The European Commission's Concerted Action on Functional Food Science in Europe, coordinated by International Life Science Institute, described functional food as "a food product that, together with the basic nutritional impact, it is satisfactorily demonstrated to affect beneficially on one or more functions of the human organism, thus either improving the general and physical conditions or/and decreasing the risk of the evolution of diseases. The amount of intake and form of the functional food should be as it is normally expected for dietary purposes. Therefore, it could not be in the form of pill or capsule just as normal food form" (Diplock, Aggett, Ashwell, Bornet, Fern, & Robertfroid, 1999). An example of different functional food types is given in the following table (Table 3)

**Table 3**. Types of functional foods (from Siró et al., 2008)

| Type of functional food | Definition | Example |
|---|---|---|
| Fortified product | A food fortified with additional nutrients | Fuit juices fortified with vitamin C |
| Enriched product | A food with added new nutrients or components not normally found in a particular food | Margarine with plant sterol ester, probiotics, prebiotics |
| Altered product | A food from which a deleterious component has been removed, reduced or replaced with another substance with beneficial effects | Fibers as fat releasers in meat or ice cream products |
| Enhanced commodities | A food in which one of the components has been naturally enhanced through special growing conditions, new feed composition, genetic manipulation, or otherwise | Eggs with increased omega-3 content achieved by altered chick feed |

The market of functional foods is very dynamic and innovative and represents undoubtedly a great potential for the food industry. This was evidenced by a rapid market growth, whose global value was estimated to at least 33 billion US$ in 2000, which was then corrected to 47.6 billion US$ in 2002 and calculated approximately to 61 billion US$ in 2004 (Hilliam, 2000; Sloan, 2002; Benkouider, 2004). In 2005, the worldwide market of functional foods generated 73.5 billion US$ and it was estimated to reach a value of at least 167 US$ after 2012 with a growth potential/year of 10 % (Granato, Branco, Nazzaro, Cruz, & Faria, 2010). United States, Europe and Japan are the largest markets for this kind of products, contributing for more than 90 % of the total sales (Benkouider, 2005). Despite these positive trends, high failure rates for this kind of products were reported as developing a functional food is expensive and requires appropriate marketing studies to gain knowledge of the products and the target consumers. Thus, the commercial success of functional products depends on taste, appearance, price and health claim that appeals to consumers (Betoret, Betoret, Vidal, & Fito, 2011; Conte, Mastromatteo, Cozzolino, Lecce, & Del Nobile, 2011).

Even though some studies indicated that people in industrialized countries are eating less meat than before (Fresco, 2009), worldwide high-value animal protein is being required more and more. The global increase in per capita consumption of meat was reported from 24.2 kg/year in 1964, to 36.4 kg/year in 1999 and, more recently, per capita meat

consumption is expected to increase to 45.3 % by 2030 and worldwide meat consumption by 72 % between 2000 and 2030 (Bruinsma, 2003; Schönfeldt, & Gibson, 2008; Fiala, 2008). Consequently, due to the increasing world population, rising incomes and urbanization, global meat production was projected to be more than double between 2000 and 2050 (Steinfeld, Gerber, Wassenaar, Castel, Rosales, & de Haan, 2006). A recent study by Olmedilla-Alonso, Jiménez-Colmenero, & Sánchez-Muniz (2013), pointed out that the increased meat consumption is highest in industrialized countries and it is positively correlated with the level of income. Table 4 shows the average daily intake of total meat, red meat and/or processed meat in several European Countries.

**Table 4.** Mean daily intake (g/day) of total meat, red meat, processed meat and red + processed meat in selected countries participating in the European Prospective Investigation into Cancer and Nutrition (EPIC) calibration study (from Williamson et al., 2005)

| | Total meat* | | Red meat | | Processed meat | | Red meat + Processed meat | |
|---|---|---|---|---|---|---|---|---|
| | Men | Women | Men | Women | Men | Women | Men | Women |
| Greece | 78.8 | 47.1 | 45.3 | 25.5 | 10.0 | 5.8 | 55.3 | 31.3 |
| Spain | 170.4 | 99.2 | 74.0 | 37.8 | 52.8 | 29.6 | 126.8 | 67.4 |
| Italy | 140.1 | 86.1 | 57.8 | 40.8 | 33.5 | 19.6 | 91.3 | 60.4 |
| Germany | 154.6 | 84.3 | 52.2 | 28.6 | 83.2 | 40.9 | 135.4 | 69.5 |
| Netherlands | 155.6 | 92.7 | 63.8 | 41.0 | 72.4 | 37.9 | 136.2 | 78.9 |
| UK | 108.1 | 72.3 | 40.0 | 24.6 | 38.4 | 22.3 | 78.4 | 46.9 |
| Denmark | 141.1 | 88.3 | 69.6 | 44.1 | 51.9 | 25.3 | 121.5 | 69.4 |

*Total meat includes pork, beef, veal, lamb/mutton, poultry, game, rabbit, horse, goat and offal

However, in the last decades, the preference of Western consumers towards food including meat and meat products has been changing consistently. In fact, they started seeing foods not only as means to provide necessary nutrients to their bodies, but also as tools to improve their physical health and mental well-being (Menrad, 2003).

Meat in itself is a major source of many nutrients, some of which are exclusive of meat or have higher bioavailability compared to other sources, thus playing a crucial role in maintaining human health. On the other hand, consumers have started associating meat and meat products with a more negative image. This is mainly due to their contents of fat, saturated fatty acids, cholesterol, sodium and nitrite, which have been associated with an increased risk to develop chronic diseases like obesity, cardiovascular disease, diabetes

mellitus and some types of cancer (Arihara, 2006). Consequently, in the last decade, the increasing demand from Western consumers towards healthier food products has pushed the meat industry to improve the image of meat and meat products through developing new strategies to optimize their nutritional composition (Toldrá & Reig, 2011). These strategies aim to affect the presence of a certain bioactive compound so that it can be assimilated or not by the organism and be present or not in significant amount in the final product, in order to provide the desired effect with the ingestion of a reasonable amount. When this effect is scientifically proved, the use of nutritional claims and claims of healthful properties are authorized (Regulation EU 1924/2006; Regulation EU 432/2012).

Regarding meat, there are three main strategies to develop functional meat and meat products:

a) Modification of the carcass and meat composition

Carcass fatness can be reduced and fatty acid profile of the meat can be improved through genetic selection of specific races and lines, or also by using genetic markers that allow to identify loci which control the expression of quantitative traits (Navajas & Simm, 2004). Moreover, feeding management in pigs demonstrated that it is possible to achieve leaner carcasses and higher proportion of lean to fat by reducing the energy level of the diet (Bee, Geber, & Messikommer, 2002). Dietary strategies can improve the fatty acid profile of the meat in monogastric animals by increasing the proportion of unsaturated fatty acids (Raes, De Smet, & Demeyer, 2004; Dalle Zotte & Szendrő, 2011). However, a higher presence of unsaturated fatty acids renders meat more prone to oxidation thus, for example, supplementing the diet with an extra dose of vitamin E could prevent this undesired phenomenon (Dalle Zotte, Cossu, & Parigi Bini, 2000; Guo, Richert, Burgess, Webel, Orr, Blair, Grant, & Gerrard, 2006). Also some useful minerals can be successfully supplemented trough diet (selenium, copper, iron), thus increasing their content in the meat (Zhang, Xiao, Samaraweera, Lee, & Ahn, 2010; Szendrő, Gerencsér, Szabó, Fébel, Szín, Radnai, Dalle Zotte, & Matics, 2012; Olmedilla-Alonso, et al., 2013). The use of growth-promoting and nutrient partitioning techniques, as well as immunization of animals against target circulation hormones or releasing factors, can modify metabolic processes that regulate the use of nutrients during growth and thus promoting, for example, protein synthesis and reducing fat deposition (Jiménez-Colmenero, Carballo, & Cofrades, 2001). Finally, also terminal sire line, age at slaughter, gender and castration can affect carcass composition (Latorre, Medel, Fuentetaja, Lázaro, & Mateos, 2003; Pauly, Spring, O'Doherty, Ampuero Kragten, & Bee, 2009).

b) Manipulation of meat raw materials

It is also possible to achieve healthier meat and meat products during transformation of muscle into meat, as well as during the handling of raw materials and preparation of meat products. This can be achieved mainly through physicochemical processes such as extensive trimming to remove the internal and external fat from the carcass, reducing the meat size particles before product making and then extract or separate lean and fat by cryoconcentration, centrifugation and decantation (Jiménez-Colmenero, et al., 2001).

c) Reformulation of meat products

During preparation of the product, it is possible to reformulate it by reducing some compounds normally present in a specific product to appropriate amounts (fat, salt, nitrites, etc), or by incorporating ingredients which are health-enhancing like fibre, antioxidants, MUFA, PUFA, vegetable proteins, probiotics and lactic acid bacteria (Zhang, et al., 2010). The possible strategies in this sense are reduction of fat, calories, sodium, cholesterol, nitrites, modification of the fatty acid profile by replacing part of the animal fat with another more suitable to health needs, and incorporation of functional ingredients (Arihara, 2006).

*4. The Ostrich: general information and digestive physiology*

The ostrich (*Struthio camelus*) belongs to the *Ratitae* order, flightless birds which includes also emu, cassowary rhea and kiwi. The ostrich is the largest of all birds living worldwide reaching 2.7 m in height and exceeding 150 kg of bodyweight in males. Females are smaller, reaching only about 2 m in height and 120 kg in live weight. Even if they can't flight, wings play several important functions such as fanning in hot days, defense and, in males, they are used during nuptial dances. Sexual dimorphism is evident in the adult animal, as males are typically black with feathers and tail ending in white whereas females are brown-grey colored (Deeming, 1999). Moreover, during breeding season, which starts in spring, an increased secretion of sexual hormones reddens regions near the beak as well as the surface of the shins. Young ostriches are fully grown at 16-18 months of age and reach sexual maturity when they are 4-5 years old.

In the wild, the ostrich spends most of the day searching food that normally is meager vegetation as they live in arid or semi-desert areas as well as grasslands (Horbańczuk, 2002). Ostriches are selective grazers/browsers and they commonly feed on succulents, grasses, seeds, new leaves and shoots from shrubs and trees, but also berries, roots, depending on feed

availability. Ostriches tend to avoid plant species rich in fat, phenolics, sodium, calcium oxalate and grasses taller than 1 m. Sometimes ostriches can eat also small insects and snails, but they seem not to be part of their common diet as ostrich was observed to lack trahalase, which is an enzyme involved in the digestion of trahalose, an insect-based carbohydrate (Cilliers & Angel, 1999). In order to supply their calcium requirements, they also ingest bones, eggshells and seashells (Sales, 2006). Despite their similarities to other birds, ostriches have evolved unique characteristics that allow them to survive in their natural habitat. As an example is the modifications of their gastrointestinal tract, which is distinct from that of other birds, and consequently of its functional characteristics which render it is very efficient in extracting nutrients and energy (Cooper, Erlwanger, & Mahroze, 2005). Like other higher animals, ostrich lacks cellulase thus they need to ferment plant fibre. The ostrich is a monogastric herbivore and, in adult age, it can utilize forage because it is a hind gut fermented like rabbit, horse or donkey (Aganga, Aganga, & Omphile, 2003). Thus, in order to let the microbes colonize and reproduce in the gastrointestinal tract, a slow rate of passage of the *digesta* is necessary. In fact, passage rate in the ostrich, which is between 39 and 48h depending on live weight, is similar to that of ruminants and they are the best post-gastric fibre fermenters among birds (Cilliers & Angel, 1999). The gastrointestinal tract of the ostrich begins with the beak, which is wide and flat, and continues with the esophagus which allows the accumulation of feed during the meal. Differently to other avian species ostriches don't have a crop, which is a feed storage organ. At the end of the esophagus the *ingesta* reaches the glandular stomach (proventriculus), which secretes enzymes and acids for the digestion. Subsequently, the muscular stomach (gizzard) grinds big particles in smaller ones thus favoring the activity of digestive enzymes. This process is favored by the presence of grits that are swallowed by the bird and normally present in this site. Afterward, the *ingesta* moves to the small intestine where intestinal juice is secreted by many glands. In that site, the presence of intestinal villi facilitates the partial absorption of nutrients. Two large caeca protrude from the end of the small intestine in which the digestive content is mixed and fermentation is stimulated. The large intestine, which can be longer than 10 meters in the adult bird, is where water is absorbed and also where the microbial fermentation takes place and allows the digestion of cellulose and hemicelluloses leading to the formation of volatile fatty acids. Volatile fatty acids, mainly acetic, propionic, butyric and valeric acid are then absorbed and metabolized as an energy source that was estimated to provide the 76 % of total metabolizable energy requirements of the growing ostrich (Swart, Mackie, & Hayes, 1993a). Overall mean passage rate is independent of live mass as well as digestibility coefficients for

NDF, hemicelluloses and cellulose which are 47, 66 and 38 %, respectively (Swart, Mackie, & Hayes, 1993b). The digestive tract ends in the cloaca, which has a special structure leading to separate defecation and urine excretion.

Within ostrich species, the commercial name of the main subspecies are Red Neck (*Struthio camelus camelus* and *Struthio camelus masaicus*), Blue Neck (*Struthio camelus molybdophanes* and *Struthio camelus australis*) and African Black (*Struthio camelus* var. *domesticus*). The latter is a strain produced by selective breeding from *Struthio camelus australis*, which was traditionally living from the south of Zambesi river to the Cape Region of South Africa, and *Struthio camelus camelus*, which ranged from the south of Atlas including Senegal, Nigeria, Sudan and Ethiopia (Horbańczuk, 2002). Adult African Blacks weigh about 115 kg, they are shorter, smaller and with darker feathers compared to the other subspecies, together with being more docile and manageable, as a result of a longer domestication process, thus being ideal candidates for ostrich farming. Even if it is the Black genotype which has higher reproduction performance and better chick survival than the other subspecies, the Zimbabwean Blue (*Struthio camelus australis*) showed the highest growth rate. The crossbreed between these two subspecies showed to produce larger birds with more meat, without negatively affecting chicks survival and the sensory quality of the meat (Hoffman, Muller, Cloete, & Brand, 2008).

### *4.1. Ostrich market*

Ostrich farming for commercial purposes began in South Africa between 1838 and 1866 (Mosenthal & Harting, 1897) when African Blacks were bred for the excellent quality of their feathers. After the collapse in the feather market in 1914 due to the First World War, the ostrich industry gradually changed to an intensively managed farming activity and in 1970 it was mainly focused on leather production (Brand & Jordaan, 2011). The interest in meat production started growing since the mid nineties, as a consequence of the outbreak of BSE (Bovine Spongiform Encephalopathy) and foot and mouth disease in Europe in 2000. The combination of these "food scandals" and an increasing demand for healthier foods by consumers, led to a great increase in the demand and consequently price for ostrich meat (Figure 2).

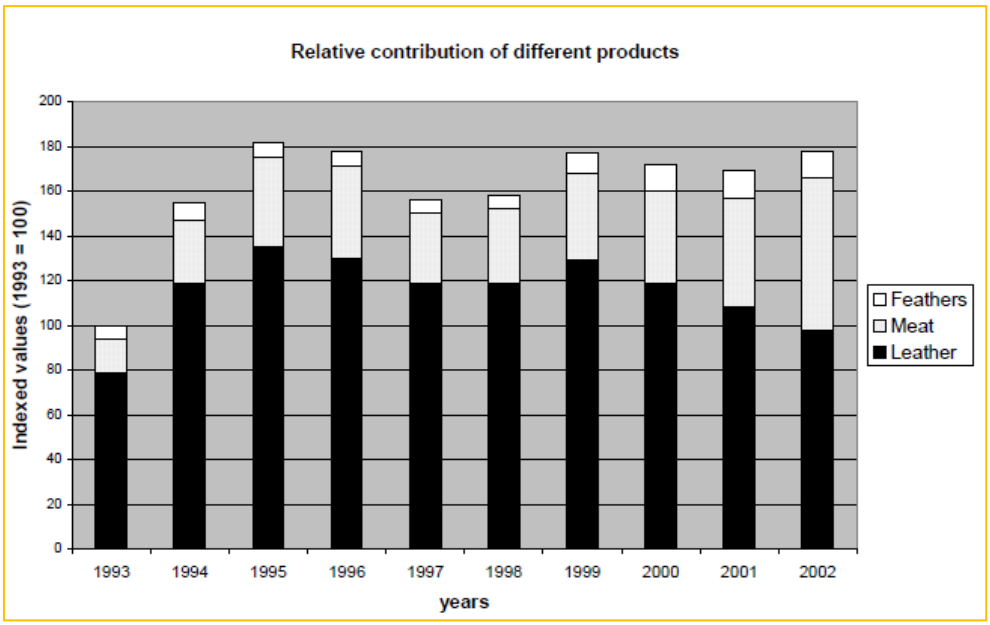



**Figure 2**. Indexed prices for ostrich skin, meat and feathers for the period 1993 - 2002 (NAMC, 2003).

Nowadays ostriches are successfully farmed all over the world (Africa, Australia, Asia, North and South America, Europe) with interesting meat production capacity that exceeds that of farmed sheep, deer and cattle, thus representing an interesting alternative red meat (Cooper & Horbańczuk, 2002). At this time, South Africa produces about 70% of all ostrich meat, leather and feathers on the world market. Western and Eastern Europe (13%) are the nearest competitors followed by Eastern countries (8%), Australasia (7%), North and South America (5%) (South African Ostrich Business Chamber, 2002) (Figure 3). In 2008 the income generated from feathers, leather and meat accounted for 5%, 50% and 45%, respectively (Hoffman et al., 2008), but meat sector has been consistently growing also in the last years and it is now the first source of profits for ostrich farming industries, accounting for about 62% of total incomes (Abolnik, Fehrsen, Olivier, van Wyngaardt, Fosgate, & Ellis, 2013). In particular, it is the huge increase in the export sector, which accounts for the 90% of all produced meat, that is leading South African meat industry to this consistent growth (Leygonie, Britz, & Hoffman, 2012). Between 60% and 70% of the exported meat is in fresh state and most of it is exported to Europe, which is responsible for the 60-70 % of global consumption of ostrich meat (Cooper, Tomasik, & Horbańczuk, 2007).

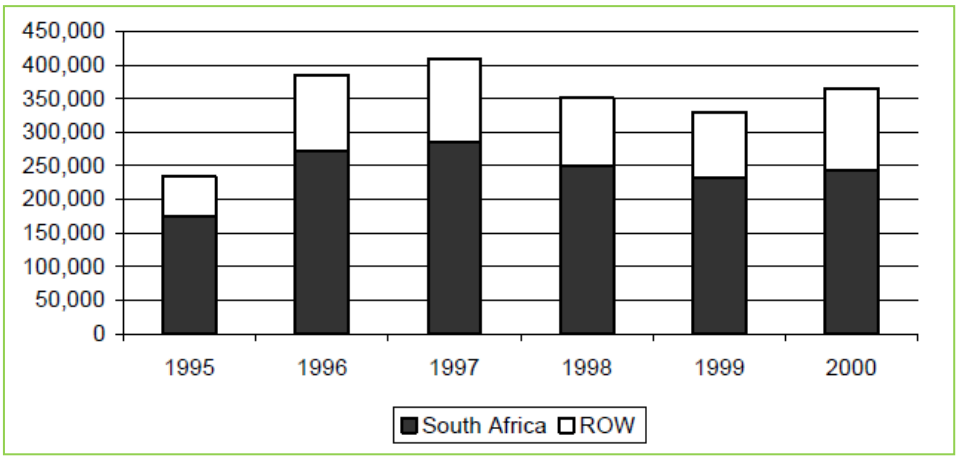



**Figure 3**. Number of ostrich slaughterings in South Africa compared to the rest of the world (ROW) for the period 1995 – 2000 (South African Department of Agriculture, 2005)

### *4.2. Ostrich farming, meat chemical composition on FA profile*

In South Africa, ostriches are mainly reared under intensive feedlot systems (80%) or under semi-intensive grazing conditions (20%), thus nutrients are provided by means of formulated feeds and concentrates (Brand & Gous, 2006). In feedlots, feed and clean water should be supplied *ad libitum* in order to aid digestion because ostriches need to feed continuously rather than in separate time slots (Aganga et al., 2003). As the successful rearing of ostriches from hatching to grower to breeder birds requires high standards of nutrition, the latter represents about the 80% of total ostrich production costs with protein source accounting for a large part of them (Brand & Olivier, 2011). For this reason, current research on ostrich farming is looking for alternative protein sources, cheaper than the common used soybean meal, that do not negatively affect growth performance of the animals (Dalle Zotte, Brand, Hoffman, Schoon, Cullere, & Swart, 2013).

Ostriches are commonly slaughtered at 12-14 months of age, when the best meat, leather and feathers quality are reached. At this stage, cold carcass yield of the ostrich is between 49 and 59 % for both males and females, depending on strain and slaughtering methods (Girolami, Marsico, D'Andrea, Braghieri, Napolitano, & Cifuni, 2003). Being ostriches flightless running birds, breast muscles are vestigial to non-existent, thus almost all the saleable meat is located in the hind limbs. Meat cuts (Figure 4) correspond to each single muscle that composes each region and they can be clearly defined as their limits are easily identifiable by muscle fascia (Balog & Almeida Paz, 2007). Fan fillet (*Iliofibularis* muscle) is the muscle with the highest income per kilogram, followed by Triangular fillet (*Iliofemoralis*

muscle) and the Rump Steak (*Iliotibialis lateralis* muscle). Other muscles which can be found in the ostrich meat market include the Big drum (*Gastrocnemius* muscle), Top Loin (*Iliotibialis cranialis* muscle), Outside strip (*Flexor cruris lateralis* muscle), Tender Loin (*Obturatorius medialis* muscle), Tip (*Femorotibialis accessorius* muscle) and Mid Leg (*Fibularis longus* muscle) (Hoffman et al., 2008).

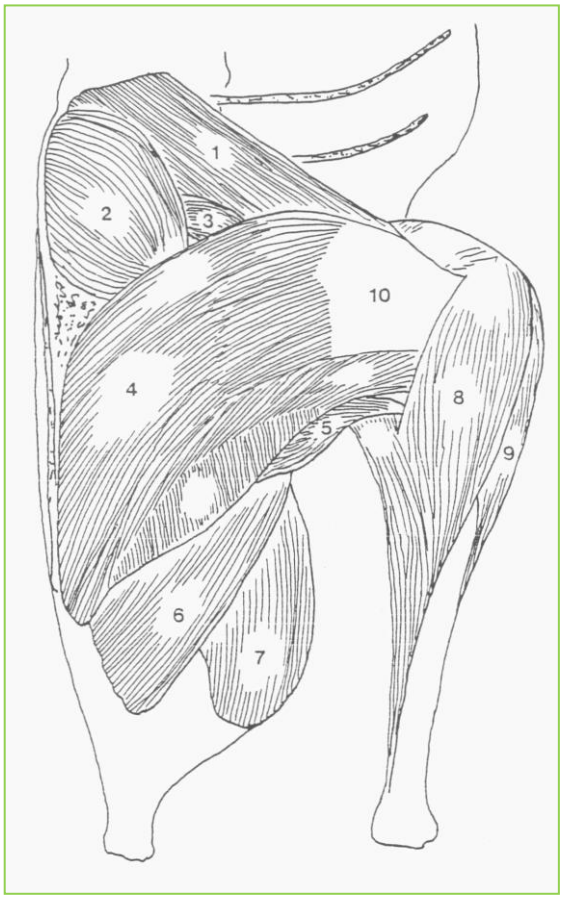



**Figure 4.** 1 List of hind limb muscles: *Iliotibialis cranialis*; 2 *Iliofemoralis externus* ; 3 *Ambiens* ; 4 *Iliotibialis lateralis* ; 5 *Iliofibularis* ; 6 *Flexor cruris lateralis* ; 7 *Obturatorius medialis* ; 8 *Gastrocnemius* ; 9 *Fibularis longus* ; 10 *Femorotibialis medius* (Mellet, 1992)

Ostrich meat is gaining more and more interest as a livestock species as it can produce high quality healthy red meat (Table 3). Compared to meat from other species, ostrich meat is characterized by a high ultimate pH which allows to classify it as an intermediate meat (between normal: pH<5.8 and high: pH>6.0) and which renders it optimal for processing thanks to the naturally high water holding capacity. On the other, hand a high pH represents a critical point for quality deterioration as ostrich meat is highly perishable (Fernández-López, Jiménez, Sayas-Barberá, Sendra, & Pérez-Alvarez, 2006).

As a consequence of the high ultimate pH and pigment content, ostrich meat is dark red colored, ranging from dark red to cherry red.

**Table 5.** Proximate composition (g/100 g edible portion) of different ostrich muscles (adapted from Majewska et al., 2009)

| Muscle | Dry matter | Protein | Intramuscular lipids | Ash |
|---|---|---|---|---|
| *Gastrocnemius pars interna* | 23.3 | 21.3 | 0.90 | 1.10 |
| *Fibularis longus* | 23.3 | 21.5 | 0.88 | 1.12 |
| *Obturatorius medialis* | 24.5 | 21.7 | 1.22 | 1.14 |
| *Flexor cruris lateralis* | 24.2 | 21.5 | 1.44 | 1.14 |
| *Iliofibularis* | 22.8 | 20.7 | 1.10 | 1.07 |
| *Femorotibialis medius* | 23.0 | 20.8 | 0.95 | 1.15 |
| *Iliotibialis lateralis* | 23.8 | 21.4 | 1.21 | 1.17 |
| *Iliofemoralis externus* | 23.8 | 20.7 | 1.22 | 1.14 |
| *Ambiens* | 24.1 | 21.3 | 1.34 | 1.09 |
| *Iliotibialis cranialis* | 24.4 | 20.6 | 1.36 | 1.13 |
| Mean | 23.7 | 21.2 | 1.16 | 1.12 |

Ostrich meat is appreciated for its very low intramuscular lipid content, with a relatively high presence of polyunsaturated fatty acids (PUFA). In addition, carcass leanness is also ensured by the easy separation of fat during processing, as it is mainly located in cavities or subcutaneous (Cooper & Horbańczuk, 2002). The fatty acid profile of ostrich meat varies among different muscles (Sales, 1998), genotypes (Hoffman, Brand, Cloete, & Muller, 2012) and age at slaughter (Girolami et al., 2003). In general, ostrich meat has lower MUFA and higher PUFA contents compared to meat of common species like beef and chicken with a relatively high content of *n*-3 (Balog, & Almeida Paz, 2007). Among SFA, palmitic (C16:0) and stearic fatty acids (C18:0) are the most abundant, whereas oleic (C18:1) and palmitoleic (C16:1) fatty acids are the most present MUFA. Considering PUFA, the content of linoleic acid (C18:3 *n*-6) is the highest, followed by that of the α-linolenic acid (C18:3 *n*-3). For this reason, Hoffman et al. (2012), observed a very low *n*-6/*n*-3 ratio, ranging from 1.6 to 2.2 in *Gastrocnemius* and *Iliofibularis* muscles of South African Black and Zimbabwean Blue Neck ostriches. In addition, being the ostrich a monogastric herbivorous, the already good fatty acid profile of its meat can be further improved by dietary means, as it was successfully demonstrated in different studies (Sabbioni, Superchi, Sussi, Quantarelli, Bracchi, Pizza, Barbieri, Beretti, Zanon, Zambini, & Renzi, 2003; Lanza, Fasone, Galofaro, Barbagallo,

Bella, & Pennisi, 2004; Polawska, Horbańczuk, Pierzchala, Strzalkowska, Jóźwik, Wójcik, Pomianowski, Gutkowska, Wierzbicka, & Hoffman, 2013). An example of the fatty acid composition of some ostrich muscles is showed in the following Table (Table 6).

**Table 6.** Fatty acid composition (% of total fatty acids) of *Gastrocnemius* and *Iliofibularis* muscles from different ostrich genotypes (adapted from Hoffman et al., 2012)

| Fatty acids | Muscles | South African Black | Zimbabwean Blue Neck | Cross |
|---|---|---|---|---|
| C16:0 | *Gastrocnemius* | 18.3 | 17.8 | 17.3 |
| | *Iliofibularis* | 16.5 | 19.9 | 19.2 |
| C18:0 | *Gastrocnemius* | 11.2 | 10.3 | 11.8 |
| | *Iliofibularis* | 11.0 | 10.6 | 10.0 |
| Σ SFA | *Gastrocnemius* | 32.9 | 33.7 | 36.4 |
| | *Iliofibularis* | 30.4 | 38.6 | 35.9 |
| C16:1 *n*-7 | *Gastrocnemius* | 5.6 | 5.0 | 4.5 |
| | *Iliofibularis* | 4.6 | 5.1 | 5.2 |
| C18:1 *n*-9ct | *Gastrocnemius* | 27.6 | 25.3 | 27.1 |
| | *Iliofibularis* | 29.5 | 26.7 | 27.3 |
| Σ MUFA | *Gastrocnemius* | 40.6 | 33.2 | 33.2 |
| | *Iliofibularis* | 41.9 | 34.2 | 34.2 |
| C18:2 *n*-6ct | *Gastrocnemius* | 14.3 | 14.0 | 14.2 |
| | *Iliofibularis* | 16.1 | 13.5 | 14.7 |
| C18:3 *n*-3 | *Gastrocnemius* | 1.7 | 1.7 | 1.5 |
| | *Iliofibularis* | 1.3 | 1.6 | 1.6 |
| C20:3 *n*-3 | *Gastrocnemius* | 7.2 | 8.9 | 6.8 |
| | *Iliofibularis* | 6.5 | 6.9 | 6.1 |
| Σ PUFA | *Gastrocnemius* | 26.5 | 33.2 | 30.4 |
| | *Iliofibularis* | 27.7 | 27.2 | 29.9 |
| *n*-6/*n*-3 | *Gastrocnemius* | 2.0 | 1.6 | 2.1 |
| | *Iliofibularis* | 2.2 | 2.2 | 2.2 |

Another positive characteristic of ostrich meat is its iron content. In a study comparing total heme and non-heme iron contents of different raw and cooked meats (Lombardi-Boccia, Martinez-Dominguez, & Aguzzi, 2002), ostrich fillet showed a higher total iron content compared to some beef cuts, veal, lamb, horse, rabbit and pork (2.43 mg/100 g meat) with heme iron content being similar to that observed in beef fillet and horse. Interestingly, differently from other species and similarly to pork chump chop, the percentage of heme iron in cooked meat was higher than that observed in raw meat (+4 %). This means that heme iron doesn't dissociate to non-heme iron during heating, thus having a higher bioavailability as a nutrient and less availability to initiate oxidative processes (Hoffman, 2008). For these reasons, ostrich meat could be an important iron source for anemic patients and for pregnant women. Ostrich meat has also a low sodium (Table 7) content, thus being an ideal red meat for all kind of consumers, including people susceptible to hypertension.

**Table 7.** Mineral composition (mg/100 g edible meat) of different ostrich muscles (adapted from Majewska et al., 2009)

| | Mineral composition | | | | | |
|---|---|---|---|---|---|---|
| Muscles | Ca | K | Mg | Na | P | Fe |
| *Gastrocnemius pars interna* | 5.45 | 243 | 24.3 | 36.1 | 216 | 2.88 |
| *Fibularis longus* | 5.94 | 245 | 23.8 | 33.4 | 220 | 3.14 |
| *Obturatorius medialis* | 5.15 | 244 | 25.3 | 38.7 | 224 | 3.04 |
| *Flexor cruris lateralis* | 5.18 | 238 | 25.4 | 33.9 | 228 | 4.02 |
| *Iliofibularis* | 5.62 | 240 | 23.8 | 32.6 | 210 | 2.32 |
| *Femorotibialis medius* | 5.01 | 256 | 24.3 | 32.1 | 222 | 2.49 |
| *Iliotibialis lateralis* | 5.40 | 254 | 24.6 | 32.0 | 218 | 3.22 |
| *Iliofemoralis externus* | 4.76 | 237 | 24.6 | 31.4 | 218 | 3.45 |
| *Ambiens* | 6.03 | 234 | 23.3 | 39.0 | 211 | 3.76 |
| *Iliotibialis cranialis* | 5.80 | 244 | 25.1 | 35.4 | 223 | 3.14 |

The low intramuscular fat content and high pHu make the sensory profile of ostrich meat very peculiar, being very tasty, mild and slightly sweet due to its high muscle glucose content (Paleari, Camisasca, Beretta, Renon, Corisco, Bertolo, & Crivelli, 1998). Moreover, the low saturated fat and collagen (0.44%) contents together with the transversal orientation of muscle fibers, explain why ostrich meat is so tender, easy to chew and to digest. For all the above mentioned reasons, ostrich meat is considered interesting to the consumer and different to that of beef (Balog & Almeida Paz, 2007).

The similar protein content of ostrich meat compared to that of other meat species (Sales & Hayes, 1996), the low intramuscular fat content and positive fatty acid profile, as well as low sodium and high heme iron contents combined with the possibility to further improve the nutritional characteristics of the meat through diet, allow ostrich meat to be marketed and sold as a delicacy all over the world.

### *4.3. Current research on the quality of ostrich meat and meat products*

As almost all ostrich meat is currently sold in its fresh state and due to the sharp growth of the ostrich meat market in the last decade, the majority of the studies in this field dealt with dietary strategies to improve the nutritional composition of the meat (Lanza *et al.*, 2004; Hoffman, Joubert, Brand, & Manley, 2005) as well as testing packaging techniques to improve its shelf-life. An example is the study by Seydim, Acton, Hall, & Dawson (2006), who observed that packaging in high oxygen reduced the shelf-life of ground ostrich meat to less than 3 days, with lipid oxidation being the limiting factor. For the same reason, also differently packaged meat (high nitrogen, air and vacuum) was below sealable quality in less than 6 days. Similar trials were performed by Fernández-López, Sayas-Barberá, Muñoz, Sendra, Navarro, & Pérez-Alvarez (2008) on fresh ostrich steaks and by Leygonie, Britz, & Hoffman (2011) on fresh ostrich *Iliofibularis* meat. In the first study, the combination of modified atmosphere packaging (MAP) with carbon monoxide (30 % $CO_2$ + 68 % argon + 0.2 % CO) provided the best results in terms visual appearance, lipid oxidation and microbial quality of the product. In the second study, MAP packaging (30 % $CO_2$ + 70 % $N_2$; 30% $CO_2$ + 70 % $O_2$) decreased the amount of drip loss, thus improving the appearance of the product. However, in oxygen MAP high oxidation degree was observed, whereas nitrogen MAP showed similar oxidative results to simply overwrapped steaks.

In order to diversify the production, as well as trying to conquer new niches in the market of "healthy" meat products, value-added products from ostrich meat are a viable option for the industry that is still mainly focused in the production of fresh meat which is then cooked, grilled or dried (biltong). In fact, as it was mentioned above, the relatively high pH of ostrich meat renders it optimal for processing thanks to its natural high water holding capacity (Fisher, Hoffman, & Mellett, 2000). Moreover, the high pH renders ostrich meat very perishable, hence the need for more research on new value-added products. This research field is so recent that the first research evaluating the quality characteristics of ostrich meat as an ingredient in quality comminuted meat products such as burgers, were those published by Fernández-López et al. (2006). The results of this study indicated that ostrich meat was a

viable option for the industry and that burgers formulated with 100% ostrich meat or mixed ostrich and beef meats were the most satisfactory in terms of healthiness of the product as well as for preference of the panelists. However, the microbial load of ostrich burgers was the negative drawback of this experiment, thus requiring further research in preservatives and antioxidants to improve the quality of the product.

The first research aiming to create a value-added meat product based on ostrich meat was that by Böhme, Mellett, Dicks, & Basson (1996). They used ostrich meat in the production of an Italian-type salami and tested the effect of two different starter cultures of lactic acid bacteria on product quality. Results of the experiment were satisfactory and sensory evaluation of the final products stated that texture and sensory quality of the different salami were very good. Moreover, panelists could clearly distinguish between salami with and without added starter culture. Subsequently, in a similar trial Dicks, Mellet, & Hoffman (2004) focused on the safety aspect of ostrich meat salami, as ostrich meat is intrinsically prone to bacterial spoilage. In this experiment, they inoculated ostrich salami with *L. monocytogenes* and tested the effectiveness of two selected bacteriocin-producing starter cultures of *Lactobacillus plantarum* and *Lactobacillus curvatus* against this pathogen. Results of the experiment showed that both starter culture, but especially that with *L. plantarum*, effectively inhibited the growth of high cell numbers of *L. monocytogenes* for 9 days at 16-18 °C. Other studies focused in establishing if processed ostrich meat products had similar acceptability scores and technological properties compared to common meat species, and dealt with chopped hams and viennas (Fisher et al., 2000), Bologna sausage (Fernández-López, Sayas-Barberá, Navarro, Sendra, & Pérez-Alvarez, 2003), low-fat ostrich meat patties formulated with either pork lard or modified corn starch, soya isolate and water (Hoffman & Mellett, 2003), ostrich liver pâté (Fernández-López, Sayas-Barberá, & Pérez-Alvarez, 2004), Spanish ostrich salchichon (Soriano, Garćia Ruiz, Gmez, Pardon, & González Viñas, 2007), traditional Thai 'Yor' sausage (Chattong, Apichartsrangkoon, & Bell, 2007), polony, ham and bacon (Schutte, 2008).

Since the mid nineties, consumers have been expressing concerns about the safety of synthetic preservatives and additives in their food, leading to a growing interest for food ingredients and additives that are organic/natural as are generally perceived as healthy and environmentally friendly (Brewer, 2011). Thus, also research has been following this trend.

The first study in this field on ostrich meat is that of Seydim, Guzel-Seydim, Acton, & Dawson (2006), which tested the separated and simultaneous addition of a rosemary extract (0.2% oleoresin) and sodium lactate (2%) in extending the shelf-life of ground ostrich meat.

They observed that the combination of the rosemary extract had synergistic effect with sodium lactate and retarded the oxidative deterioration of the product and limiting the microbial growth, thus extending the shelf-life of ostrich meat by 3 days. However, color attributes of the product were worst than that made with the single application of the rosemary extract, which nevertheless didn't provide satisfactory results in controlling the growth of microbial flora. As visual appearance (color and loss of exudates) is the first attribute that determines how consumers perceive quality thus influencing purchasing behaviour (Resurreccion, 2003), high quality, healthy and safe meat products that meet consumers expectancies are requisites of utmost importance. Another research studied, for the first time, the application of unfermented and fermented rooibos (*Aspalathus linearis*), a South African tea shrub with health-promoting properties due to its unique polyphenolic compounds, in preventing lipid oxidation in ostrich meat products (Cullere, Hoffman, & Dalle Zotte, 2013). Results of this study showed that unfermented rooibos extract (2%) prolonged the shelf-life of ostrich meat patties and fermented rooibos extract (0.5% and 1%) retarder lipid oxidation of ostrich salami until 15 days of ripening, thus being a promising natural antioxidant in meat products. In addition, Hoffman, Jones, Muller, Joubert, & Sadie (2014) investigated the effect of the same fermented rooibos extract of the above mentioned experiment in ensuring lipid, protein stability and evaluating sensory scores of ostrich droëwors. In this case, even if panelists judged rooibos tea extract a positive attribute, the application of this natural extract didn't simultaneously provide lipid and protein stability, probably due to type and duration of drying method applied to the product, thus requiring further research on this topic.

*5. The rabbit: general information and digestive physiology*

The rabbit (*Oryctolagus cuniculus*) belongs to the *Leporidae* family and *Lagomorpha* order. Compared to the other livestock species, rabbits have been domesticated far later. The wild rabbit of Southern Europe and North Africa is thought to have been discovered by Phoenicians when they reached Spain around 1000 BC. However, the first evidences of controlled breeding dated back to the sixteenth century. At that time, rabbit breeding diffused in France, Italy, Flanders and England and several breeds were known. At the beginning of the nineteenth century, after the abolition of seigneurial privileges, rabbit rearing spread to rural western Europe and, with the European colonial expansion, also to Australia and New Zealand (FAO, 1997).

During the nineteenth century and more intensely during the twentieth century, hutch rearing allowed selection, protection and multiplications of breeds that were unadapted to the wild. Breeders formed associations and breeding techniques and hutch hygiene improved. Following the Second World War, in order to cope with meat shortages, rabbit production massively grew all over Europe and in Japan. Later on, from 1950, rabbit production almost stopped in Japan and northern Europe, as meat with better more acceptable sensory profile was again available. Differently, in Latin countries rabbits were still farmed as their meat had become a traditional dish. In the late fifties, New Zealand White rabbits were introduced from the United States to France and Italy and, for the first time, hutches were put in close buildings. Between 1960 and 1970, rearing techniques improved and New Zealand White rabbits, as well as Californian rabbit, rapidly took place of the traditional European breeds that weren't adapted to live on the mesh floor of cages. French and Italian breeders further improved these two breeds and, in France, the French National Institute for Agricultural Research (INRA) designed the combination of the two breeds to form new specialized hybrid strains. At the end of 1970, these hybrids were diffused to Italy, Spain, Belgium and the Federal Republic of Germany. In the same time, other hybrid strains from the same breeds were produced in Hungary and United Kingdom. Little by little, rabbit farming techniques improved more and more leading to a very rationale and intensive production system, aiming to fully exploit their prolific nature as well as their capability to convert plant proteins into high-value animal proteins.

Rabbits are herbivores with post-gastric digestion, they can utilize a wide variety of feed resources and, considering their feeding behaviour, they are classified as "browsers" or concentrate selectors. Because of their small size, the metabolic rate is high and this limits their ability to live on a low energy concentration diet.

Consequently, the rabbit evolved a complex gastrointestinal physiology that allows a high food intake (65-80 g/kg of body weight), and a rapid transit of feed through the digestive system to meet nutritional requirements. The key factor of the digestive system is the separation of the digestible and easily fermentable part of the diet and the slowly fermentable fibrous waste in the proximal colon, thus the rabbit doesn't need a high absorptive surface area in the large intestine (Davies & Davies, 2003). However, in order to reach its full functional capacity, the digestive system of the growing rabbit must go through a period of adaptation from milk to solid feed (Carabaño, Piquer, Menoyo, & Badiola, 2010). In order to function properly, the digestive system of adult rabbits needs indigestible fibre made of long particle length (>0.5 mm) and many of the gastrointestinal problems of this species derive from unbalanced diets (low fibres, high proteins, high carbohydrates) (Gidenne, 1997). The first important compartment of the digestive system of the rabbit is the stomach, which is never empty and acts as a storage cavity for caecotrophs. In the stomach, which during digestion has a pH between 1 and 2, the hydrolysis of proteins begins. The only exception in this sense is the digestion of the mucin covering of the caecotroph. As they are swallowed without mastication, caecotrophs remain intact within the stomach for several hours after ingestion. This is possible because mucinous coat protects microbes inside the caecotroph that produces lactic acid, which in turn buffers the pH of the stomach that raises to 3 (Davies & Davies, 2003).

The stomach is linked with a coiled caecum by a small intestine approximately 3 m long, where the secretion of bile, digestive enzymes and buffers occurs. As the rabbit has a constantly active digestive system and low protein and carbohydrate intakes, the pancreas is small. However it is fundamental as it completes protein digestion through secretion of various enzymes (Debray, Le Huerou-Luron, Gidenne, & Fortun-Lamothe, 2003). Pancreas also secretes lipases and bicarbonate ions which serve to neutralize the acidic chime entering the small intestine from the stomach. The small intestine, which has a pH close to 7, is the site where the greater part of digestion and absorption take place by passive or active transportation throughout the mucosa. In the duodenum and jejunum of the small intestine most of the digestion of carbohydrates and simple proteins takes place and nutrients are further absorbed. Here also amino acids, volatile fatty acids, vitamins and digested microbial organisms of the caecotroph material are absorbed. Lysis of the microbes within the caecotrophs also releases microbial enzymes, notably amylase, which enhances the rabbit's own digestive process.

Digestibility at the end of the ileum accounts for 0.8-1 of the total dietary amino acid and starch. The rabbit's caecum, whose content is slightly acid (pH 5.4-6.8), is proportionally the largest of any mammal and represents approximately 0.49 of the total capacity of the gastrointestinal tract. The caecum is an anaerobic fermentation chamber for organisms where the presence of the *ingesta* and the secretion of mucopolysaccarides contribute to fermentation (Davies & Davies, 2003). The microbial flora of the caecum breaks down ammonia, urea, proteins, enzymes and cellulose and metabolizes also xylans and pectins. As a result, the protein and enzymes structures of the microbes themselves will then be digested as cecotrophs. In addition, also volatile fatty acids (VFA) are generated from fermentation (acetic, formic, propionic and butyric acids) and absorbed through the caecum and colon walls which will then serve as energy sources. The proportion among different VFA is quite different in the rabbit compared to other animals with butyric acid which normally exceeds propionic acid: 60-70 % acetic, 15-20 % butyric, 10-15 % propionic volatile fatty acid. At the appendix of colon, water is continually secreted and added to the cecal contents and then water is re-absorbed across the wall.

Caecotrophy in rabbits does not occur as a response to a nutritional imbalance, but represents a specialized digestive strategy. Caecotrophy begins at 3–4 weeks of age, when rabbits begin to consume solid food. In post-weaned rabbits (4 weeks old), soft faeces (caecotrophs) production linearly increases with age, reaching a maximum at 63–77 days old (25 g DM day−1). This period corresponds to the maximum growth requirements and to the greatest increment in feed intake (Carabaño, Piquer, Menoyo, & Badiola, 2010).

Caecotrophes, which are produced from the partially fermented matter of the cecum, are ingested by the rabbit directly from the rectus as a result of a neurologic licking response, and are swallowed without being chewed. Caecotrophy is affected by light, ingestive patterns and varies between wild and captive rabbits. As feed intake patterns are inversely related to caecotroph production, in captive rabbits caecotrphy occurs during the night and it usually follows about 4 hours after ingestion of food (Davies & Davies, 2003). Caecotrophs have about 50% of the crude fiber level of the faeces and, regardless of the level of fiber in the diet, the ratio between hard faeces and caecotrophes is quite constant. If dietary protein is reduced, the protein level in the hard faeces drops whereas that of the caecothrophs remains the same. The fundamental part of the rabbit's digestive process is the regulation of colon and caecum motility which allows the separation of the intestinal content in fermentable substrates (caecotrophs or soft faeces) and indigestible wastes (hard feces).

The secretion of water by the proximal colon helps the mixing and separation of these two contents which is driven by different contraction types. As a result, indigestible fibers greater than 0.5 mm length accumulate in the central lumen of the proximal colon, whereas smaller particles are moves to the periphery where they congregate. Fibers wastes in the central lumen of the proximal colon, passes rapidly distally and forms feces by the physical compression of the *fusus coli*. Subsequently, they are expelled as small, hard and dry feces. Differently, digestible components and fluid are passed by retrograde peristalsis back up the colon and caecum for fermentation. Then caecal content, which is rich in semi-digested food material and microorganisms, passes rapidly through the colon. In this case, *fusus coli* contractions are gentle and don't expel the fluid from the pellets which are then added with lysozyme and covered with mucus in the distal colon. Caecotrophes arrive in the anus and they are directly ingested by the rabbit as a response of rectal mechanoreceptor stimulation, olfactory stimuli and blood concentrations of various metabolites and hormones (Davies & Davies, 2003).

### *5.1. Rabbit farming and diet*

Since 1970, in the main European rabbit producing Countries (Italy, Spain and France), rabbit farming progressively became an intensive system organized as vertical integration, which was characterized by intensive breeding programs and which lead to a general improvement of nutrition and management practices (Petracci & Cavani, 2011). Moreover, at the end of each productive cycle, it was possible to properly clean cages and structures which allowed a better control of diseases and this significantly improved the average health status of the during the productive cycle. In addition, feed could be easily provided and animal growth could be better monitored. Finally, this farming system was positive also from the economical point of view, as it ensured high fertility and productivity rates, satisfactory conversion indexes, relatively low use of antibiotics and it allowed sales planning (Marongiu, 2008). This intensification led to a sharp increase in the size of rabbit companies and a parallel decrease in the number of rabbit producers (Lebas, Coudert, Rochambeau, & Thébault, 1997).

Nowadays, rabbit can be farmed in "close cycle" or "specialized" rabbitries. In the first ones, as it suggests the term, the whole farming process takes place within the same rabbitry: males are kept for artificial insemination, rabbit does give birth to kits, and weaned rabbits which are reared until they reach the slaughter weight. Differently, in specialized farming, only one phase of the productive cycle (reproduction or fattening) is present in the

same farm (Marongiu, 2008). In Italy and Hungary, meat producing rabbits are typically fattened in cages by pair, whereas in France, Spain, Portugal and USA rabbits are usually reared in small groups of 6 to 10 rabbits (Dalle Zotte, Princz, Metzeger, Szabó, Radnai, Biró-Németh, Orova, & Szendrő, 2009).

For what concern the diet, fibers are the main constituent of a feed for growing rabbits and it can range from 15 to 50 %. Specifically, a certain amount of low-digestible fibre as well as digestible fibre such as pectins and hemicelluloses is necessary in order to prevent digestive disorders (Gidenne, Kerdiles, Jehl, Arveux, Briens, Eckfendler, Fortune, Montessuy, Muraz, & Stéphan, 2001). Thus, in order to provide an adequate nutrients intake ensuring both satisfactory productive performances and gastrointestinal health, a diet must contain a minimum quantity (from 140 to more than 200 g/unit) and quality (lignin/cellulose ratio) of lignocelluloses, an appropriate digestible fibers/lignocelluloses ratio (from 30 to more than 40) and the right quantity of starch (fless than 140 g/unit until 45 days and less than 180 g/unit at the end of fattening). Interestingly, some studies showed that digestible fibers such as hemicelluloses and pectins are highly utilized for growth by rabbits and that, when the diet is equilibrated also for lignocelluloses, they can improve the digestive health of the animal (Perez, Gidenne, Bouvarel, Arveux, Bourdillon, Briens, La Naour, Messager, & Mirabito, 2000; Gidenne, 2003).

### *5.2. The market of rabbit meat*

Rabbits are mainly used a meat source even if they can also be used to produce fur or as companion rabbits. Europe is the first producer of rabbit meat in the world with more than 528,000 tons/year but, if considering single Countries, China is the World leader with 440,000 tons/year (Table 8). In Europe the distribution of the production is very heterogeneous as well as farming systems. Italy, Western France, Mediterranean Spain, Northern Portugal, Belgium and The Netherlands have very intensive and specialized productions (500-1000 does/farm), whereas in other Countries like Southern France, Southern Portugal and Northern Spain farms are small and semi-intensive in which the farmer often takes care also of the reproduction. In Europe there are also Countries where only rural farming is present like Germany, Eastern and Middle France and Southern Spain. Finally, in Northern Europe, rabbit farming is almost non-existent, mainly due to cultural reasons as rabbit is more associated to a pet rather than a meat source. In Europe, the first producer is Italy (44% of the whole European production), followed by Spain, France, Czech Republic and Slovakia, Germany and others (Table 9).

Europe accounts for the 80% of the world imports for rabbit meat, with Italy, Belgium, France and Germany being the main importers. Differently, rabbit meat is mainly exported by China and Eastern-Europe. Rabbit meat is consumed in many European (Malta, Cyprus, Italy, Czech Republic, Spain, Belgium, Luxembourg; Portugal and France) and in certain North African (Egypt and Algeria) Countries (Dalle Zotte & Szendrő, 2011). Even though in the Mediterranean area rabbit meat has a rich history of traditions, still nowadays there are few traditional further-processed products. The reason for this situation can be found in historical traditions that associate rabbit meat consumption with special occasions. As a consequence of the concentration of the population in urban centers and the resulting development of large scale retailing, rabbit meat started to be sold as pre-packed entire carcasses and also portioned in main cuts (loin and hind legs). However, rabbit meat market never developed to further-processed meat products, like it happened for poultry products (Cavani, Petracci, Trocino, & Xiccato, 2009).

**Table 8.** Major producers of rabbit meat worldwide (retrieved from FAO, ISTAT, AVITALIA)

| Country | Production (t/year) | Total production (%) |
|---|---|---|
| China | 440,000 | 44 |
| Italy | 230,000 | 19 |
| Spain | 115,000 | 9 |
| France | 85,000 | 7 |
| Egypt | 70,000 | 6 |
| Czech Republic + Slovakia | 39,000 | 3 |
| Germany | 33,000 | 3 |
| Ukraine | 23,000 | |
| USA | 17,000 | |
| Morocco | 12,000 | |
| Hungary | 11,000 | 9 |
| Belgium | 10,000 | |
| Greece | 5,000 | |
| Others | 7,000 | |

**Table 9.** Major producers of rabbit meat in the EU (retrieved from FAO, ISTAT, AVITALIA)

| Country | Total production (%) |
|---|---|
| Italy | 44 |
| Spain | 22 |
| France | 16 |
| Czech Rep. + Slovakia | 7 |
| Germany | 6 |
| Others | 5 |

When white meat started to be seen as a healthy alternative to red meat, poultry industry made significant investments in the processing area to produce many different processed products such as salami, ham and sausages (Fletcher, 2004). The great advantage for poultry products compared to other meat species, is the absence of cultural and religious obstacles that could limit its consumption, as well as the intrinsic ideal properties of the meat for further processing (bland flavour and soft texture). Moreover, also thanks to the use of mechanically deboned meat which is very economical and suitable for product-making, poultry industry can offer meat at lower and more competitive prices compared to other meat species.

Differently, only few processed meat products from rabbit are marketed (hamburgers, rolls and baby foods) because its cost/price is not competitive compared to poultry products due to higher production costs. Moreover, it is very difficult to produce mechanically deboned meat from rabbit carcasses because the skeleton is susceptible to lose bone fragments. Furthermore, even if there are no religious barriers for rabbit meat consumption, in many Countries rabbits are considered as companion animals (González-Redondo, & Contreras-Chacón, 2012). As a result, nowadays rabbit meat is averagely still considered a niche product because a lot of time is typically needed for correct preparation before cooking, being thus time-consuming and overall not optimal for the modern consumer (Dalle Zotte, 2002).

Thus, in order increase the availability of rabbit meat in the market and meet the demand of consumers, the industry has started focusing on ready-to-eat meals (Cavani & Petracci, 2004). This resulted in slight change in the type of marketed rabbit meat: in 1986, the 98% of rabbit meat was sold as whole carcasses, whereas in 2011 the offer was more diversified.

The market provided 70% of meat that was sold as whole carcasses, 25% were portioned cuts and the remaining % were ready-to-cook products (Petracci & Cavani, 2011). However, as rabbit meat is more and more becoming a marginal food also in traditionally meat consuming countries, in the next future new strategies to increase or at least maintain rabbit meat consumption will be necessary. Such strategies should focus on the introduction of rabbit meat as an ingredient in ready-to-cook meals to meet the demand of modern consumers, as well as to design new further-processed meat products which should focus also in improving the appearance and sensory traits of the meat (Dalle Zotte, 2002). In addition, rabbit meat should be produced and marketed with added functionality, thus reinforcing and fully exploiting its naturally healthy profile (Dalle Zotte & Szendrő, 2011).

*5.2. Rabbit meat chemical composition and FA profile*

The commercial weight at which rabbits are slaughtered varies depending on the Country and sometimes, within Country, on the region. In Italy the live weight for slaughtering rabbits ranges from 2.2 kg of Naples to 3 kg of Cuneo, with an average carcass weight of 2.5 kg. Differently, in Spain carcass weight is between 2.0-2.2 kg, whereas in France it is 2.3-2.5 kg. From the rabbit carcass four main cuts can be obtained, three are first retail cuts and one is a second retail cut. The most quantitatively important are the hind legs, the leanest meat cut is the *Longissimus dorsi* muscle (loin prime cut), the fore legs are the fattest portion (8.8 g/ 100 g meat) (Hernández & Dalle Zotte, 2010), whereas the thoracic cage is the second retail cut (Figure 5).

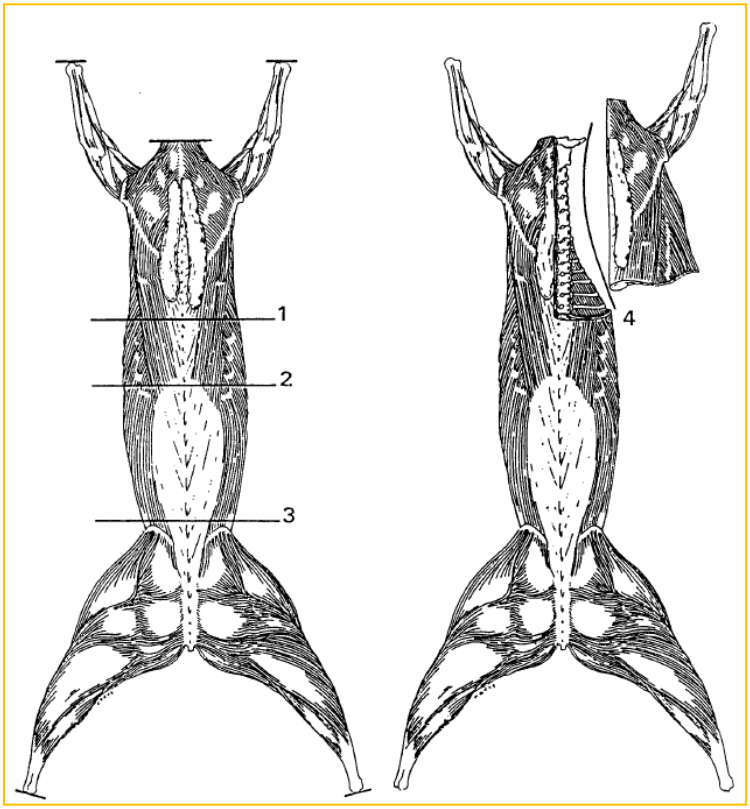



**Figure 5**. Carcass separation according to anatomical division (cutpoints 2 and 3) and technological division (cutpoints 1, 3 and 4). **Cutpoint 1**: section between the 7$^{th}$ and 8$^{th}$ thoracic vertebra, following the prolongation of the ribs when cutting the thoracic wall. **Cutpoint 2**: Section bwtween the last thoracic and the first lumbar vertebra, following the prolongation of the 12$^{th}$ rib when cutting the thoracic wall. **Cutpoint 3**: section between the 6$^{th}$ and 7$^{th}$ lumbar vertebra, cutting the abdominal wall transversally to the vertebral column. **Cutpoint 4**: Separation of fore legs, including insertion and thoracic muscles (from Blasco & Ouhayoun, 1993)

Rabbit meat provides excellent nutritive and dietetic properties as it rich in proteins (18.2-23.7 g/100 g meat) and B vitamins. Particularly, it provides the greatest quantity of vitamin $B_{12}$ among the most common meats such as pork, beef, veal and chicken. Rabbit meat is also an important source of minerals P, K, Zn and Se and it is favorably low in Na (37-47 mg /100 g edible fraction). Rabbit meat has also relatively high energy values (144-215 kcal /100 g meat, depending on the cut) which derives almost exclusively from the high protein content, which represents the 80% of the total energy value (Dalle Zotte & Szendrő, 2011). In fact, rabbit can also be considered a lean meat source (about 8.5 % of fat, considering the whole carcass) with a low cholesterol content (45 mg/100 g of edible fraction, whole carcass) and a favorable fatty acid profile which can be further improved through diet (Dal Bosco, Castellini, Bianchi, & Mugnai, 2004; Trebušak, Levart, Salobir, & Pirman, 2014). In addition, lipids of rabbit meat are highly unsaturated (60% of total fatty acids) with a relatively high content of PUFA (Dalle Zotte, 2002). For all the above mentioned positive characteristics, the nutritional profile of rabbit meat is perfect for all kind of consumers and, thanks to its low sodium and cholesterol contents, it also suitable for people suffering from hypertension and for cholesterol-lowering diets (Dalle Zotte & Szendrő, 2011).

### *5.2. Functional rabbit meat and processed meat products*

The development of new further processed rabbit meat as well as meat with added functionality, seems the key for the survival of the industrial-scale rabbit production system. For this reason, starting from the last decade but speeding-up in the last years, current research is directed towards adding functionality to fresh rabbit meat by dietary strategies (see review of Dalle Zotte & Szendrő, 2011).

In fact, being the rabbit a monogastric animal, diet is an effective tool to modify its intramuscular fatty acid composition (Petracci, Bianchi, & Cavani, 2009). As it was demonstrated by Dal Bosco et al. (2004), the addition of 0.08 kg/kg diet of flaxseed to the diet of growing rabbits, provided a significant increase in PUFA and decrement in SFA contents in the *Logissimus dorsi* meat compared to a control group which was fed with sunflower. Particularly, the total *n*-3 content of the meat was about twice that of the control group leading to a *n*-6/*n*-3 ratio of 2.95, which is within the recommended values (British Nutrition Foundation, 1993). Moreover, despite the high presence of PUFA would render meat particularly susceptible to oxidation, the addition of 200 mg/kg of α-tocopheryl-acetate to rabbits diet was effective in protecting the meat and this resulted in very high sensory quality of the cooked meat. The same positive effect of a dietary supplementation with vitamin E on

the shelf-life and sensory acceptance of rabbit meat was observed also by Dalle Zotte, Cossu, & Parigi Bini (2000). Interestingly, the direct dietary addition of long chain *n*-3 PUFA to rabbits diet (Berbardini, Dal Bosco, Castellini, & Miggiano, 1996), provided a higher level of long chain *n*-3 PUFA (LCP) than that obtained by the addition of flaxseed, which provides their precursor (Dal Bosco et al., 2004). However, in the first study the oxidative stability of the meat was low, even when vitamin E was supplemented to the diet. This result was probably due to the fact that rabbits fed directly with LCP *n*-3, couldn't develop a parallel antioxidant response, as it was observed with the precursor. A similar study (Petracci et al., 2009) evaluating the dietary fortification of rabbit meat with *n*-3 PUFA and α-tocopheryl-acetate, confirmed to have a positive effect on fatty acids profile and sensory stability of the meat (*Longissimus lumborum* muscle). Vitamin E was also tested alone on the qualitative characteristics of cooked meat and it provided protection against oxidation during cooking (boiling, frying and roasting). Specifically, 200 mg/kg of α-tocopheryl-acetate improved the fat quality by increasing *n*-3 PUFA and lowering SFA (Dal Bosco, Castellini, & Bernardini, 2001).

Other recent studies focused on testing natural sources in order to achieve an effective protection of rabbit meat from oxidative deterioration and/or improving other meat quality characteristics. A work by Simonová, Chrastinová, Mojto, Lauková, Szabóová, & Rafay (2010), found that the dietary supplementation with 10 μl/animal/day of an oregano extract didn't have any negative effect on the carcass traits and it improved the energy value and amino acid composition of rabbit meat (*Longissimus dorsi* muscle). The effect of dietary supplementations with oregano essential oil on the quality of rabbit meat was tested also by Botsoglu, Florou-Paneri, Christaki, Giannenas, & Spais, (2004) and Soultos, Tzikas, Christaki, Papageorgiou, & Steris (2009). The first research work observed that oregano essential oil improved the oxidative stability of muscle tissues, whereas the second study found that the microbial quality of rabbit carcasses during refrigerated storage was enhanced in supplemented animals compared to the control group. Differently from the positive results of all the above mentioned literature, other natural additives didn't provide such evident and positive results. It's the case of chia seed (Meineri, Cornale, Tassone, & Peiretti, 2010) and tannins (Dalle Zotte, Balzan, Novelli, Bohatir, Matics, & Szendrő, 2010) whose dietary supplementation to growing rabbits didn't slower lipid oxidation rate of rabbit meat.

*Spirulina platensis* is a blue-green alga that can provide interesting amounts of protein, vitamins, essential amino acids, minerals and it is one of the few sources γ–linolenic acid (GLA) which showed important effects on different aspects of human health (Fan & Chapkin,

1998). When this alga was supplemented to the diet of growing rabbits (50 g/kg of diet) it seemed to improve the atherogenic and thrombogenic indexes of the meat, thus improving its nutritional quality (Peiretti & Meineri, 2011).

Results of a recent work by Dal Bosco, Mugnai, Roscini, Mattioli, Ruggeri, & Castellini (2014), showed that increasing the presence of natural bioactive compounds through alfalfa as complementary feed in rabbit diets, the nutritional quality of rabbit meat (higher α-linolenic, EPA and DHA fatty acids compared to a control diet without alfalfa) successfully improved. Finally, when 10 g/kg of reishi mushroom (*Ganoderma lucidum*) or 10 g/kg of olive leaves (*Olea europaea*) were included in rabbit diets, no significant effect on the oxidative stability of meat lipids was observed (Trebušak, Levart, Salobir, & Pirman, 2014).

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# CHAPTER 2

## General aims

Modern consumers are becoming more and more aware of the strict association between diet and health status and see food not only as a tool to satisfy their hunger and to provide necessary nutrients, but also a way to prevent nutrition-related diseases. For this reasons, in recent years consumers of Western Countries have started perceiving meat and meat products in a more negative view, as overconsumption is involved in many chronic diseases such as obesity, cardiovascular diseases and cancer. This is mainly attributed due to their fat, cholesterol and sodium contents, as well as for the presence of synthetic additives. Consequently, in order to meet consumers expectancies, since the last decade meat industry has been focused in producing healthier meats and processed meat products, namely functional. The increasing demand of such products can be explained by increasing costs of healthcare, increase in the life expectancy and, more in general, as a tool to improve well-being.

For all the above mentioned considerations, this PhD thesis aimed to study functional meat and meat products obtained from unconventional meat species, namely the ostrich and the rabbit. These two meat sources have been chosen because of their positive nutritional profile and because they represent an alternative to the most common farmed species. Specifically, Chapters 3 and 4 of the present thesis tested different dietary strategies to improve the nutritional profile of rabbit meat, whithout impairing growth and health performances. Differently, Chapters 5 and 6 studied the reformulation of meat products derived from ostrich, in order to achieve a better nutritional profile as well to ensure product quality and safety.

# CHAPTER 3a

Running Head: Dietary Spirulina and Thyme to growing rabbits

# Effect of Dietary supplementation of Spirulina (*Arthrospira platensis*) and Thyme (*Thymus vulgaris*) on apparent digestibility and productive performances of growing rabbits

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## ABSTRACT

The aim of this study was to evaluate the effect of dietary supplementation with Spirulina (*Arthrospira platensis*) or/and Thyme (*Thymus vulgaris*) on total tract apparent digestibility of nutrients and on the growing rabbits' performances. At weaning (5 weeks of age) the rabbits were randomly allotted to 7 groups (42 rabbits/group, 3 rabbits/cage). Rabbits of the control group (C-C) received a control pelleted feed throughout the experiment (5-11 weeks of age) without any supplementation (CP: 176 g $kg^{-1}$, NDF: 325 g $kg^{-1}$). In the other groups, the control diet was supplemented with 5% Spirulina (S, mainly in substitution of soybean meal), or 3% Thyme (T, mainly in substitution of alfalfa meal) or by both 5% S and 3% T (ST) for the whole (5-11 weeks of age; groups: S-S, T-T, ST-ST), or part of the growing period (8-11 weeks of age; groups: C-S, C-T, C-ST). Supplementations had no effect on apparent digestibility of dry matter, organic matter, ADF, gross energy and digestible energy. The CP TTAD was lowest in rabbits fed the S diet whereas it was highest in C fed rabbits, being the other two treatments intermediate ($P<0.001$). The starch TTAD was lowest for S-fed rabbits (98.3%) and highest for ST fed rabbits (99.4%), being the other 2 dietary groups intermediate ($P<0.001$). Differently, the EE TTAD was higher in T than ST and C dietary groups (70.4 *vs* 67.7 % on average, respectively; $P<0.001$), showing S fed rabbits an intermediate value (69.1%). The NDF TTAD of the ST diet was lower than that of the other three groups (16.4 *vs.* 21.0; $P<0.001$, respectively). The TTAD of Ca reached the lowest value for the S diet (53.5%) compared with the other three diets (59.1% on average; $P<0.001$). Also for K and P the S diet had the lowest digestibility ($P<0.001$), but in this case the C group always showed the highest values ($P<0.001$), exhibiting T and ST rabbits intermediate results. Spirulina and/or Thyme dietary supplementation had no effect on feed intake (133 g/d), daily weight gain (38.3 g/d), morbidity (9.9 %) or mortality (1.8 %). Significant differences were only found for feed conversion ratio, which was lower for the C-T group (3.39) than for the C-C group (3.54, $P<0.05$). Based on these results, Spirulina and Thyme included separately or combined in growing rabbit diets did not exhibit substantial effects on the growth performance or health status.



**Key words:** Spirulina, Thyme, rabbits, growth performance, total tract apparent digestibility

## 1. Introduction

Spirulina *(Arthrospira platensis)* is generally regarded as a rich source of protein, vitamins, essential amino acids, minerals, essential fatty acids (FA) and antioxidant pigments like carotenoids (Belay, Kato, & Ota 1996; Dalle Zotte & Szendrő, 2011). Feeding Spirulina-enriched diets (5, 10 and 15%) to rabbits from 9 to 13 weeks of age (Peiretti & Meineri, 2008, 2011) did not result in significant effects on productive performances, despite the fact that the meat lipids content increased and their FA profile changed (polyunsaturated FA and n-6/n-3 increased; total n-3 FA decreased). As no mortality or health problems were observed (Peiretti & Meineri, 2008) the effect of Spirulina supplementation on health status could not be assessed.

Thyme *(Thymus vulgaris)* is rich in essential oils (2.17-4.73%), which are known to have antioxidant properties and antimicrobial activity against foodborne pathogens (Jordán, Martínez, Goodner, Baldwin, & Sotomayor 2006; Rota, Herrera, Martínez, Sotomayor, & Jordán, 2008). Özkan, Sari, Bayezit, Doğan, Akpulat, & Erdağ (2010) examined the effect of a methanolic extract (essential oil) from wild Thyme *(Thymus serpyllum)* on coccidiosis in rabbits. The treatment (100 mg/kg body weight) decreased the faecal oocyst counts and increased growth rate until 24 d post treatment. Investigation in animal feeding tested the Thyme essential oils inclusion widely and found beneficial effects on gut microflora (Hernández, Madrid, García, Orengo, & Megías, 2004; Amad, Männer, Wendler, Neumann, & Zentek, 2011) and on growth performance (Abd El-Hakim, Cherian, & Ali, 2009) in growing broiler chickens. Thyme dry leaves are also supposed to have a certain effect on health and growth performance. In rabbits, the only study where Thyme dry leaves at 2.5% of dietary inclusion level was used did not find beneficial effects neither on live performances nor on health status (Dalle Zotte, Sartori, Bohatir, Rémignon, & Ricci, 2013), likely due to the low inclusion level or the optimal health status of rabbits in this trial.

Available data on mineral digestibility, especially in rabbits, are scarce. Spirulina is a good source of minerals but the effect of its supplementation on mineral digestibility has been tested only once, in companion dwarf rabbits (Dalle Zotte et al., 2013). Thyme has never been evaluated in this sense: tannins presence (polyphenols, plant materials) may negatively affect mineral digestibility, as it was reported by Al-Mamary, Al-Habori, Al-Aghbari, & Al-Obeidi (2001) when evaluating dietary sorghum tannins on rabbits digestive enzymes and mineral absorption.

Therefore, this study aimed to evaluate the effect of the dietary supplementation and the length of supplementation (between the ages of 5-11 or 8-11 weeks) of Spirulina (5%) or/and Thyme leaves (3%) on total tract apparent digestibility of nutrients, productive performance and health status in growing rabbits.

## 2. Materials and Methods

*2.1 Animals and experimental design*

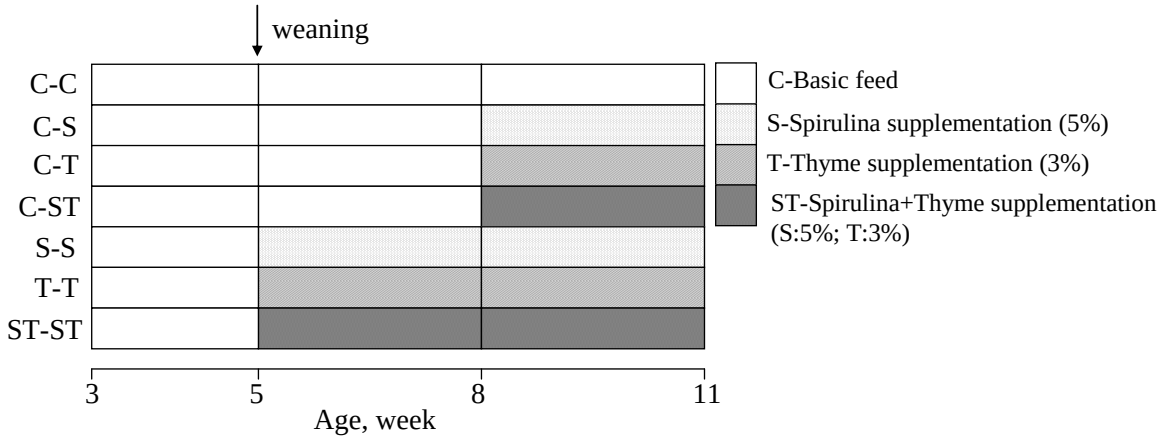


**Figure 1:** Experimental design

The experiment was conducted at the experimental rabbit farm of Kaposvár University (Hungary) using a maternal line (selected for litter size, adult body weight: 4.0 to 4.5 kg) rabbits. All animals were treated according to the principles stated by EC Directive 86/609/EEC regarding the protection of animals used for experimental and other scientific purposes. All rabbits received a pelleted control diet (C) from three weeks of age onwards. After weaning (at five weeks of age) the individually marked rabbits were housed in wire net cages (0.61 x 0.32 m, 3 rabbits/cage).

The environmental temperature was kept from 15 to18 °C and daily lighting in the rabbitry was 16 hours long (6:00-22:00).

Two hundred and ninety four weaned rabbits weighing 952±81 g were randomly allotted to 7 groups (42 rabbits/group, no littermates were allowed within the groups, 3 rabbits/cage, 14 cages/treatment). Rabbits of the control group (C-C) received a pelleted diet without any supplementation throughout the experiment (5-11 weeks of age). In the other groups the diet was supplemented with 5% Spirulina (*Arthrospira platensis*) (S diet, mainly in substitution of soybean meal), or with 3% Thyme (*Thymus vulgaris*) leaves (T diet, mainly in substitution of alfalfa meal) or by both supplements (ST diet) before pelleting and fed to rabbits for the whole experimental period (5-11 weeks of age; groups: S-S, T-T, ST-ST,

respectively) or during the last three weeks of the growing period (8-11 weeks of age; groups: C-S, C-T, C-ST, respectively, Figure 1).

**Table 1.** Chemical composition (g $kg^{-1}$) of Spirulina (*Arthrospira platensis*) and Thyme (*Thymus vulgaris*)

| | *Arthrospira platensis* | *Thymus vulgaris* |
|---|---|---|
| Dry matter (DM) | 944 | 889 |
| Crude protein (CP) | 658 | 52.3 |
| Ether extract (EE) | 8.6 | 31.9 |
| Crude fibre (CF) | nd[1] | 181 |
| Ash | 65.1 | 65.9 |
| Neutral detergent fibre (NDF) | 2.4 | 298 |
| Acid detergent fibre (ADF) | 4.8 | 210 |
| Acid detergent lignin (ADL) | 0.6 | 68.1 |
| Starch | 35.6 | 58.4 |
| Ca | 2.2 | 13.6 |
| P | 9.2 | 0.7 |
| Ca/P | 0.2 | 18.7 |
| Gross energy (GE), MJ/kg | 19.5 | 15.7 |

[1]nd: not detected.

Supplements (Spirulina powder and Thyme dried leaves) were purchased from a commercial supplier. In order to obtain diets balanced in energy and nutrient content, the chemical composition and energy content of the supplements were preliminarily analysed (Table 1). Then, with the aim to formulate isonitrogenous, iso-energetic and isofibrous diets, soybean meal represented the main ingredient that has been substituted with the supplements studied. The diets contained no medication, and no coccidiostatics. Water and feed were available *ad libitum*. The ingredients and the chemical composition and mineral profile of the experimental diets are shown in Table 2 and 3, respectively.

**Table 2.** Ingredients of the experimental diets

| | Experimental diets | | | |
|---|---|---|---|---|
| | C | S | T | ST |
| Ingredient, as-fed basis (g $kg^{-1}$) | | | | |
| Dehydrated alfalfa meal 170 g/kg CP | 400 | 398 | 370 | 398 |
| Barley meal | 247 | 262 | 237 | 262 |
| Soybean meal | 130 | 55 | 140 | 60 |
| Wheat straw | 120 | 110 | 120 | 90 |
| Dried apple pomace | 40 | 40 | 40 | 40 |
| Spirulina | - | 50 | - | 50 |
| Thyme leaves | - | - | 30 | 30 |
| Fat powder[1] | 35 | 35 | 35 | 35 |
| Sodium Chloride | 5 | 5 | 5 | 5 |
| Monocalcium phosphate | 3 | 3 | 3 | 3 |
| DL-methionine | 1 | 1 | 1 | 1 |
| L-lysine | 4 | 6 | 4 | 6 |
| Premix[2] | 5 | 5 | 5 | 5 |
| Zeolite | 10 | 30 | 10 | 15 |

[1] Fat content: 40%.

[2] Premix provided per kg of complete diet: vitamin A, 12,000 IU; vitamin D3, 1000 IU; vitamin E acetate, 50 mg; vitamin K3, 2 mg; biotin, 0.1 mg; thiamin, 2 mg; riboflavin, 4 mg; vitamin B6, 2 mg; vitamin B12, 0.1 mg; niacin, 40 mg; pantothenic acid, 12 mg; folic acid, 1 mg; choline chloride, 300 mg; iron, 100 mg; copper, 20 mg; manganesium, 50 mg; cobalt, 2 mg; iodine, 1mg; zinc, 100 mg; selenium, 0.1 mg.

**Table 3.** Chemical composition of the experimental diets

| | Experimental diets | | | |
|---|---|---|---|---|
| | C | S | T | ST |
| Analysed composition (g $kg^{-1}$ as fed) | | | | |
| Dry matter (DM) | 896 | 898 | 898 | 896 |
| Crude protein (CP) | 176 | 170 | 175 | 172 |
| Ether extract (EE) | 25 | 26 | 27 | 28 |
| Ash | 86 | 75 | 84 | 77 |
| Crude Fibre (CF) | 160 | 162 | 157 | 158 |
| Neutral detergent fibre (NDF) | 323 | 316 | 314 | 301 |
| Acid detergent fibre (ADF) | 212 | 205 | 208 | 195 |
| Acid detergent lignin (ADL) | 53 | 45 | 53 | 46 |
| Hemicelluloses (NDF-ADF) | 111 | 111 | 106 | 106 |
| Cellulose (ADF-ADL) | 158 | 160 | 155 | 149 |
| Starch | 163 | 181 | 170 | 178 |
| NFE[1] | 463 | 478 | 471 | 471 |
| NNCC[2] | 325 | 352 | 341 | 356 |
| Gross energy (GE), MJ/kg | 16.3 | 16.5 | 16.4 | 16.4 |
| Ca | 9.6 | 9.3 | 9.4 | 10.6 |
| P | 4.0 | 3.9 | 4.0 | 4.3 |
| K | 10.5 | 9.4 | 9.7 | 10.6 |
| Na | 2.7 | 2.6 | 2.4 | 2.7 |
| Fe | 0.52 | 0.48 | 0.53 | 0.54 |

[1]NFE: Nitrogen Free Extracts; [2]NNCC: Non nitrogenous cellular content = Organic matter – CP – NDF.

### *2.2. Digestibility trial*

The total tract apparent digestibility (TTAD) of dry matter (DM), organic matter, crude protein, ether extract, starch, NDF, ADF, cellulose, hemicelluloses, gross energy (GE), Ca, K and P of the experimental diets (C, T, S and TS) was measured in an *in vivo* digestibility trial conducted at the experimental farm of Padova University (Italy). The digestibility trial was approved by the Italian Ministry of Education, University and Research and by the Ethical Committee of the Padova University. The digestibility trial was carried out on thirty-two (8 animals per diet) 62 day-old hybrid rabbits according to the European standardized method (Perez, Lebas, Gidenne, Maertens, Xiccato, et al., 1995). Rabbits were equally distributed by gender and live weight into the four dietary groups and individually caged. After a week of adaptation to the new diets, faeces were collected for a 4-day period.

*2.3. Collection of data and data management*

Body weight (BW) was measured at 5, 8 and 11 weeks of age and feed intake (FI) for 5-8 and 8-11 week period, daily weight gain (DWG) and feed conversion ratio (FCR) were then calculated. BW and DWG were evaluated based on individual data (n=42 rabbits/group), whereas FI and FCR based on the cage unit (n=14 cages/group). When calculating FI, it was assumed that morbid rabbits did not consume any pellet for the 2 days preceding their death. The morbidity and the mortality were recorded weekly and daily, respectively. Rabbits suffering from diarrhoea and/or with a negative or very low DWG during a one-week period were considered as morbid. When the same individual was registered with diarrhoea at several subsequent examinations morbidity was registered only once within the same period.

*2.4. Chemical analyses*

Analyses of Spirulina and Thyme supplements as well as those of the experimental diets and faeces were carried out in duplicate using AOAC (2000) methods in order to determine the concentrations of DM (934.01), crude protein (CP; 2001.11), crude fibre (CF; 978.10), ash (967.05) and starch (amyloglucosidase- α-amylase method, 996.11). Ether extract (EE) was determined after acid-hydrolysis (EC, 1998). Neutral detergent fibre (NDF, without sodium sulphite), acid detergent fibre (ADF), and acid detergent lignin (ADL) were analyzed according to Mertens (2002), AOAC (2000, procedure 973.187) and Van Soest, Robertson, & Lewis (1991), respectively, using the sequential procedure and the filter bag system (Ankom Technology, New York).

The gross energy (GE) was measured with an adiabatic bomb calorimeter (ISO, 1998). Mineral analyses were performed on Spirulina and Thyme supplements (Ca, P), on the experimental diets (Ca, P, K, Na, Fe) and faeces (Ca, P, K) by ICP-OES (Spectro Ciros Vision EOP) after microwave digestion (AOAC 2000, 999.10).

*2.5. Statistical analysis*

The productive traits and the digestibility data were analysed by one-way ANOVA, morbidity and mortality by chi-square test using SPSS 10.0 software package, with diet as fixed effect. Probability values were considered significant when <0.05.

## 3. Results and Discussion

### *3.1. Total tract apparent digestibility*

**Table 4.** Effect of dietary Spirulina (*Arthrospira platensis*) and Thyme (*Thymus vulgaris*) on total tract apparent digestibility (TTAD) and nutritive value (8 rabbits/diet)

| | Experimental diets | | | | *P*-value | RSD[1] |
|---|---|---|---|---|---|---|
| | C | S | T | ST | | |
| Live Weight, g | 1812 | 1897 | 1842 | 1778 | ns | 223 |
| Dry matter (DM) intake, g /d | 128.6 | 139.1 | 139.6 | 127.8 | ns | 17.8 |
| TTAD, %: | | | | | | |
| DM | 61.4 | 60.6 | 60.7 | 61.0 | ns | 1.49 |
| Organic matter | 62.5 | 62.0 | 62.2 | 62.0 | ns | 1.44 |
| Crude protein | $76.0^{c}$ | $73.1^{a}$ | $74.6^{bc}$ | $74.4^{ab}$ | < 0.001 | 0.97 |
| Ether extract | $67.8^{a}$ | $69.1^{ab}$ | $70.4^{b}$ | $67.7^{a}$ | < 0.001 | 1.18 |
| NDF | $23.2^{b}$ | $19.1^{b}$ | $20.9^{b}$ | $16.4^{a}$ | 0.002 | 3.07 |
| ADF | 13.58 | 9.69 | 11.92 | 9.39 | ns | 3.39 |
| Hemicelluloses (NDF-ADF) | $41.5^{b}$ | $36.5^{b}$ | $38.5^{b}$ | $29.2^{a}$ | < 0.001 | 2.47 |
| Cellulose (ADF-ADL) | 19.3 | 16.5 | 17.1 | 15.6 | ns | 3.16 |
| Starch | $99.1^{b}$ | $98.3^{a}$ | $99.2^{b}$ | $99.4^{c}$ | < 0.001 | 0.04 |
| Gross energy | 62.3 | 61.6 | 62.0 | 61.7 | ns | 1.45 |
| Ca | $60.7^{b}$ | $53.5^{a}$ | $58.0^{b}$ | $58.7^{b}$ | < 0.001 | 1.60 |
| P | $45.6^{c}$ | $36.1^{a}$ | $41.5^{b}$ | $43.2^{b}$ | < 0.001 | 2.22 |
| K | $86.6^{d}$ | $80.5^{a}$ | $82.9^{b}$ | $85.4^{c}$ | < 0.001 | 0.62 |
| Nutritive value: | | | | | | |
| Digestible protein (DP), g/kg | $133.3^{d}$ | $124.2^{a}$ | $130.5^{c}$ | $127.6^{b}$ | < 0.001 | 1.72 |
| Digestible energy (DE), MJ/kg | 10.14 | 10.19 | 10.19 | 10.11 | ns | 0.21 |
| DP to DE ratio, g/MJ | $13.16^{c}$ | $12.19^{a}$ | $12.81^{b}$ | $12.62^{b}$ | < 0.001 | 0.13 |

[1]RSD: Residual Standard Deviation.

Dietary supplementation of Spirulina, Thyme and the combination of the two natural supplements did not affect rabbits DM intake (Table 4). Differently, TTAD of nutrients showed great differences among dietary groups (Table 4). The CP TTAD was lowest in rabbits fed the S diet whereas it was highest in C fed rabbits, being the other two treatments intermediate (P<0.001). The low levels of CP TTAD for S and ST diets might be related to a lower CP digestibility of Spirulina and Thyme compared to soybean meal (Table 3).

The starch TTAD was lowest for S-fed rabbits (98.3%) and highest for ST fed rabbits (99.4%), being the other 2 dietary groups intermediate (P<0.001).

Differently, the EE TTAD was higher in T than ST and C dietary groups (70.4 *vs* 67.7 % on average, respectively; P<0.001), showing S fed rabbits an intermediate value (69.1%).

As far as fibre fractions TTAD are concerned, only the TTAD of NDF and hemicelluloses were found to be different among dietary groups. The value of the ST diet was lower than that of the other three groups (16.4 *vs.* 21.0 % for NDF; P<0.01, and 29.2 *vs* 38.8 % for hemicelluloses; P<0.001, respectively).

The TTAD of the macroelements Ca, K and P also differed among dietary treatments (Table 4). The TTAD of Ca reached the lowest value for the S diet (53.5%) compared with the other three diets (59.1% on average; P<0.001); analogous results were found for K and P (P<0.001). Regarding these last two macroelements, the C group always showed the highest value, T and ST rabbits exhibiting intermediate digestibility coefficients (P<0.001). The only other study on mineral TTAD in rabbits fed both supplements was recently conducted by Dalle Zotte et al. (2013) on dwarf rabbits supplemented with 3% Spirulina and/or 2.5% Thyme and no significant effect was evidenced. However, based on the results obtained in these two studies, some differences emerged. In our work the TTAD of Ca was 57.7% on average (Table 4), definitely higher than that (36.8%) found by Dalle Zotte et al. (2013). A similar pattern was noticed for the average TTAD of K and P in the two studies (83.9% *vs* 69% and 41.6% *vs.* 13.3% for K and P, respectively). In the two studies rabbits' age was similar and they were in perfect sanitary condition during the digestibility trial, but the dietary EE content differed, being lower in the current study. However, no negative influence on minerals TTAD attributable to dietary EE content has notwithstanding been reported in literature (Fernández, Cobos, & Fraga, 1994).

As a direct consequence of the CP TTAD trend, the dietary DP content was the highest for rabbits fed C diet and the lowest for those fed S diet (P<0.001). Thus, also the DP to DE ratio exhibited the same trend (P<0.001).

Our results did not confirm those reported by Peiretti & Meineri (2009) who observed an improvement in the TTAD of the CP caused by dietary inclusion of 1% Spirulina. However, in the study of Peiretti a& Meineri (2009) the ingredients inclusion level was not provided, the rabbits were much older than ours, they were feed-restricted, and the dietary CP content varied between control and supplemented diets. Thus, the two studies followed very different protocols and the results obtained for nutrients digestibility are scarcely comparable. However, it is assumed that the CP TTAD seems to be dependent on the inclusion levels of Spirulina. In fact, whereas the 3% Spirulina inclusion produced no adverse effect on CP TTAD (Dalle Zotte et al., 2013), its increase to 10% and 15% (in substitution of soybean meal

and alfalfa meal) resulted in a negative TTAD trend for CP but also for the other nutrients (Peiretti & Meineri, 2008).

Another hypothesis for such results on TTAD of nutrients could be deducted from zeolite presence in the diets, especially for S diet, where a higher inclusion level was found. This natural clay was added in order to produce diets with similar chemical composition. Several studies evaluated the effect of dietary zeolite supplementation on growth, digestive metabolism and nutrients digestibility of livestock, especially ruminants and pigs. In a study on growing pigs, retention of Ca, P, Mg, Na, K and Fe were linearly reduced by increasing zeolite's dietary supplementation levels (Shurson, Ku, Miller, & Yokoyama, 1984).

Based on the results obtained so far, the effect of dietary Spirulina and/or Thyme supplementation on nutrient digestibility in rabbits is still unclear. Especially for what concerns Spirulina, results were surprising as Alvarenga, Rodrigues, Cantarelli, Zangeronimo, Júnior, et al., (2011) showed that *Arthrospira platensis* had higher nutritional value compared to soybean meal in terms of crude protein content, mineral matter, apparent metabolisable energy and amino acids profile. Thus further investigation is needed to elucidate S and T mode of action in the rabbit digestive system, and the feed processing technology should be taken also into account, as probably it plays a role as a source of variation in feed digestibility. Caecal microbiota and fermentation of rabbits fed S and T supplements were also considered in another study (Vántus Bònai, Zsolnai, Dal Bosco, Szendrő, et al., 2012), but no substantial effects were observed.

Dietary 3% Thyme inclusion level slightly impaired nutritive value, whereas synergic effect of S and T supplements was not evidenced; additional research is thus needed as this has been the first scientific attempt to test this combination in rabbits reared for meat purposes.

### *3.2. Productive performances*

Despite the fact that the digestive efficiency of some nutrients was different among control and treated groups, separated or combined dietary inclusion of Spirulina and Thyme did not affect BW, average DWG or FI of growing rabbits throughout the trial (Table 5).

**Table 5.** Effect of the dietary supplementation with Spirulina (*Arthrospira platensis*) and Thyme (*Thymus vulgaris*) on live performance of rabbits from 5 to 11 weeks of age

| | Experimental diets | | | | | | | *P*-value | RSD[1] |
|---|---|---|---|---|---|---|---|---|---|
| | C-C | C-S | C-T | C-ST | S-S | T-T | ST-ST | | |
| No. rabbits | 42 | 42 | 42 | 42 | 42 | 42 | 42 | | |
| Daily weight gain, g/d | | | | | | | | | |
| 5-8 weeks | 41.9 | 42.2 | 40.5 | 43.2 | 42.2 | 43.9 | 41.7 | ns | 0.36 |
| 8-11 weeks | 33.6 | 33.0 | 35.9 | 34.5 | 34.9 | 34.3 | 34.5 | ns | 0.33 |
| 5-11 weeks | 37.8 | 37.8 | 38.2 | 38.8 | 38.6 | 39.1 | 38.1 | ns | 0.23 |
| Feed intake, g/d | | | | | | | | | |
| 5-8 weeks | 115 | 114 | 109 | 119 | 113 | 118 | 115 | ns | 0.96 |
| 8-11 weeks | 152 | 148 | 151 | 154 | 152 | 151 | 154 | ns | 1.02 |
| 5-11 weeks | 134 | 131 | 130 | 137 | 133 | 135 | 134 | ns | 0.80 |
| Feed conversion rate | | | | | | | | | |
| 5-8 weeks | 2.75 | 2.72 | 2.69 | 2.76 | 2.69 | 2.68 | 2.76 | ns | 0.01 |
| 8-11 weeks | $4.57^{b}$ | $4.50^{ab}$ | $4.21^{a}$ | $4.50^{ab}$ | $4.37^{ab}$ | $4.42^{ab}$ | $4.49^{ab}$ | 0.033 | 0.03 |
| 5-11 weeks | $3.54^{b}$ | $3.48^{ab}$ | $3.39^{a}$ | $3.52^{ab}$ | $3.44^{ab}$ | $3.44^{ab}$ | $3.53^{ab}$ | 0.032 | 0.01 |
| Live weight at 11 weeks, g | 2542 | 2539 | 2551 | 2582 | 2569 | 2594 | 2555 | ns | 11.6 |

[1]RSD: Residual Standard Deviation; No. of replicates:14 cages/treatment for feed intake and feed conversion ratio and 42 rabbits/treatment for body weight and daily weight gain.

Differently, FCR from 8 to 11 weeks of age and that of the whole period (5-11 weeks of age) were affected by dietary treatments ($P<0.05$). Specifically, C-T rabbits exhibited better FCR than C-C ones ($P<0.05$), whereas the other feeding groups presented intermediate values.

Similar to the results of the present trial, other studies did not observe differences in rabbits live performances when fed Spirulina supplements at levels of 0.5% (Colla, Muccillo-Baisch, & Costa, 2008), 1% (Peiretti & Meineri, 2009), 3% (Dalle Zotte, et al., 2013) or 5, 10 and 15% (Peiretti & Meineri, 2008). In poultry, sewage-grown Spirulina was firstly tested as a replacer for ground nut cake (Saxena, Ahmad, Shyam, & Amla, 1983) and results were promising because the 6-week weight gain and FI were improved with increasing dietary Spirulina inclusion level. In another study on weanling pigs (Grinstead, Tokach, Dritz, Goodband, & Nelssen, 2000), dietary supplementation with 1 and 2% Spirulina (in substitution of soybean meal) showed slight improvements in growth performance and feed efficiency, but only if considering certain phases of the cycle.

The effect of Thyme supplementation on rabbit performance has not been clearly demonstrated yet, thus requiring further investigation. In a study (Ibrahim, El-Ghamry, & El-Mallah, 2000) Thyme supplementation promoted an increase in BW, DWG and FI in rabbits.

Differently, a more recent experiment by Dalle Zotte, et al. (2013), reported that 2.5% Thyme supplementation in diets fed to dwarf rabbits for 14 weeks did not produce any difference in live performances. The only exception regarded the period 8-9 weeks of age, when diet supplemented with Thyme showed higher daily weight gain compared to those fed simultaneously with Spirulina and Thyme.

Rabbit morbidity and mortality were satisfactory along the study period and no effects attributable to the dietary treatments were observed (Table 6).

**Table 6.** Effect of the dietary supplementation with Spirulina (*Arthrospira platensis*) and Thyme (*Thymus vulgaris*) on rabbit morbidity and mortality rates (%) from 5 to 11 weeks of age

| | Experimental diets | | | | | | | *P*-value |
|---|---|---|---|---|---|---|---|---|
| | C-C | C-S | C-T | C-ST | S-S | T-T | ST-ST | |
| Morbidity: | | | | | | | | |
| 5-8 weeks | 7.1 | 11.9 | 14.3 | 4.8 | 9.5 | 4.8 | 14.3 | ns |
| 8-11 weeks | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2.4 | 0.0 | ns |
| 5-11 weeks | 7.1 | 11.9 | 14.3 | 4.8 | 9.5 | 7.1 | 14.3 | ns |
| Mortality: | | | | | | | | |
| 5-8 weeks | 2.4 | 0.0 | 2.4 | 0.0 | 2.4 | 0.0 | 0.0 | ns |
| 8-11 weeks | 0.0 | 4.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | ns |
| 5-11 weeks | 2.4 | 4.8 | 2.4 | 0.0 | 2.4 | 0.0 | 0.0 | ns |

Morbidity ranged from 4.8% (C-ST group) to 14.3% (C-T and ST-ST group) in the whole growth period (5-11 weeks of age). Mortality was very low as well, ranging from zero (C-ST, T-T, ST-ST groups) to 4.8% (C-S group). These results support those in literature (Peiretti & Meineri, 2008). In both studies, the favourable environmental conditions and then the excellent health status of the rabbits did not permit to verify the assumed positive effects of Spirulina and/or Thyme to prevent digestive disorders.

Optimal DP to DE ratio for growing rabbits, suggested in order to guarantee both high performance and good sanitary conditions, ranges between 9.5 to 10 g $MJ^{-1}$ (Carabaño, Villamide, García, Nicodemus, & Llorente, 2009; De Blas & Mateos, 2010). However, even if the DP to DE ratio obtained in our study was higher and varied between 12.19 and 13.16 g $MJ^{-1}$, no negative effects neither on growth performance (Table 5) nor on morbidity and mortality (Table 6) were observed.

When growing rabbits' diet was supplemented with a blend of formic acid, lactic acid and essential oil from rosemary, thyme and cinnamon, mortality was lower compared to those fed only with formic and lactic acid, but similar to that of a control group without any additive and to another one supplemented with antibiotics (Cesari, Toschi, Pisoni, Grilli, & Cesari, 2008). Dietary inclusion of *Thymus serpyllum* decreased the faecal oocysts' count in rabbits, which could be considered as a possible indicator of the rabbits' health (Özkan, Sari, Bayezit, Doğan, Akpulat, & Erdağ, 2010).

In a recent study (Vántus et al., 2012) the S and/or T dietary supplementations had no substantial effect on the volatile FA content of caecal digesta, even though by means of Quantitative PCR it was demonstrated that ST supplementation exerted a antimicrobial effect on the investigated bacterial groups in the caecum.

## 4. Conclusions

Supplementing diets with 5% Spirulina (in substitution of soybean meal) or 3% Thyme (in substitution of alfalfa meal) to growing rabbits for 3 or 6 weeks reduced the nutritive value of the diets. Particularly, Spirulina lowered CP, starch and mineral TTAD, suggesting a lower protein digestibility than soybean meal. Despite this, no substantial effect on rabbits performance and health status, was observed. Spirulina and Thyme combination did not provide the advisable synergistic effect. Future studies should take into consideration feed processing technology, pelleting, storage and packaging conditions, as they may reduce or nullify the nutrient and functional compounds' availability. Spirulina and/or Thyme effect on health status should be tested under poorer sanitary conditions.

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# CHAPTER 3b

Running Head: Dietary Spirulina and Thyme to growing rabbits

# Effect of Dietary supplementation of Spirulina (*Arthrospira platensis*) and Thyme (*Thymus vulgaris*) on carcass composition, meat physical traits, and vitamin $B_{12}$ content on growing rabbits

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## ABSTRACT

The aim of this study was to compare the effect and duration of the dietary inclusion of 5% Spirulina (*Arthrospira platensis*) and/or 3% Thyme (*Thymus vulgaris*) on growing rabbit carcass composition, meat and bone rheological traits, and the vitamin $B_{12}$ content of *Longissimus dorsi* (LD) meat. The study involved 294 maternal line growing rabbits of the Pannon breeding program. At weaning (5 weeks), animals were randomly divided by dietary treatment into 7 groups of 42 rabbits each. A control group (C-C) received a pellet with no supplementation throughout the trial (5-11 weeks of age), whereas the other groups were fed diets supplemented with 5% Spirulina (S), 3% Thyme (T) or with both ingredients (ST) for either the entire growing period (5-11 weeks of age; groups: S-S, T-T, ST-ST, respectively), or its final part only (8-11 weeks of age; groups: C-S, C-T, C-ST, respectively). Results showed that regardless of the duration of supplementation, Spirulina and Thyme provided no effect on the traits examined, except for scapular fat content, whose value was higher in the S-S group than in the C-T group ($P<0.05$). Spirulina was confirmed as a rich source of vitamin $B_{12}$ that was successfully transferred into LD meat, thus demonstrating its value as an effective natural supplement in producing food fortified with this vital element. Further studies are necessary to clarify the effect of Spirulina on carcass fat deposition, bone development, and mineralization.



**Key words:** *Spirulina platensis*, *Thymus vulgaris*, Rabbit meat, Vitamin $B_{12}$

## 1. Introduction

Since the European Union first limited the use and then definitively banned the use of antibiotics as growth promoters in animal feeding (Anadón, 2006) and public opinion on antibiotic use by humans in the USA has changed progressively, more and more scientific studies have been dedicated to natural alternatives (Montesissa & Calini, 2006; Falcão-e-Cunha, 2007; Franz, Baser, & Windisch, 2010; Hashemi & Davoodi, 2011). The EU decision stemmed from the concern that low-continuative dosage of antibiotics to either enhance animal performance or simple prophylaxis purposes could lead to the formation of resistant strains of human pathogens that pose a real sanitary risk to the population (Wegener, 2003).

Furthermore, the growing need to reduce the environmental impact of livestock combined with higher consumer pressure for more natural food production systems has increased the interest of the industry in natural feed supplements. In this context, essential oils and aromatic plants have become more and more widely-used as natural feed additives to increase feed palatability, positively affect gastrointestinal flora, exert a coccidiostatic effect, ensure optimal productive performance, and achieve antimicrobial action on chilled meat (Dickens, Berrang, & Cox, 2000; Hernández, Madrid, García, Orengo, & Megías, 2004; Cross, McDevitt, Hillman, & Acamovic, 2007).

Thyme (*Thymus vulgaris*) is a well known Mediterranean shrub traditionally used as an appetizing substance, sensory additive, and flavouring agent. Most studies conducted as yet have investigated its antimicrobial and antioxidant actions with thymol and carvacrol as its major phenolic compounds (Yanishlieva, Marinova, & Pokorny´, 2006; Al-Turki, 2007; Solomakos, Govaris, Koidis, & Botsoglou, 2008; Hoffman-Pennesi & Wu, 2010).

Another set of natural products that might prove useful in animal production to enhance the nutritional value of conventional food and improve the health status of consumers through diet are microalgae (Gouveia, Batista, Sousa, Raymundo, Bandarra, 2008), one of the most promising of which is Spirulina (*Arthrospira platensis*), a filamentous blue-green microalgae once consumed by the Aztecs in Mexico and still consumed in the Lake Chad area in Africa (Belay, 2002). Today, Spirulina is widely-known and appreciated for its high protein content and for being an important source of β-carotene, vitamin $B_{12}$, whose dietary deficiency in vegetarians represents a growing concern (Stabler & Allen, 2004), and minerals. The organic source of Ca and P provided by Spirulina suggests its use in poultry and rabbit feeding to guarantee correct lifelong bone development and higher bone strength, thus reducing carcass downgrade.

Moreover, it has been shown to be anticarcinogenic and to have many positive health properties, such as the mitigation of hyperlipidemia and the control of hypertension and high serum glucose levels (Belay, Ota, Miyakawa, & Shimamatsu, 1993). Despite having a higher production cost than common animal feeds, it represents an interesting alternative thanks to its ability to grow under alkaline and saline conditions that are unsuitable for most traditional crops (Carlos, Sassano, João, Carvalho, Luiz, et al., 2004). Spirulina is normally produced in outdoor ponds that leave small environmental footprints and minimize the utilization of land, which can be placed to other purposes (Belay, 2002). Research has also shown that Spirulina can prove useful in recycling nutrients through organic waste treatment processes (Ahsan, Habib, Parvin, Huntington, & Hasan, 2008). Thanks to all these positive aspects, Spirulina is currently being produced worldwide, with half of its production used in feeding fish and livestock.

In poultry feeding, incorporation with Spirulina has provided satisfactory results in terms of productive performance and as a substitute for mineral-vitamin premixes (Venkataraman, Somasekaran, & Becker, 1994; Belay, Kato, & Ota, 1996). It has also been shown effective in improving carcass colour, and in lowering total cholesterol content when its effect on egg quality was tested (Holman & Malau-Aduli, 2013). Research in some species like pigs and rabbits is still in the earliest phases however, and therefore a wider scope of research is required before the previous results can be confirmed (Grinstead, Tokach, Dritz, Goodband, & Nelssen, 2000).

The objective of this study was therefore to evaluate the effect of dietary Thyme and Spirulina supplementation on growing rabbit carcass composition, vitamin $B_{12}$ absorption into meat cuts, meat rheological traits, and bone development. The results presented in this article are part of a wider study that has involved productive performance, the health status and apparent digestibility of the diets (Gerencsér, Szendrő, Matics, Radnai, Kovács, et al., 2013), microbial diversity in the caecum and caecal fermentation (Vàntus, Bónai, Zsolnai, Dal Bosco, Szendrő, et al., 2012), and the fatty acid profile of the meat and its oxidative status during retail display (Dal Bosco, Gerencsér, Szendrő, Mugnai. Cullere, et al., 2013). To our knowledge, this is the first study that evaluates the synergic effect of Spirulina and Thyme on animal productive performance, health, and meat quality.

## 2. Materials and Methods

### *2.1 Animals and experimental design*

For this study, a total of 294 maternal line growing rabbits of the Pannon breeding program were used. Animals were reared at the experimental farm of Kaposvár University (Hungary) and received a control pelleted diet (C) from the age of 3 weeks. At weaning (5 weeks of age), animals were randomly divided by dietary treatment into 7 groups and housed by 3 in wire net cages (0.61 x 0.32 m). Control group rabbits (C-C) received a pelleted diet with no supplementation throughout the trial (from 5 to 11 weeks of age). The other groups received pelleted diets supplemented with 5% Spirulina (S diet, mainly in substitution of soybean meal), 3% Thyme leaves (T diet, mainly in substitution of alfalfa meal) or with both ingredients (ST) for the entire period (groups: S-S, T-T, ST-ST) or for only the last 3 weeks of fattening (8-11 weeks of age; groups: C-S, C-T, C-ST, Figure 1). These two different durations of supplementation were planned in a perspective of cost-reduction. Experimental diets were isonitrogenous, isoenergetic, and did not include coccidiostatics. Water and feed were available *ad libitum*, and the temperature and photoperiod in the rabbitry were 15-18 °C and 16L:8D, respectively. Ingredients and chemical composition of the experimental diets are reported by Gerencsér et al. (2013).

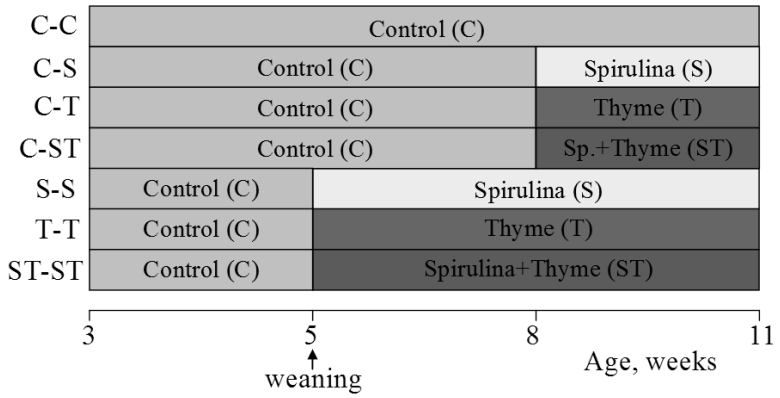


**Figure 1.** Experimental design and dietary treatment

*2.2. Slaughter, carcass dissection and meat sampling*

At 11 weeks of age, the rabbits were transported to a slaughterhouse located 200 km from the experimental farm (n=35, 34, 34, 36, 35, 36 and 36 rabbits for C-C, C-S, C-T, C-ST, S-S, T-T and ST-ST groups, respectively) and slaughtered by cutting the carotid arteries and jugular veins after electro-stunning. The slaughtering and carcass dissection procedures followed the World Rabbit Science Association (WRSA) recommendations described by Blasco & Ouhayoun (1996), and all the steps taken to obtain offal (head, heart+lung+thymus+trachea+oesophagus – HLTTO – liver, kidneys, perirenal and scapular fat), weights (slaughter weight – SW –, chilled carcass – CC –weight and reference carcass – RC– weight), and yields (carcass yield and reference carcass yield) are detailed in a previous study (Dalle Zotte, Princz. Metzger, Szabó, Radnai, et al., 2009). CC was recorded after 24h chilling in a ventilated room at 4 °C.

Subsequently, the *Longissimus dorsi* (LD) muscle and hind legs (HL) were dissected from 15 and 10 rabbits per dietary group, respectively, and then weighed. Once pHu (pH measured at 24 h *post mortem*) was measured at the $5^{th}$ lumbar vertebra level, the right and left sides of the LD muscle were individually packed and frozen at -80 °C until further analysis. HL were individually frozen at -80 °C immediately after dissection.

*2.3. Thawing, cooking loss, bone trait and vitamin $B_{12}$ determination*

Left LD were allowed to thaw overnight at room temperature, weighed, and subsequently used for thawing and cooking loss measurements. Samples were individually packed under-*vacuum* in PVC bags and cooked in a water bath at 80 °C for 1h. Right LD of the animals belonging to C-C, S-S and ST-ST groups (6 samples per group) were ground at frozen state with a Retsch Grindomix GM 200 grinder at 4000 rpm for 10 seconds, then freeze dried and subsequently used for vitamin B12 determination (AOAC 2006, Method no. 952.20). Diets C, S and ST were also analyzed for vitamin B12 content. Right and left HL were thawed overnight and, after weighing, right legs were deboned in order to determine the meat/bone ratio (Blasco & Ouhayoun, 1996). Femur and tibia were separately weighed, then length and minor diameter were measured with a digital caliper (JUWEL *Digital-Schieblehre Rostfrei* H4215/5X A12).

Femur fracture toughness (FT) was calculated at the average bone length point using a dynamometer Texture TA-HD (SMS- *Stable Micro System*) with a 6 cm wide cell and a load rate of 0.5 mm/sec. Left HL were individually packed under-vacuum in PVC bags and cooked in a water bath at 80 °C for 2.5 hours for cooking loss determination.

### *2.4. Statistical Analysis*

Data were analyzed using the General Linear Model procedures of the statistical analysis software SAS 9.1 for Windows (SAS, 2004). A one-way analysis of variance (ANOVA) tested the diet as fixed effect and the significance level was calculated at the 5% confidence level. Normality of data was analysed with a Shapiro-Wilk confidence level of 85%.

## 3. Results and Discussion

Dietary supplementation with 5% Spirulina, 3% Thyme, or both had no affect on rabbit slaughter weight, carcass yields or retail cut percentages (Table 1). The only exception was scapular fat content, which differed between S-S and C-T groups (0.56 *vs* 0.39 % of the Chilled Carcass –CC-, $P<0.05$). In literature, Spirulina has been reported to possess hypotriglyceridemic action by stimulating lipoprotein lipase activity (Belay, 2002). This property has been observed under pathogenic conditions, however, such as in rabbits fed high cholesterol diets in which 0.5 g/day of Spirulina increased serum levels of HDL, the latter representing a protective factor against atherosclerosis (Colla, Muccillo-Baisch, & Costa, 2008). In a similar trial, 1% and 5% Spirulina supplementation were shown effective in reducing serum total cholesterol and LDL levels in hypercholesterolemic rabbits (Cheong, Kim, Sok, Hwang, & Kim, 2010). On the other hand, contrary to as observed in serum parameters, Spirulina did not appear to lower carcass fatness in rabbits fed high fat diets when compared to those fed low-fat diets (Meineri, Ingravalle, Radice, Aragno, & Peiretti, 2009). The addition of 150 mg Spirulina/kg to the diet of growing rats, in fact, increased the visceral fat content compared to a Control group (Sixabela, Chivandi, Badenhorst, & Erlwanger, 2011).

Generally speaking, Spirulina seems effective in improving serum cholesterol status in pathogenic conditions, and although this suggests its use as a protective factor against atherosclerosis, its effect at lipid deposit level requires further investigation because the results obtained thus far preclude definitive assumptions.

As regards Thyme, our results support those found in literature, which considered its essential oil, however. Abdominal fat content was significantly reduced when Thyme essential oil was supplemented to Japanese quails diets either at 60 or 200 mg/kg (Denli, Okan, & Uluocak, 2004; Khaksar, van Krimpen, Hashemipour, & Pilevar, 2012) and to broiler chicks diets at 1 g/kg inclusion level (Al-Kassie, 2009). These results might be attributable to the positive effect of the Thyme compounds on digestive efficiency, which leads to improved feed conversion rate, as observed in our study (Gerencsér et al., 2013). In another recent study on growing dwarf rabbits, however, a lower Thyme leave dietary inclusion level (2.5%) was unable to modify digestive efficiency and animal growth (Dalle Zotte, Sartori, Bohatir, Rémignon, & Ricci, 2013), in this way hypothesizing a dose-related effect.

Overall carcass weight, yields, dissectable fat, and meatiness results (Table 1) were satisfactory and comparable to results provided in literature (Dal Bosco, Castellini, & Mugnai, 2002; Metzger, Kustos, Szendrő, Szabó, Eiben, et al., 2003; Gondret, Larzul, Combes, & de Rochambeau, 2005; Gidenne, Combes, Feugier, Jehl, Arveux, et al., 2009).

**Table 1.** Effect of the dietary Spirulina (*Arthrospira platensis*) and Thyme (*Thymus vulgaris*) supplementation on carcass traits

| | Experimental groups | | | | | | | *P*-value | SEM[1] |
|---|---|---|---|---|---|---|---|---|---|
| | C-C | C-S | C-T | C-ST | S-S | T-T | ST-ST | | |
| No. of rabbits | 35 | 34 | 34 | 36 | 35 | 36 | 36 | | |
| Slaughter weight (SW), g | 2474 | 2471 | 2480 | 2516 | 2497 | 2536 | 2492 | ns | 12.6 |
| Chilled Carcass (CC), g | 1502 | 1502 | 1504 | 1525 | 1527 | 1543 | 1514 | ns | 7.95 |
| Reference Carcass (RC), g | 1228 | 1226 | 1233 | 1248 | 1250 | 1268 | 1238 | ns | 6.73 |
| Carcass yield, % SW | 60.7 | 60.8 | 60.7 | 60.6 | 61.1 | 60.9 | 60.8 | ns | 0.08 |
| RC yield, % CC | 81.7 | 81.6 | 82 | 81.8 | 81.9 | 82.2 | 81.7 | ns | 0.07 |
| Drip loss, % | 2.33 | 2.26 | 2.17 | 2.32 | 2.21 | 2.18 | 2.27 | ns | 0.23 |
| As % of chilled carcass: | | | | | | | | | |
| Head | 9.35 | 9.52 | 9.40 | 9.25 | 9.32 | 9.30 | 9.25 | ns | 0.03 |
| HLTTO[2] | 1.69 | 1.62 | 1.62 | 1.57 | 1.62 | 1.26 | 1.66 | ns | 0.02 |
| Liver | 5.66 | 5.65 | 5.20 | 5.72 | 5.57 | 5.38 | 5.75 | ns | 0.06 |
| Kidneys | 1.07 | 1.03 | 1.09 | 1.10 | 1.03 | 1.02 | 1.05 | ns | 0.01 |
| Perirenal fat | 1.51 | 1.47 | 1.35 | 1.60 | 1.55 | 1.59 | 1.57 | ns | 0.03 |
| Scapular fat | $0.46^{ab}$ | $0.51^{ab}$ | $0.39^{a}$ | $0.51^{ab}$ | $0.56^{b}$ | $0.48^{ab}$ | $0.45^{ab}$ | 0.029 | 0.01 |
| Dissectable fat | 1.97 | 1.98 | 1.74 | 2.11 | 2.11 | 2.07 | 2.03 | ns | 0.04 |
| As % of RC: | | | | | | | | | |
| Fore part | 28.0 | 28.4 | 28.4 | 28.5 | 28.0 | 28.2 | 28.1 | ns | 0.06 |
| Intermediate part | 31.7 | 31.4 | 31.7 | 31.4 | 31.5 | 31.3 | 31.7 | ns | 0.07 |
| Hind part | 37.9 | 37.8 | 37.8 | 37.5 | 37.9 | 37.9 | 37.8 | ns | 0.07 |
| Perirenal fat | 1.85 | 1.81 | 1.65 | 1.95 | 1.9 | 1.94 | 1.93 | ns | 0.04 |

[1] SEM: Standard Error of the Least Squares Means; [2] HLTTO: Heart, lung, thymus, trachea and oesophagus; Means in the same row with unlike superscripts differ; a, b: $P<0.05$

Neither the dietary inclusion of Spirulina and/or Thyme nor the duration of their supplementation influenced the traits of LD and HL meat portions (Tables 2 and 3, respectively), thus confirming previous results that considered dietary inclusions of 5, 10 and 15% of Spirulina (Peiretti & Meineri, 2011).

The ratio of LD and HL portions on the reference carcass (RC) were on average 10.9% and 34.9%, respectively. Comparing these ratios with those obtained in a study that used the same genetic line as we did (Metzger, Odermatt, Szendrõ, Mohaupt, Romvári, et al., 2006), in our study rabbits had lighter LD and heavier HL, thus resulting in different incidences on RC, which were, however, most likely attributable to the successful CT based selection scheme over the years performed to increase hind leg muscle volume (Szendrő, Matics, Gerencsér, Radnai, Lengyel , et al., 2009).

Rabbit LD pHu was unaffected by dietary treatment, and the average value of 5.9 observed was within the range reported in literature (Ouhayoun & Dalle Zotte, 1993; Hernández & Dalle Zotte, 2010). Even if differences were not statistically significant, the S-S group showed a numerically lower thawing loss percentage than the average of the other dietary treatments (10.4 *vs* 11.7 %, respectively) and thus numerically lower total losses (33.1 *vs* 34.9 %). An initial explanation of this trend suggests that Spirulina might have positively affected cell membrane integrity during freezing-thawing phases, but this theory was not confirmed by HL thawing losses.

**Table 2.** Effect of the dietary Spirulina (*Arthrospira platensis*) and Thyme (*Thymus vulgaris*) supplementation on traits of *Longissimus dorsi* (LD) muscle

| | Experimental groups | | | | | | | *P*-value | SEM[1] |
|---|---|---|---|---|---|---|---|---|---|
| | C-C | C-S | C-T | C-ST | S-S | T-T | ST-ST | | |
| No. of samples | 15 | 15 | 15 | 15 | 15 | 15 | 15 | | |
| LD[2], g | 133 | 132 | 136 | 134 | 140 | 138 | 139 | ns | 1.65 |
| LD[2], % RC[3] | 10.9 | 10.7 | 11.0 | 10.7 | 11.2 | 10.8 | 11.1 | ns | 0.08 |
| pHu | 5.90 | 5.97 | 5.94 | 5.88 | 5.92 | 5.84 | 5.84 | ns | 0.08 |
| Thawing losses, % | 11.4 | 11.3 | 11.8 | 12.1 | 10.4 | 12.1 | 11.2 | ns | 0.02 |
| Cooking losses, % | 24.4 | 22.1 | 22.6 | 22.8 | 22.6 | 24.3 | 23.4 | ns | 0.27 |
| Total losses, % | 35.8 | 33.4 | 34.3 | 34.9 | 33.1 | 36.4 | 34.6 | ns | 0.27 |

[1] SEM: Standard Error of the Least Squares Means; [2]Two LD muscles; [3] RC: Reference Carcass

**Table 3.** Effect of the dietary Spirulina (*Arthrospira platensis*) and Thyme (*Thymus vulgaris*) supplementation on hind legs (HL) traits

| | Experimental groups | | | | | | | *P*-value | SEM[1] |
|---|---|---|---|---|---|---|---|---|---|
| | C-C | C-S | C-T | C-ST | S-S | T-T | ST-ST | | |
| No. of rabbits | 10 | 10 | 10 | 10 | 10 | 10 | 10 | | |
| HL[2], g | 427.4 | 438.5 | 443.7 | 446.0 | 451.3 | 459.3 | 442.9 | ns | 3.94 |
| HL[2], % RC[3] | 34.9 | 35.2 | 35.2 | 34.8 | 34.6 | 35.4 | 34.4 | ns | 0.12 |
| Thawing loss, % | 4.15 | 4.05 | 3.93 | 4.04 | 3.95 | 4.35 | 3.74 | ns | 0.10 |
| Cooking loss % | 18.1 | 17.9 | 18.3 | 19.0 | 19.0 | 19.9 | 18.9 | ns | 0.20 |
| Total losses, % | 26.2 | 26.1 | 26.9 | 27.0 | 27.1 | 28.9 | 27.4 | ns | 0.28 |

[1] SEM: Standard Error of the Least Squares Means; [2] Two hind legs; [3] RC: Reference Carcass

Spirulina and/or Thyme dietary supplementation did not affect HL bone traits (Table 4), and mean values were in accordance with those reported in literature (Dalle Zotte et al., 2009). Femur and tibia presented average lengths of 91.4 and 70.2 mm, respectively, and the meat/bones ratio was 5.65, which was higher than the value reported by Metzeger et al. (2003) in a study on 13 week-old New Zealand White rabbits.

As reviewed by Holman and Malau-Aduli (2013), Spirulina is an important source of Ca (1200 mg/kg) and P (13000 mg/kg), thus the mineral content of the experimental diets used in our study were balanced by taking this aspect into account. Unlike the mineral premix, however, Spirulina is an organic source, and for this reason we hypothesized a higher mineral bioavailability in S-supplemented diets than in the others, as well as a possible effect on rabbit bone traits. Unexpectedly, the results of the total tract apparent digestibility of the diets showed S diet mineral digestibility to be the lowest (Gerencsér et al., 2013) and no differences in bone traits were observed as a result. Tibia length, which is an indicator of linear growth (Masoud, Shapiro, Kent, & Moses, 1986; Fritton, Myers, Wright, & van der Meulen, 2005), was found to be greater in rats fed 150 and 1500 mg/kg supplemented with Spirulina than those fed a control diet (Sixabela, et al., 2011). In another work on ovariectomised rats and hindlimb-unloaded mice, 0.08, 0.8, and 4 g/kg body weight/day of Spirulina determined trabecular bone loss. The component responsible for this depletion has yet to be identified, however (Ishimi, Sugiyama, Ezaki, Foujioka, & Wu, 2006).

**Table 4.** Effect of the dietary Spirulina (*Arthrospira platensis*) and Thyme (*Thymus vulgaris*) supplementation on rabbit hind leg (HL) bones traits

| | Experimental groups | | | | | | | *P*-value | SEM[1] |
|---|---|---|---|---|---|---|---|---|---|
| | C-C | C-S | C-T | C-ST | S-S | T-T | ST-ST | | |
| No. of samples | 10 | 10 | 10 | 10 | 10 | 10 | 10 | | |
| HL bones, g | 30.6 | 31.4 | 31.8 | 30.5 | 31.2 | 31.7 | 31.9 | ns | 0.31 |
| Femur, g | 13.0 | 13.5 | 13.8 | 13.1 | 13.3 | 14.0 | 13.3 | ns | 0.11 |
| Femur length, mm | 91.1 | 91.8 | 92.4 | 91.0 | 91.7 | 90.6 | 91.3 | ns | 0.29 |
| Femur minor Ø, mm [2] | 6.48 | 6.50 | 6.60 | 6.54 | 6.54 | 6.72 | 6.45 | ns | 0.04 |
| Femur fracture toughness, kg | 25.3 | 26.8 | 31.3 | 29.0 | 27.2 | 27.8 | 28.3 | ns | 0.56 |
| Tibia, g | 7.56 | 7.54 | 7.61 | 7.57 | 7.86 | 7.88 | 7.78 | ns | 0.07 |
| Tibia minor Ø, mm [2] | 5.38 | 5.54 | 5.58 | 5.28 | 5.45 | 5.77 | 5.27 | ns | 0.05 |
| Tibia length, mm | 70.5 | 70.0 | 71.4 | 70.5 | 70.6 | 68.9 | 69.7 | ns | 0.35 |
| HL bones, % HL | 15.3 | 15.3 | 15.2 | 14.7 | 14.7 | 15.1 | 15.3 | ns | 0.08 |
| Meat to bones ratio | 5.56 | 5.55 | 5.61 | 5.83 | 5.8 | 5.66 | 5.57 | ns | 0.04 |

[1] SEM: Standard Error of the Least Squares Means; [2]Ø= diameter

Spirulina is known to be an important source of vitamins, especially vitamin $B_{12}$, which is an almost exclusive prerogative of animal-origin foods (Dalle Zotte & Szendrő, 2011). Rabbit meat naturally contains this micronutrient, as may be seen in the LD meat of the C-C group (Table 5). In this study, Spirulina was demonstrated to be an effective fortifier, given that the LD meat of rabbits fed the S-S diet showed a significantly ($P<0.05$) higher vitamin $B_{12}$ content compared to rabbits fed the C-C diet, whereas the ST-ST fed animals presented an intermediate value (0.6620 *vs* 0.9539 *vs* 0.8051 mcg vitamin $B_{12}$/100 g meat for C-C, S-S and ST-ST groups, respectively). The group-dependent trend of vitamin $B_{12}$ presence in LD meat corresponded directly to that of the diets (0.509 *vs* 0.841 *vs* 0.697 mcg/ 100 g feed, for C-C, S-S and ST-ST groups, respectively).

**Table 5.** Vitamin B12 content in feeds (mcg/100 g feed) supplemented with Spirulina (*Arthrospira platensis*) and its effect on vitamin B12 content (mcg/100 g meat) in raw *Longissimus dorsi* (LD) meat

| | Experimental groups | | | *P*-value | SEM[1] |
|---|---|---|---|---|---|
| | C-C | S-S | ST-ST | | |
| No. of samples | 6 | 6 | 6 | | |
| Vitamin $B_{12}$ in feeds | 0.509 | 0.841 | 0.697 | - | - |
| Vitamin $B_{12}$ in LD meat | $0.662^{a}$ | $0.954^{b}$ | $0.805^{ab}$ | 0.012 | 0.03 |

1 SEM= Standard Error of the Least Squares Means; Means in the same row with unlike superscripts differ; a,b: $P<0.05$

In accordance with literature, vitamin $B_{12}$ content in the feed and its absorption percentage are inversely proportional (95 *vs* 85 *vs* 86 % for C-C, S-S and ST-ST groups, respectively), even if rabbits showed higher absorption capacity than what has been reported for humans (Allen, 2009).

This represents the first scientific evaluation of the dietary fortification of vitamin $B_{12}$ and its absorption and consequent content in rabbit meat by means of dietary raw materials. Although other examples of vitamin $B_{12}$ fortification in pig-meat products via dietary vitamin $B_{12}$ supplementation are reported in literature (Sahlin & House, 2006), said supplementation consisted in synthetic vitamin $B_{12}$. Vitamin $B_{12}$ content of meat and meat products is not often reported in studies evaluating meat quality, and when it is quantified, great differences can be noted: vitamin $B_{12}$ content of lean beef meat ranges between 0.8 and 3.9 mcg/100 g meat, that of lean pork meat between 0.3 and 2 mcg/100g, and that of lamb between 0.9 and 3.5 mcg/100 g (Giguère, Girard, & Matte, 2005; Ortigues-Marty, Micol, Prache, Dozias, & Girard, 2005; Sahlin & House, 2005; Truswell, 2007; Williams, Droulez, Levy, & Stobaus,

2007; Schönfeldt van Heerden, Sainsbury, & Gibson, 2011). Rabbit meat is reported to be very rich in vitamin $B_{12,}$ ranging from 8.7 to 11.9 mcg/100 g (review by Dalle Zotte & Szendrő, 2011), which is clearly higher than the values obtained in our study. The reason for these wide ranges could be attributed to the fact that vitamin $B_{12}$ exists in different forms (cobalamins and cobalamin analogs), thus the sample preparation method and the analysis technique applied are crucial to final content quantification. Several methods are reported in literature (Baker & Miller-Ihli, 2000; Heudi, Kilinç, Fontannaz, Marly, 2006; Indyk, Persson, Caselunghe, Moberg, & Filonzi, 2002), all of which present different sensitivity, detection specificity, precision, selectivity and reliability, and for this reason different results are not surprising. For example, one study comparing a microbial and a chemiluminescence method in estimating the vitamin $B_{12}$ content of Spirulina tablets (Watanabe, Takenaka, Abe, Tamura, & Nakano, 1998) obtained extremely different results (147.5 *vs* 17.35 mcg/100 g for microbial and chemiluminescence methods, respectively), therefore suggesting that the commonly-known and accepted values for this microelement could easily be over- or under-estimated. The development of laboratory techniques that allow reliable and comparable vitamin $B_{12}$ quantification in food for more precise nutritional information to consumers is therefore desirable.

## 4. Conclusions

Dietary supplementation with 5% Spirulina and/or 3% Thyme and the duration of treatment had no effect on rabbit carcass composition, on LD and HL traits, nor on HL bone traits. Spirulina was effective in fortifying vitamin $B_{12}$ content of LD meat, even if the percentage of absorption decreased as dietary vitamin $B_{12}$ increased. Weak signs of carcass fatness change were observed with dietary Spirulina/Thyme supplementation, thus requiring further studies to demonstrate if and how these supplements affect lipid metabolism.

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# CHAPTER 3c

Running Head: Dietary Spirulina and Thyme to growing rabbits

# Effect of Dietary supplementation of Spirulina (*Arthrospira platensis*) and Thyme (*Thymus vulgaris*) on rabbit meat appearance, oxidative stability and fatty acid profile during retail display

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## ABSTRACT

The objective of this study was to evaluate the effect of Spirulina and Thyme supplementation on rabbit meat during retail display. At weaning 294 rabbits were allocated to 7 different treatments (42 rabbits/treatment). Rabbits of the control group (C) received a diet without any supplementation throughout the experiment (5-11 weeks of age). The other groups were fed diets containing 5% Spirulina (S), 3% Thyme (T) or both supplements (ST) for the whole trial (5-11 weeks; treatments S, T and ST), or for a part of the growing period (8-11 weeks; treatments C-S, C-T and C-ST). Colour parameters, pH, water holding capacity and drip loss were determined on fresh and stored *Longissimus dorsi* muscle of 5 rabbits/treatment. Spirulina- and Thyme-supplemented diets had a significant effect on redness and yellowness of *Longissimus dorsi*. Drip loss was significantly reduced in C-T and T groups that also showed the highest content of α-tocopherol and n-3 fatty acids content and the lower lipid oxidation.



**Key words:** Spirulina, Thyme, Rabbit meat, Physical traits, Lipid oxidation

## 1. Introduction

Different studies show that the main quality aspects of consumer's choice for the purchase of meat are taste, tenderness, juiciness, freshness, leanness, healthiness and nutritious (Grunert, 1997). Appearance, in particular colour and loss of exudates, determines how consumers perceive quality and influences purchasing behaviour (Resurreccion, 2003). In the specific case of rabbit meat, which is very rich in unsaturated fatty acids (Dalle Zotte, 2002), a certain degree of lipid oxidation, mainly during processing and storage (Castellini, Dal Bosco & Bernardini, 1998; Dalle Zotte, 2002; Cavani & Petracci, 2004), is expected with a detrimental effect on its physical characteristics, like colour and water holding capacity. To counteract this process, together with meat appropriate modified atmosphere packaging, there has been an increasing interest in the use of antioxidants in rabbit feed formulas (Corino, Pastorelli, Pantaleo, Oriani & Salvatori, 1999; Dal Bosco, Castellini, Bianchi & Mugnai, 2004). Synthetic antioxidants were widely used in the meat industry, but consumer concerns over safety and toxicity pushed the food industry to find natural sources (Coronado, Trout, Dunshea & Shaha, 2002).

In rabbit, many studies were carried out to evaluate the effect of different antioxidants derived from olive oil (Lopez-Bote, Rey, Sanz, Gray & Buckley, 1997), oats (Lopez-Bote, Sanz, Rey, Castaño & Thos, 1998), soy-isoflavones (Yousef, Kamel, Esmail & Baghdadi, 2004), oregano-essential oils (Botsoglou, Florou-Paneri, Christaki, Giannenas & Spais, 2004), grape polyphenols (Sgorlon, Stradaioli, Stefanon, Altimer & Della Loggia, 2005), grape pomace (Eid, 2008), olive pomace (Dal Bosco, Mourvaki, Cardinali, Servili, Sebastiani, Ruggeri, Mattioli, Taticchi, Esposto & Castellini, 2012), red quebracho tannins (Dalle Zotte & Cossu, 2009), chestnut hydrolysable tannins (Dalle Zotte, Matics, Bohatir, Sartori, Gerencsér & Szendrő, 2012), alfalfa polysaccharides (Liu, Dong, Tong, Xu & Zhang, 2011), algae (Peiretti & Meineri, 2011) and green tea (Eid, Zeweil, Ahmed, Basyony & Farok, 2011).

Spirulina (Atrhrospira platensis) is a rich source of phycocyanin, an antioxidant biliprotein pigment, and carotenoids (Cheong, Kim, Sok, Hwang, Kim, Kim, Lee, Kim & Kim, 2010; Belay, Kato & Ota, 1996). Thyme (Thymus vulgaris) essential oil contains more than 60 ingredients, which are known to have antioxidant properties and antimicrobial activity (Rota, Herrera, Martínez, Sotomayor & Jordán, 2008).

On the basis of these considerations, in this trial the main rabbit meat traits for assessing the consumer choices (colour, drip loss during a simulated retail display) at time of

purchase was investigated. This study is a part of an extensive research aimed to evaluate the effect of the dietary supplementation (between 5 to 11 or 8 to 11 weeks of age) of Spirulina (*Arthrospira platensis*, 5%) powder and/or Thyme (*Thymus vulgaris*, 3%) dried leaves on live performance, health status, and carcass and meat quality of growing rabbits, where this part focused on the oxidative status and fatty acid profile of rabbit meat during a simulated retail display. The dietary levels of the two used antioxidants were selected on the basis of the few previous experiments on this issue (Nieto, Díaz, Bañón & Garrido, 2010; Peiretti & Meineri, 2008; Peiretti & Meineri 2009;Peiretti & Meineri, 2011).

## 2. Materials and methods

### *2.1. Animals and experimental design*

**Table 1**. Ingredients (g/kg), chemical composition (g/kg) and gross energy (MJ//kg) of the experimental diets

| | Control (C) | Spirulina (S) | Thyme (T) | Spirulina+Thyme (ST) |
|---|---|---|---|---|
| Dehydrated alfalfa meal | 379 | 390 | 355 | 380 |
| Barley | 260 | 275 | 250 | 260 |
| Soybean meal | 145 | 65 | 155 | 70 |
| Wheat straw | 120 | 130 | 120 | 120 |
| Dried beet pulp | 56.2 | 56.2 | 56.2 | 56.2 |
| Spirulina | 0 | 50 | 0 | 50 |
| Thyme leaves | 0 | 0 | 30 | 30 |
| Sunflower seed oil | 10 | 10 | 10 | 10 |
| Dicalcium phosphate | 4 | 4 | 4 | 4 |
| NaCl | 5 | 5 | 5 | 5 |
| DL-Methionine | 1.8 | 1.8 | 1.8 | 1.8 |
| L-Lysine | 3 | 3 | 3 | 3 |
| Vit.-Min. premix[1] | 5 | 5 | 5 | 5 |
| Zeolite | 11 | 5 | 5 | 5 |
| Chemical composition: | | | | |
| Dry matter | 895.72 | 897.94 | 898.16 | 895.87 |
| Crude protein | 175.79 | 169.87 | 174.96 | 171.51 |
| Ether extract | 25.39 | 26.46 | 26.87 | 27.68 |
| Ash | 85.62 | 75.26 | 84.01 | 76.62 |
| Starch | 163.3 | 180.9 | 169.8 | 178.0 |
| Gross Energy, MJ/kg | 16.32 | 16.53 | 16.44 | 16.40 |

[1] Premix provided per kg of complete diet: vitamin A, 12,000 IU; vitamin D3, 1000 IU; vitamin E acetate, 50 mg; vitamin K3, 2 mg; biotin, 0.1 mg; thiamin, 2 mg; riboflavin, 4 mg; vitamin B6, 2 mg; vitamin B12, 0.1 mg; niacin, 40 mg; pantothenic acid, 12 mg; folic acid, 1 mg; choline chloride, 300 mg; iron, 100 mg; copper, 20 mg; manganesium, 50 mg; cobalt, 2 mg; iodine, 1mg; zinc, 100 mg; selenium, 0.1 mg.

The experiment was conducted at the experimental rabbit farm of Kaposvár University (Hungary) using maternal line rabbits (n=294). Rabbits received a control diet (C) from the age of 3 weeks. After weaning (5 weeks of age), they were randomly sorted to 7 groups (42 rabbits/group) and housed in wire net cages (0.61 x 0.32 m, 16 rabbits/$m^2$) until 11 weeks of age, when they were slaughtered. Rabbits of the control group received a diet throughout the experiment without any supplementation (C diet). In the other groups the diets were completed by 5% Spirulina (S), 3% Thyme (T) or by both (ST) for the whole (5-11 weeks of age; groups: S, T, ST), or for a part of the growing period (8-11 weeks of age; groups: C-S, CT, C-ST) (Table 1). The latter was conceived to evaluate a short-period of natural additives supplementation to reduce the feeding costs. Water and feed were available *ad libitum* for every group and the diets did not contain medication. The applied temperature and lighting schedule in the rabbitry were 15-18 °C and 16L:8D, respectively.

*2.2. Collection and analytical determinations*

At 11 weeks of age 5 rabbits per group, with a live weight close to the average of the group (2562 g + 10%) (Gerencsér, Szendrő, Matics, Radnai, Kovács, Nagy, Dal Bosco, & Dalle Zotte, 2012) were selected and, after 12 hours feed withdrawal, slaughtered; animals did not undergo transport. Following electro-stunning, rabbits were killed by cutting the carotid arteries and jugular veins. After 24 h carcass refrigeration at +4 °C, the two sides of *Longissimus dorsi* (LD) muscle were removed and carefully freed from connective and adipose tissues. The same day, samples were transported refrigerated to the Department of Applied Biology (Perugia, Italy) to be analyzed. The day after, on the left LD muscle side, pH, colour parameters, water holding capacity (WHC), antioxidants content, oxidative processes (TBARs) and fatty acid (FA) profile were determined as described below.

The right side of LD muscle was weighed and left whole for the determination of drip loss. All the samples were successively placed on plastic foams, over-wrapped with PVC film (600 $cm^2$) and displayed at +4 °C under continuous cool white fluorescent illumination (2,300 lux). All the analyses were conducted at day 1 and again at days 3, 6 and 9, whereas the FA profile determination was repeated only at the end of the storage period (day 9).

The pH was measured with a Knick digital pHmeter (Broadly Corp., Santa Ana, CA, USA) after grinding 1 g of muscle into 10 mL of distilled water for 30 sec (Korkeala, Mäki-Petais, Alanko & Sorvettula, 1986).

The colour parameters (L*, a*, b*) were evaluated using a tristimulus analyser (Minolta Chroma Meter CR-200; Azuchi-Machi Higashi-Ku, Osaka 541, Japan) with the CIElab (1976). The L*a*b* color system consists of a luminance or lightness component (L*) and two chromatic components: the a* which goes from green (-a*) to red (+a*) and the b* which ranges from blue (-b*) to yellow (+b*). The colorimeter was calibrated using a standard pink plate. It has an 8 mm diameter measuring area and uses diffuse illumination and 0° viewing angle (spectral component included) for accurate measurement of a wide variety of subjects. The WHC was estimated by centrifuging 1 g of muscle for 4 min at 1,500 x g and determining the residual water by drying the sample at 70 °C overnight (Cyril, Castellini & Dal Bosco, 1996). Meat tocopherol ($\alpha$-tocopherol and its isoform $\beta$+$\gamma$ and $\delta$) and retinol contents were quantified by HPLC according to Hewavitharana, Lanari & Becu (2004). In particular, 500 μL of distilled water and 1 mL of ethanol were added to 500 □g of sample and then vortexed for 10 sec. Successively, 0.2 mL hexane and butylhydroxytoluene (0.01%) were added and the mixture was carefully shaken and centrifuged. An aliquot of supernatant (0.8 mL) was taken and injected into the HPLC (CM 4000, Milton Roy, Riviera Beach, FL, USA), using a silica column (Beckman, Fullerton, CA, USA). Fluorescence detection was performed with a spectrofluorimeter (excitation and emission wavelengths of 292 nm and 330 nm, respectively).

The extent of muscle lipid peroxidation was evaluated by a spectrophotometer (set at 532 nm, Hitachi U-2000, Theodor - Heuss - Anlage 12, Mannheim, F.R. Germany), which measured the absorbance of thio-barbituric acid-reactive substances (TBARs), and a tetraethoxypropane calibration curve in sodium acetate buffer (pH=3.5, Dal Bosco, Mugnai, Mourvaki, Cardinali, Moscati, Paci & Castellini, 2009). Oxidation products were quantified as malondialdehyde (MDA) equivalents (mg MDA/kg muscle).

The FA profile of meat was determined by gas-chromatography (Fisons mega 2, equipped with a flame ionization detector; Fisons Instruments S.p.A., Rodano, Milano, Italy) after lipid extraction (Folch, Lees & Stanley, 1957) and consecutive hot derivatization with a methanolic solution of sulfuric acid (3%). Separation of the resulting fatty acid methyl esters (FAME) was carried out on an Agilent (J&W) capillary column (30 m x 0.25 mm ID) coated with a DB-Wax stationary phase (film thickness of 0.25 mm). The individual FAME were identified by reference to the retention time of authentic FAME standards. The FA composition of the samples was expressed as a percentage of total FA and calculated with Chrom-Card software.

*2.3. Statistical Analysis*

Meat characteristics were evaluated with a linear model for the analysis of repeated measures estimating the interactive effect of time (1..9 days) x treatment (StataCorp, 2005 – GLM procedure). The statistical significance of differences was assessed by a multiple t-test.

## 3. Results and discussion

In Table 2, the physical characteristics of the LD muscle are presented. Independently to the dietary treatment, storage at simulated retail display conditions significantly increased pH ($P<0.0001$; data not shown). During storage, pH variations depend on two opposite events: the hydrolysis of proteins, with $NH_3$ release, and the hydrolysis of lipids with release of FA (Cabanes, Ouhayoun & Gilbert, 1996). We observed the maximum pH value in all groups after six days of storage and successively a reversal trend (average pH values = 5.83, 5.88, 5.93 and 5.90, for day 1, 3, 6 and 9, respectively), probably for the above mentioned release of free FA. Concerning WHC it should be noted that the refrigeration for long periods should reduce it due to membrane breakage; however we observed an opposite situation with an improvement of WHC during storage, probably due to the higher pH and to the lower water content of the meat.

**Table 2.** Effect of the dietary Spirulina (*Arthrospira platensis*) and Thyme (*Thymus vulgaris*) supplementation on some physical characteristics of the *Longissimus dorsi* muscle during display

| | Experimental groups | | | | | | | *P*-value | Pooled SE |
|---|---|---|---|---|---|---|---|---|---|
| | C | C-S | C-T | C-ST | S | T | ST | | |
| Day 1 | | | | | | | | | |
| pH | 5.81 | 5.90 | 5.80 | 5.85 | 5.90 | 5.82 | 5.83 | ns | 0.22 |
| L* | 56.1 | 56.0 | 57.0 | 57.0 | 58.0 | 55.4 | 56.9 | ns | 3.10 |
| a* | 3.61 | 4.40 | 3.20 | 3.74 | 3.60 | 3.02 | 3.41 | 0.042 | 1.46 |
| b* | 1.72 | 0.90 | 0.90 | 0.78 | 1.10 | 1.64 | 0.89 | 0.003 | 0.88 |
| WHC (%) | 56.2 | 57.0 | 58.0 | 56.7 | 57.0 | 58.9 | 57.2 | ns | 4.30 |
| Day 3 | | | | | | | | | |
| pH | 5.88 | 5.90 | 5.90 | 5.89 | 5.90 | 5.86 | 5.86 | ns | 0.28 |
| L* | 56.9 | 57.0 | 57.0 | 57.6 | 58.0 | 56.3 | 57.5 | ns | 2.92 |
| a* | 3.80 | 4.50 | 3.40 | 3.70 | 3.80 | 3.28 | 3.50 | 0.031 | 1.02 |
| b* | 1.79 | 1.20 | 1.50 | 1.26 | 1.50 | 1.66 | 1.46 | ns | 0.54 |
| WHC (%) | 56.8 | 58.0 | 58.0 | 56.1 | 57.0 | 59.0 | 57.6 | 0.027 | 1.85 |
| Day 6 | | | | | | | | | |
| pH | 5.92 | 5.90 | 5.90 | 5.92 | 5.90 | 5.93 | 5.95 | ns | 0.19 |
| L* | 56.9 | 56.0 | 59.0 | 57.6 | 59.0 | 56.1 | 58.4 | ns | 4.52 |
| a* | 3.80 | 5.00 | 3.10 | 3.51 | 4.10 | 3.12 | 4.95 | 0.046 | 0.98 |
| b* | 1.79 | 1.60 | 1.50 | 1.65 | 1.90 | 1.74 | 1.89 | ns | 0.61 |
| WHC (%) | 56.8 | 59.0 | 58.0 | 57.9 | 57.0 | 59.9 | 56.9 | 0.005 | 1.78 |
| Day 9 | | | | | | | | | |
| pH | 5.89 | 5.90 | 5.90 | 5.90 | 5.90 | 5.90 | 5.92 | ns | 0.18 |
| L* | 58.3 | 59.0 | 59.0 | 58.2 | 58.0 | 58.9 | 61.1 | ns | 4.51 |
| a* | 4.85 | 5.20 | 4.50 | 5.32 | 4.80 | 4.44 | 5.54 | 0.035 | 1.30 |
| b* | 1.90 | 1.70 | 1.70 | 1.74 | 2.30 | 1.80 | 2.25 | 0.004 | 0.92 |
| WHC (%) | 57.90 | 58.00 | 59.00 | 57.50 | 57.00 | 59.70 | 57.8 | ns | 3.50 |

No.=35 per day; ns: not significant.

The LD meat colour was affected by the storage time with an increase of L*, a* and b* values in accordance with the findings of Cabanes-Roiron, Ouhayoun & Gilbert (1994). Concerning the effect of Spirulina and Thyme, meat colour differences were mainly observed on a* (redness) and b* (yellowness), even if it did not always reach the statistical significance. In particular, C-T and T treatments showed the lower values for a* at day 1 of display, as well as at the end of storage.

The a* value lowering observed only in rabbits fed T diet, could be explained by a less intense oxidation of myoglobin with consequent lower levels of metmyoglobin. This could also explain the slight higher WHC of C-T and T groups compared to the average value of C, C-S, C-ST, S and ST groups (58.7 and 59.7 *vs* 57.7%).

As expected, TBARs values increased during storage in all groups (Table 3). At day 1, the highest value was recorded in the S group (even if not significant) and the lowest in the T one. The rate of oxidative processes during storage was not similar for all treatments. In fact, at the end of trial, the highest MDA content was observed in C and the lowest in T treatment, followed by the C-T one ($P<0.001$). This is quite surprising because of the demonstrated *in vitro* antioxidant activity of Spirulina (Wang, Pan, Sheng, Xu & Hu, 2007) and the reason of the lack of the same positive effect in muscle tissue is unclear. Eid et al., (2011) reported that feeding rabbits with diets containing 0.5% of green tea (very rich in catechins), significantly decreased TBARs of the thigh and loin rabbit meat stored for two months, but did not affect Total Reactive Antioxidant Potential values of the rabbit serum. These results would confirm the hypothesis of different mechanisms of action exerted by the different antioxidants in various vegetal essences (scavenger *in vivo*, chain-breaking in membrane, etc.). Concerning Thyme, it has a strong antioxidant activity related to the high content of thymol and carvacrol; indeed biphenyl compounds, dimerization products of thymol and carvacrol and a flavonoid (eriodicytol), have also been isolated as efficient antioxidants inhibiting superoxide anion production in the xanthine/xanthine oxidase system and mitochondrial and microsomal lipid peroxidation (Kahkonen, Hopia, Vuorela, Rauha, Pihlaja, Kujala & Heinonen, 1999).

**Table 3.** Effect of the dietary Spirulina (*Arthrospira platensis*) and Thyme (*Thymus vulgaris*) supplementation on antioxidant contents (□g/g) and TBARs level (mg MDA/100 g) of the *Longissimus dorsi* muscle during display

| | Experimental groups | | | | | | | *P*-value | Pooled SE |
|---|---|---|---|---|---|---|---|---|---|
| | C | C-S | C-T | C-ST | S | T | ST | | |
| Day 1 | | | | | | | | | |
| α-toc | 305.6 | 234.8 | 472.3 | 284.0 | 236.8 | 423.2 | 256.2 | <0.001 | 109.1 |
| γ+β-toc | 2.70 | 2.12 | 5.14 | 2.46 | 2.25 | 3.72 | 2.23 | <0.001 | 1.62 |
| δ-toc | 37.6 | n.d. | n.d | n.d. | n.d. | n.d. | 43.4 | 0.035 | 5.46 |
| Retinol | 12.8 | 15.9 | 15.2 | 13.0 | 17.2 | 11.8 | 11.3 | 0.002 | 3.88 |
| TBARs | 0.15 | 0.15 | 0.15 | 0.15 | 0.18 | 0.14 | 0.16 | ns | 0.08 |
| Day 3 | | | | | | | | | |
| α-toc | 250.2 | 213.0 | 372.3 | 261.8 | 225.3 | 400.1 | 242.3 | <0.001 | 120.5 |
| γ+β-toc | 2.14 | 2.00 | 4.85 | 2.40 | 2.20 | 3.00 | 2.11 | <0.001 | 1.23 |
| δ-toc | 27.0 | n.d. | n.d | n.d. | n.d. | n.d. | 30.1 | 0.031 | 3.40 |
| Retinol | 12.7 | 15.1 | 14.8 | 13.2 | 17.0 | 11.1 | 11.1 | <0.001 | 0.88 |
| TBARs | 0.23 | 0.21 | 0.19 | 0.20 | 0.22 | 0.15 | 0.24 | 0.0270 | 0.06 |
| Day 6 | | | | | | | | | |
| α-toc | 175.8 | 184.2 | 350.1 | 239.1 | 216.2 | 315.7 | 174.9 | <0.001 | 109.1 |
| γ+β-toc | 1.78 | 1.52 | 3.26 | 2.26 | 1.65 | 3.12 | 1.54 | 0.004 | 1.62 |
| δ-toc | 15.4 | n.d. | n.d | n.d. | n.d. | n.d. | 21.5 | 0.035 | 1.46 |
| Retinol | 11.1 | 13.0 | 11.9 | 12.7 | 15.7 | 10.9 | 10.8 | ns | 0.88 |
| TBARs | 0.26 | 0.22 | 0.2 | 0.22 | 0.24 | 0.17 | 0.27 | 0.005 | 0.05 |
| Day 9 | | | | | | | | | |
| α-toc | 125.1 | 157.4 | 257.9 | 225.6 | 212.5 | 269.9 | 157.1 | <0.001 | 95.4 |
| γ+β-toc | 1.51 | 1.13 | 2.90 | 2.08 | 1.35 | 2.71 | 1.37 | ns | 1.12 |
| δ-toc | 15.9 | n.d. | n.d. | n.d. | n.d. | n.d. | 15.2 | 0.012 | 6.80 |
| Retinol | 10.9 | 12.2 | 10.5 | 12.5 | 15.7 | 10.7 | 10.5 | ns | 4.52 |
| TBARs | 0.30 | 0.24 | 0.23 | 0.28 | 0.29 | 0.20 | 0.27 | <0.001 | 0.05 |

No.=35 per day; ns: not significant.

Lee, Umano, Shibamoto & Lee (2005) identified twelve aroma constituents of Thyme and evaluated their antioxidant activities. Eugenol, thymol, carvacrol, and 4-allylphenol showed strong antioxidant activities that were comparable or higher to those of the standard antioxidants, α-tocopherol and butylated hydroxy toluene. These claims are also supported by a comparison with the results of our previous study, which was carried out to assess the effect of the combined action of dietary vitamin E and ascorbic acid on the oxidative status of rabbit meat, using the same storage condition protocol of the present trial (Castellini, Dal Bosco & Bernardini, 2000). Surprisingly, T feeding group showed a meat TBARs value similar to that of meat whose rabbits fed a diet supplemented with 200 IU of α-tocopheryl acetate and 1000 mg/L of ascorbic acid.

Concerning the amount of some antioxidants in the LD muscle during the trial, time had a significant effect in all groups ($P<0.0001$; data not shown), showing a decreasing trend in antioxidants amount during the display period. In agreement with the above mentioned results, the groups supplemented with Thyme only (C-T and T) showed the highest content of α-tocopherol, at the beginning and at the end of the storage period ($P<0.001$). This situation can be explained considering that Thyme is, as already said, a good source of powerful antioxidants such as phenols, but also such as ascorbic acid and tocopherols (Youdim & Deans, 2000; Lee et al., 2005; Barros, Heleno, Carvalho & Ferreira, 2010).

The FA profile of the LD meat (Table 4) differed on the basis of the inclusion level of T and S supplements, that was characterized by amounts of total *n*-3 and *n*-6 FA of 3.13 and 11.35, and 31.7, and 21.08 %, respectively (data not shown). Thus, Thyme presence (C-T and T-T groups) determined a significant increase in meat of *n-3* FA both at the beginning and at the end of the trial ($P<0.001$), whereas, at day 1, S group showed the highest amount of *n-6* FA and thus PUFA levels ($P<0.01$). Specifically, the meat of S and ST animals presented the highest amount of C18:2*n*-6, and S, C-ST and ST the highest values of C18:3*n*-6 ($P<0.001$). The total *n*-6 FA increase in LD meat of rabbits fed S diets was also evidenced by Peiretti & Meineri (2011) who investigated the effects of four inclusion levels (0, 5, 10, or 15 %) of Spirulina on meat quality of growing rabbits.

Further confirmation on the positive action of Thyme have been obtained from the analysis of the percentage increase of the TBA-Rs and leakages of n-3 FA during the period of display (Table 5). C-T and T treatments induced significantly ($P<0.05$) lower increases of lipid oxidation and at the same time the lower losses of *n-3* FA acid. Only the ST treatment caused a non-linear trend between increase of TBARs and loss of *n-3* FA, with a high development of oxidative process not accompanied by significant reductions of polyunsaturated fatty acids.

In agreement with the above mentioned statements, Thyme-supplemented diets (C-T and T) showed a significant reduction of the drip loss during display and such improvement was probably

due to the positive effect of Thyme antioxidants on the integrity of muscle fibres, thus implementing their capability to retain water. With regards to poultry meat, Asghar, Lin, Gray, Buckley, Booren, Crackel & Flegal (1989) suggested that antioxidants preserve the functionality of membranes and thus improve their role as semi permeable barriers against exudative loss. According to Cheah, Cheah & Krausgrill (1995) the beneficial effect of dietary antioxidants on drip loss is due to their ability to stabilize membranes, presumably

achieved by inhibiting the phospholipase $A_2$ activity and by lowering $Ca^{++}$ release, determining in turn, a reduction in the rate of post-mortem glycolysis with a subsequently higher pH. An analogous positive action on drip loss was reported by Monahan, Buckley, Gray & Morrissey (1990) and Mitsumoto, Arnold, Schaefer & Cassens (1995) in pork and beef meat, respectively. Contrary to our expectations, supplementing the diet with Spirulina had no substantial effect on the membrane integrity of rabbit muscle.

Different hypothesis could be advanced to explain such trend. Firstly, the bioactive compounds of Spirulina have a demonstrated scavenger effect in the reduction of free radicals *in vivo*, but the effect in the reduction of oxidation processes in meat membrane phospholipids is not demonstrated. Another possibility is related to the eventual pro-oxidant effect of these compounds at certain levels. Some Authors investigated this issue, and concluded that the phycocyanin, which is abundant in Spirulina, can become pro-oxidant at certain conditions and concentrations (Macari, Putin, Gudumac, Rudic, Macari & Pavlicenco, 2011).

**Table 4.** Effect of the dietary Spirulina (*Arthrospira platensis*) and Thyme (*Thymus vulgaris*) supplementation on fatty acid profile (% total FAME) of the *Longissimus dorsi* muscle during display

| | Experimental groups | | | | | | | *P*-value | Pooled SE |
|---|---|---|---|---|---|---|---|---|---|
| | C | C-S | C-T | C-ST | S | T | ST | | |
| Day 1 | | | | | | | | | |
| Saturated | 41.62 | 39.01 | 41.20 | 41.67 | 39.37 | 39.38 | 39.43 | ns | 4.30 |
| C14:0 | 2.49 | 2.33 | 2.63 | 2.59 | 2.07 | 2.13 | 2.05 | ns | 0.22 |
| C16:0 | 29.62 | 28.9 | 30.4 | 30.0 | 29.9 | 29.85 | 30.09 | ns | 0.58 |
| C18:0 | 7.56 | 6.33 | 6.39 | 7.16 | 5.90 | 6.04 | 6.01 | ns | 1.41 |
| Others | 1.95 | 1.47 | 1.78 | 1.90 | 1.46 | 1.36 | 1.28 | ns | 0.18 |
| Monounsaturated | 28.43 | 30.31 | 29.93 | 29.56 | 28.39 | 29.38 | 29.44 | ns | 2.62 |
| C16:1 *n*-7 | 5.31 | 5.93 | 5.57 | 5.63 | 5.08 | 5.14 | 4.93 | ns | 0.59 |
| C18:1 *n*-9 | 22.63 | 23.8 | 23.9 | 23.4 | 23.0 | 23.42 | 23.87 | ns | 2.10 |
| Others | 0.49 | 0.62 | 0.48 | 0.54 | 0.31 | 0.82 | 0.64 | ns | 0.14 |
| Polyunsaturated | 29.60 | 30.52 | 28.93 | 28.87 | 32.32 | 31.20 | 31.30 | 0.0066 | 3.35 |
| C18:2 *n*-6 | 22.15 | 23.40 | 21.55 | 21.60 | 25.19 | 23.53 | 24.29 | <0.001 | 3.09 |
| C18:3 *n*-6 | 0.25 | 0.29 | 0.33 | 0.58 | 0.79 | 0.22 | 0.54 | <0.001 | 0.73 |
| C20:4 *n*-6 | 3.51 | 3.72 | 2.91 | 3.15 | 3.60 | 2.99 | 3.39 | ns | 0.34 |
| C18:3 *n*-3 | 2.10 | 1.58 | 2.50 | 2.03 | 1.29 | 2.72 | 1.49 | <0.001 | 0.44 |
| C20:5 *n*-3 | 0.12 | 0.12 | 0.23 | 0.14 | 0.19 | 0.69 | 0.48 | <0.001 | 0.05 |
| C22:5 *n*-3 | 0.57 | 0.43 | 0.54 | 0.51 | 0.17 | 0.14 | 0.11 | <0.001 | 0.07 |
| C22:6 *n*-3 | 0.01 | 0.04 | 0.28 | 0.30 | 0.28 | 0.25 | 0.14 | ns | 0.05 |
| Others | 0.90 | 0.94 | 0.59 | 0.56 | 0.81 | 0.77 | 0.86 | 0.0245 | 0.25 |
| $\Sigma$ *n*–6 | 25.91 | 27.41 | 24.79 | 25.33 | 29.58 | 26.74 | 28.22 | <0.001 | 3.62 |
| $\Sigma$ *n*–3 | 3.00 | 2.17 | 3.69 | 3.09 | 1.96 | 3.79 | 2.26 | <0.001 | 0.81 |

| | Experimental groups | | | | | | | *P*-value | Pooled SE |
|---|---|---|---|---|---|---|---|---|---|
| | C | C-S | C-T | C-ST | S | T | ST | | |
| Day 9 | | | | | | | | | |
| Saturated | 43.24 | 39.70 | 41.40 | 43.70 | 41.70 | 40.90 | 41.03 | 0.0009 | 3.21 |
| C14:0 | 2.97 | 2.86 | 3.14 | 3.52 | 3.84 | 3.39 | 3.26 | ns | 0.60 |
| C16:0 | 30.33 | 29.90 | 30.60 | 31.20 | 30.30 | 28.61 | 29.65 | ns | 1.94 |
| C18:0 | 7.59 | 5.04 | 5.78 | 6.28 | 5.81 | 7.30 | 6.30 | <0.001 | 1.36 |
| Others | 2.35 | 1.88 | 1.86 | 2.74 | 1.72 | 1.60 | 1.82 | 0.0287 | 0.35 |
| Monounsaturated | 27.36 | 32.90 | 31.30 | 29.30 | 28.30 | 28.41 | 28.89 | <0.001 | 4.25 |
| C16:1 *n*-7 | 4.74 | 7.37 | 6.74 | 6.14 | 5.38 | 5.52 | 5.82 | 0.0001 | 1.10 |
| C18:1 *n*-9 | 22.23 | 24.90 | 24.00 | 22.80 | 22.70 | 22.64 | 22.74 | 0.0219 | 2.06 |
| Others | 0.39 | 0.62 | 0.49 | 0.39 | 0.25 | 0.25 | 0.33 | 0.0421 | 0.14 |
| Polyunsaturated | 28.76 | 27.10 | 27.43 | 27.15 | 29.77 | 30.77 | 30.90 | <0.001 | 3.40 |
| C18:2 *n*-6 | 21.38 | 21.00 | 20.20 | 20.60 | 24.10 | 23.88 | 23.64 | <0.001 | 3.97 |
| C18:3 *n*-6 | 0.59 | 0.33 | 0.52 | 0.41 | 0.57 | 0.11 | 0.63 | ns | 0.11 |
| C20:4 *n*-6 | 3.45 | 3.15 | 2.81 | 2.97 | 2.95 | 2.53 | 3.09 | ns | 0.26 |
| C18:3 *n*-3 | 2.33 | 1.50 | 2.86 | 2.20 | 1.28 | 2.92 | 1.88 | <0.001 | 0.64 |
| C20:5 *n*-3 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.5 | 0.1 | <0.001 | 0.01 |
| C22:5 *n*-3 | 0.22 | 0.29 | 0.39 | 0.33 | 0.14 | 0.12 | 0.08 | 0.0031 | 0.15 |
| C22:6 *n*-3 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.10 | 0.10 | 0.0039 | 0.00 |
| Others | 0.69 | 0.79 | 0.55 | 0.52 | 0.63 | 0.64 | 1.38 | <0.001 | 0.30 |
| $\Sigma$ *n*–6 | 25.42 | 24.43 | 23.48 | 24.00 | 27.60 | 26.52 | 27.36 | <0.001 | 4.14 |
| $\Sigma$ *n*–3 | 2.80 | 1.96 | 3.56 | 2.75 | 1.84 | 3.66 | 2.21 | <0.001 | 0.92 |

No.=35 per day. ns: not significant.

**Table 5**. Trend of TBARs, n-3 fatty acids and drip loss (%) in the *Longissimus dorsi* muscle during retail display (Day 1 *vs* Day 9)

| | Δ TBARs | Δ n-3 FA during display | Drip loss |
|---|---|---|---|
| C | $48.99^{c}$ | $- 6.67^{b}$ | $6.87^{b}$ |
| C-S | $36.02^{b}$ | $- 9.09^{c}$ | $7.05^{b}$ |
| C-T | $31.44^{a}$ | $- 9.68^{c}$ | $3.96^{a}$ |
| C-ST | $47.55^{c}$ | $- 2.70^{a}$ | $9.68^{c}$ |
| S | $38.83^{b}$ | $- 5.00^{b}$ | $7.80^{b}$ |
| T | $29.80^{a}$ | $- 4.35^{b}$ | $3.90^{a}$ |
| ST | $44.24^{bc}$ | $- 2.63^{a}$ | $8.80^{bc}$ |
| $X^2$ | 6.32 | 1.52 | 2.13 |

No.=35 per day; a..c: P<0.05.

## 4. Conclusions

In conclusion, Thyme improved colour parameters and reduced exudative losses during a simulated retail display, also considering a shorter supplementation period (C-T group). This situation could determine a better impact on consumers at the time of purchase, and the shorter supplementation period would be a good compromise between Thyme's efficacy and farmers demand to limit production costs.

Contrarily to our expectations, also considering the encouraging results obtained in our related study on the effect of these supplements on bacterial community in the caecum and caecal fermentation of rabbits (Vántus, Bónai, Zsolnai, Dal Bosco, Szendrő, Tornyos, Bodnár, Morsy, Pósa, Toldi, Bóta, Kovács & Dalle Zotte, 2012), dietary supplementation of Spirulina had no effect on oxidative stability of rabbit meat, maybe for the poor absorption from the gut as a result of interference on uptake of antioxidants by Spirulina. It is conceivable that the dietary level of Spirulina was not adequate for rabbit requirements for the reduction of tocopherol deposition and its failure to decrease TBARs and drip loss. However, from the scientific viewpoint the mechanism of the disconnect between TBARs and protection of *n-3* FA is worthy of further investigations.

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# CHAPTER 3d

Running Head: Dietary Spirulina and Thyme to growing rabbits

# Dietary Spirulina (*Arthrospira platensis*) and Thyme (*Thymus vulgaris*) supplementation to growing rabbits: effects on raw and cooked meat quality, nutrient true retention and oxidative stability

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## ABSTRACT

The aim of this experiment was to study the effect of Spirulina and Thyme supplementation on raw and cooked rabbit meat quality, nutrient true retention and protection against oxidative stress conditions. With this purpose, at weaning, a total of 294 rabbits were assigned to 7 dietary treatments: rabbits in the control group (C-C) received a pellet without any supplementation throughout the experiment (5-11 weeks of age). In the other groups, the pellet contained 5% Spirulina (S), 3% Thyme (T), or both (ST) for either the entire (5-11 weeks: groups S-S, T-T, ST-ST) or only the final part of the growing period (8-11 weeks: groups C-S, C-T, C-ST). For this trial, *Longissimus thoracis et lumborum* (LTL) and hind leg (HL) cuts were considered. The 5% S supplement was effective in increasing the γ-linolenic acid (GLA) and dihomo-GLA contents of LTL and HL rabbit meat. Thyme improved the oxidative stability of raw and freeze-dried HL meat but not that of cooked meat.



**Keywords:** Spirulina, Thyme, Rabbit meat, True Retention, GLA, TBARS

## 1. Introduction

Meat in itself can be considered a functional food to the extent that it naturally contains many nutritive elements essential for humans, such as protein, fats, vitamins, essential amino acids, and minerals (Zhang, Xiao, Samaraweera, Lee, & Ahn, 2010). Within the heterogeneous scenario of fresh meat, rabbit meat is an interesting white meat ideal for modern consumers who are increasingly aware of the link between diet and health and see it as a valuable way to improve the quality of their lives (Olmedilla-Alonso, Jiménez-Colmenero, & Sánchez-Muniz, 2013). Rabbit meat is appreciated for its vitamin $B_{12}$ content, considered the highest among all the most common animal meat species, for its low sodium (37.0 and 49.5 mg/100 g of meat, in *Longissimus thoracis et lumborum* and hind leg meat, respectively), and low cholesterol (47.0 and 61.2 mg/100 g meat in *Longissimus thoracis et lumborum* and hind leg, respectively), with proteins providing 80% of its total energy value (Dalle Zotte & Szendrő, 2011).

Meat in general can be also fortified with functional ingredients to further improve its nutritional value and thus meet consumers' nutritional expectations (Arihara, 2006). One potential functional ingredient currently being adopted in human and animal nutrition, especially in aquaculture, is Spirulina (*Arthrospira platensis*), a blue-green microalgae well known for being rich in highly digestible protein and essential amino acids, minerals, vitamins (β-carotene and carotenoids, provitamin A, $B_{12}$, and other B vitamins), and PUFA (Belay, 2002). Specifically, it is one of the few sources of γ–linolenic acid (GLA) known to possess serum triglycerides and low-density lipoprotein lowering action, anti-inflammatory and immune-regulatory effects, and down-regulation of atherogenic potential by enhancing macrophage series-1 prostaglandin biosynthesis (Dawczynski, Hackermeier, Viehweger, Stanger, Springer, & Jahreis, 2011; Fan & Chapkin, 1998). Furthermore, *in vitro* and *in vivo* studies on rats and mice (Deng & Chow, 2010) have shown Spirulina to provide a certain antioxidant activity, even if Dal Bosco, Gerencsér, Szendrő et al., (2014) observed no protective effect by Spirulina dietary supplementation against lipid oxidation in rabbit meat during retail display. Spirulina naturally grows in high-salt alkaline conditions and is commercially available in liquid mediums with light energy, air, and mineral requirements (Mahajan & Kamat, 1995). It provides high yields and can be produced by exploiting desalinated waste water, and animal faecal waste, and then safely fed back to livestock, in this way promoting high land-use efficiency (Holman & Malau-Aduli, 2013). Despite all these positive characteristics, research into potential application of Spirulina to monogastric feed rations has been directed almost exclusively towards productive performance, whereas studies concerning rabbit meat quality are still limited (Peiretti & Meineri, 2009; Peiretti & Meineri, 2011).

One negative drawback to fortifying meat with higher PUFA content is the resulting greater susceptibility to lipid oxidation, which is one of the leading causes of meat deterioration in both nutritive and visual value and overall sensory quality (Cullere, Hoffman, & Dalle Zotte, 2013). Antioxidants have been added to cope with the problem, but because synthetic compounds like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are linked to health risks and consumers prefer more natural ingredients, efforts have been made in the last decade to find alternative functional supplements. Those most subjected to increasing study are herbal plants and derivatives, with many works demonstrating their protective effect against oxidation when directly added to meat and meat products (Formanek, Kerry, Higgins, Buckley, Morrissey, & Farkas, 2001; Estévez, Ventanas, & Cava, 2006). One is Thyme (*Thymus vulgaris*), a perennial Mediterranean herb in the *Lamiaceae* family. It is rich in PUFA, widely known, and appreciated for its proven antimicrobial, antifungal, antibacterial, and antioxidant actions principally due to the presence of phenolic compounds thymol and carvacrol (Kykkidou, Giatrakou, Papavergou, Kontominas, & Savvaidis, 2009; Jabri-Karoui, Bettaieb, Msaada, Hammami, & Marzouk, 2012). Despite the fact that thyme has been widely studied in poultry farming as a growth promoter alternative to antibiotics, its effect as a functional ingredient in improving meat fatty acid profile (FA) and oxidative stability has only been evaluated in ewes (Nieto, Banñón, & Garrido, 2011; Martínez, 2013). In light of the above, the objective of this study was to introduce Spirulina (S) powder and/or Thyme (T) dried leaves in the diet of growing rabbits in order to enhance the nutritional characteristics of their meat and improve its stability under severe oxidative stress conditions. This study focuses on *Longissimus thoracis et lumborum* (LTL) meat, the leanest cut in the rabbit carcass, and hind leg (HL) meat, the most quantitatively important (Dalle Zotte, 2002).

This study is part of a wider research that has also considered rabbit productive performance, health status, the apparent digestibility of the diets (Gerencsér, Szendrő, Matics, et al., 2014), microbial diversity in caecum and caecal fermentation (Vàntus, Bonai, Zsolnai, et al., 2012), carcass composition, vitamin $B_{12}$ absorption into meat cuts, meat rheological traits, bone development (Dalle Zotte, Cullere, Sartori, et al., 2014), the FA profile of the meat, and its oxidative status during retail display (Dal Bosco et al., 2014).

## 2. Materials and Methods

### *2.1. Analyses of Spirulina, Thyme and experimental diets*

The Spirulina powder and Thyme dried leaves adopted were commercial products bought from a private company and supplemented to rabbit feed just before pelleting. Chemical composition of Spirulina and Thyme supplements (Table 1) was determined in duplicate using AOAC (2000) methods in order to measure the concentrations of dry matter (DM; procedure 934.01), crude protein (CP; procedure 2001.11), crude fibre (CF; procedure 978.10), ash (procedure 967.05), and starch (amyloglucosidase- α-amylase method, 996.11). Ether extract (EE) was determined after acid-hydrolysis (EC, 1998). Neutral detergent fibre (NDF, without sodium sulphite), acid detergent fibre (ADF), acid detergent lignin (ADL) and acid-insoluble ash (AIA) were analyzed according to Mertens (2002), AOAC (2000, procedure 973.187) and Van Soest, Robertson, & Lewis (1991) using the sequential procedure and filter bag system (Ankom Technology, New York) respectively. GE was measured with an adiabatic bomb calorimeter (ISO 1998). Mineral analyses (Ca, P) were performed on Spirulina and Thyme supplements by ICP-OES (Spectro Ciros Vision EOP) after microwave digestion (AOAC 2000, 999.10). The fatty acid extraction of Spirulina and Thyme supplements and that of the experimental diets (Tables 2 and 3) was conducted by Accelerated Solvent Extraction (M-ASE) in which petroleum ether was the solvent used for extraction. Subsequently, fatty acid methyl esters (FAMEs) were determined following the same procedure below described for raw and cooked rabbit meat samples.

### *2.2. Rabbits and experimental design*

A total of 294 maternal line rabbits of the Pannon breeding program were farmed at the experimental rabbitry of Kaposvár University (Hungary), where they were fed a control pelleted diet (C) until weaning, at 35 days of age. Animals were subsequently randomly allocated into 7 dietary groups (No. = 42 rabbits/group) and housed by 3 in wire net cages (0.61 x 0.32 m). Rabbits in the control group (C-C) received a pellet without any supplementation throughout the experiment, from 5 to 11 weeks of age. In the other groups, the pellet contained 5% Spirulina (S), or 3% Thyme (T), or both (ST) for either the entire (5-11 weeks: groups S-S, T-T, ST-ST), or only the end of the growing period (8-11 weeks: groups C-S, C-T, C-ST). These two supplementation lengths were planned for cost-reduction purposes.

Diets were isonitrogenous, isoenergetic, and did not include coccidiostatics (Gerencsér, et al., 2014). Water and pellets were available *ad libitum;* the temperature and photoperiod in the rabbitry were 15-18 °C and 16L:8D, respectively.

*2.3. Sample collection, cooking procedure and freeze-drying*

At the end of the feeding period, at 11 weeks of age, rabbits (n=35, 34, 34, 36, 35, 36 and 36 rabbits for C-C, C-S, C-T, C-ST, S-S, T-T and ST-ST groups, respectively) were electrically stunned, slaughtered by cutting the carotid arteries and jugular veins, and carcasses were chilled for 24 h at +3 °C. Subsequently, according to the procedures of the World Rabbit Association (Blasco & Ouhayoun, 1996), *Longissimus thoracis et lumborum* (LTL) muscles and hind legs (HL) were excised from 15 and 10 randomly selected rabbits per dietary treatment, respectively. The right side of each LTL was cut and meat was ground using a Retsch Grindomix GM 200 (10 seconds at 4000 rpm). Subsequently, each sample was divided in two sub-samples: the first was packed (fresh) in plastic bags and put at -20 °C until further analysis, whereas the second was freeze-dried (FD) and then stored in the same conditions as the fresh sub-sample. The same procedure described for right LTL muscle was adopted also for right HL. Left LTL and HL were individually vacuum-sealed in cooking PVC bags and cooked in a water bath. The cooking procedures were 80 °C for 1h and 85 °C for 2.30 h for LTL and HL, respectively. Afterwards, each sample was chilled with cold water, removed from its PVC bag, and separated into two sub-samples: the first was placed directly into a new plastic bag (fresh), whereas the second was FD. Both sub-samples were then stored at -20 °C until subsequent analysis.

*2.4. Proximate composition, cholesterol and heme iron contents, nutrient True Retention and TBARS*

Proximate composition (AOAC, 1995) was determined on raw (right part) and cooked (left part) freeze dried LTL (No.=15 samples/group) and HL (No.=10 samples/group) meat samples, with protein content calculated by difference. Cholesterol content was determined as specified by Casiraghi, Lucisano, Pompei, & Dellea (1994) and heme iron content measured using the method described by Hornsey (1956). Nutrient true retention (TR) was calculated using the formula provided by Murphy, Criner, & Gray (1975). The extent of muscle lipid peroxidation in raw, cooked, fresh, and FD rabbit meat was evaluated by a spectrophotometer (set at 532 nm, Hitachi U-2000, Theodor - Heuss - Anlage 12, Mannheim, F.R. Germany),

which measured the absorbance of thio-barbituric acid-reactive substances (TBARS), and a tetraethoxypropane calibration curve in sodium acetate buffer (pH=3.5, Dal Bosco, Mugnai, Mourvaki, et al., 2009). Oxidation products were quantified as malondialdehyde (MDA) equivalents (mg MDA/kg muscle).

*2.5. Lipid extraction, FA trans esterification and Chromatographic conditions*

Lipid extraction was performed on raw and cooked FD samples (n=7 and n=10 samples/dietary group for LTL and HL, respectively) combining the traditional Folch method (Folch, Lees, & Stanley, 1957) with that provided by Lee, Trevino, & Chaiyawat (1996) and the Accelerated Solvent Extraction (M-ASE), in which chloroform/methanol (1:2) was the binary solvent mixture used for extraction. The sample was shaken in a saline solution (0.5 % NaCl in water) equal to one-fourth of the total extracted volume and allowed to stand for 1 hour in order to obtain a biphasic separation. The lower phase was filtered through filter paper (Whatman No. 1) provided with a layer of anhydrous sodium sulphate. Subsequently, the filter paper was washed with 5 ml of chloroform. Total lipid content was determined gravimetrically after the removal of the solvent (chloroform) by evaporation under nitrogen stream at 50 °C.

In order to determine fatty acid methyl esters (FAMEs), samples were then transmethylated using a methanolic solution of $H_2SO_4$ (4 %). Biphasic separation was obtained by adding 0.5 ml of distilled water and 1.5 ml of N-Heptane to each sample. FAME were quantified by gas chromatography (Shimadzu GC17A, equipped with an Omegawax 250 column (30 m x 0.25 μm x 0.25 μm) and FID detector. Helium was used as carrier gas at a constant flow of 0.8 mL/min. Injector and detector temperatures were 260 °C. Peaks were identified based on commercially available FAME mixtures (37-Component FAME Mix, Supelco Inc., Bellefonte, PA, USA) and the data obtained were expressed as % of total detected FAME.

*2.6. Statistical Analysis*

Data were analyzed using SAS 9.1 statistical analysis software for Windows (SAS, 2008) General Linear Model (GLM) procedures. A One-way ANOVA tested the diet as fixed effect on proximate composition and raw and cooked meat FA profile. Another ANOVA tested the diet, the state condition (raw, cooked) and the storage condition (fresh, freeze-dried) as fixed effects on the TBARS content of LTL and HL meat. A preliminary analysis considered also double (Diet x State; Diet x Storage) and triple (Diet x State x Storage) interactions. As no significant effects were found, only main effects were considered. Least square means were obtained using the Bonferroni test. *P* values were considered significant when <0.05.

## 3. Results and Discussion

*3.1. Spirulina and Thyme chemical composition and FA profile*

The chemical composition and fatty acid (FA) profile of the Spirulina powder and Thyme dried leaves used in this trial are presented in Tables 1 and 2. Spirulina was confirmed as an important source of protein (65.8 g/kg) with 8.6 g/kg of ether extract. Starch accounted for 35.6 g/kg; the Ca/P ration was 0.2. The most abundant FA was palmitic acid (27.8 % of total FA), followed by γ-linolenic acid (21.08 % of total FA). Spirulina contained a high percentage of polyunsaturated FA (PUFA; 38 % of total FA), mainly *n*-6 PUFA, which determined a high *n*-6/*n*-3 ratio (11.15), but also a consistent amount of saturated FA (SFA; 31.8 % of total FA).

The Spirulina chemical composition shown in this study differed markedly from that defined in a work evaluating Spirulina as a possible replacement to soybean meal in diets for broilers (Alvarenga, Rodrigues, Cantarelli, et al., 2011). Particularly, the Spirulina powder used in our study had higher dry matter content than in the other study (94.4 *vs* 88.1 %, respectively) due to its higher crude protein (65.8 *vs* 58.2 %,) and ether extract (8.6 *vs* 2.6 %) contents. Phosphorus content was similar in the two studies, whereas the Ca of the above-mentioned trial was twice that of our work (0.48 *vs* 0.22 %). Different temperatures, *inoculum* age and concentration, UV-B radiation and, even if to a lesser extent, nitrogen concentrations, can vary Spirulina crude protein content considerably, from 46 to more than 70 % of total DM, and can also cause ether extract values to vary from 2.6 to 16 % (Oliveira, Monteiro, Robbs, & Leite, 1999; Pelizer, Danesi, Rangel, et al., 2003; Colla, Bertolin, & Costa, 2004;

Gupta, Bhadauriya, Chahuan, & Bisen, 2008). Regarding the FA profile of *Arthrospira platensis*, observations shared with other studies include the fact that palmitic acid was the most abundant FA, followed by γ-linolenic and linoleic fatty acids. However, as observed for chemical composition, wide variations also in regard to FA profile have been reported and ascribed to the cultivation system in different studies (Ötles & Pire, 2001; Colla et al., 2004).

**Table 1.** Chemical composition (g/kg as is) of Spirulina (*Arthrospira platensis*) and Thyme (*Thymus vulgaris*)

| | *Arthrospira platensis* | *Thymus vulgaris* |
|---|---|---|
| Dry matter (DM) | 943.9 | 889.5 |
| Crude protein (CP) | 658.1 | 52.3 |
| Ether extract (EE) | 8.6 | 31.9 |
| Crude fibre (CF) | nd[1] | 181.5 |
| Ash | 65.1 | 65.9 |
| Neutral Detergent Fibre (NDF) | 2.4 | 298.1 |
| Acid Detergent Fibre (ADF) | 4.8 | 209.6 |
| Acid Detergent Lignin (ADL) | 0.6 | 68.1 |
| Acid Insoluble Ash (AIA) | 0.0 | 4.9 |
| Starch | 35.6 | 58.4 |
| Ca | 2.2 | 13.6 |
| P | 9.2 | 0.7 |
| Ca/P | 0.2 | 18.7 |
| Gross Energy (GE), MJ/kg | 19.5 | 15.7 |

[1]not detected

Thyme chemical composition differed widely from that of Spirulina, and showed 52.3 g/kg of crude protein, 31.9 g/kg of ether extract, and 181.5 g/kg of crude fibre, with a high Ca/P ratio (18.7). PUFA accounted for 43 % of total FA, with linoleic (31.6 % of total FA) and α-linolenic acids (10.25 % of total FA) being the most abundant PUFA. SFA and monounsaturated FA (MUFA) accounted for 18.4 and 32.1 % of total FA, respectively. As regards Thyme, because its essential oil is the active part of the plant responsible for most of its health-promoting properties, research has focused on its composition, also as it varies during different phases of its vegetative cycle (Hudaib, Speroni, Di Pietra, & Cavrini, 2002; Jabri-Karoui et al., 2012). Given the perspectives of potential application in commercial rabbit farming and reducing already expensive feeding costs, we decided to test Thyme dried leaves.

**Table 2.** Fatty Acid profile (% of total FAME) of Spirulina (*Arthrospira platensis*) and Thyme (*Thymus vulgaris*)

| | *Arthrospira platensis* | *Thymus vulgaris* |
|---|---|---|
| C12:0 | 0.84 | 0.07 |
| C14:0 | 0.43 | 0.00 |
| C15:0 | 0.02 | 0.15 |
| C16:0 | 27.8 | 14.1 |
| C17:0 | 0.24 | 0.18 |
| C18:0 | 2.38 | 3.6 |
| C20:0 | 0.02 | 0.00 |
| C22:0 | 0.07 | 0.29 |
| Total SFA | 31.8 | 18.4 |
| C14:1 | 0.87 | 0.00 |
| C16:1 *n*-9 | 6.93 | 0.48 |
| C17:1 | 0.55 | 0.11 |
| C18:1 *n*-9 (Oleic) | 2.19 | 31.3 |
| C18:1 *n*-11 (trans Vaccenic) | 0.87 | 0.06 |
| C20:1 *n*-9 | 0.00 | 0.16 |
| Total MUFA | 11.4 | 32.1 |
| C18:2 *ct n*-6 (Linoleic) | 13.8 | 31.6 |
| C18:3 *n*-6 (γ-Linolenic) | 21.1 | 0.05 |
| C18:3 *n*-3 | 2.97 | 10.3 |
| C20:2 | 0.0 | 0.07 |
| C20:3 *n*-6 | 0.0 | 0.05 |
| C20:3 *n*-3 | 0.0 | 0.17 |
| C20:5 *n*-3 (EPA) | 0.16 | 0.53 |
| C22:5 *n*-3 | 0.0 | 0.35 |
| Total PUFA | 38.0 | 43.1 |
| *n*-6 | 34.9 | 31.7 |
| *n*-3 | 3.13 | 11.3 |
| *n*-6/*n*-3 | 11.2 | 2.80 |

*3.2. FA profile of the experimental diets*

**Table 3**. Fatty acids profile (% of total FAME) of the experimental diets

| | Experimental diets | | | |
|---|---|---|---|---|
| | C | S | T | ST |
| C12:0 | 0.01 | 0.00 | 0.06 | 0.00 |
| C14:0 | 0.32 | 0.26 | 0.29 | 0.29 |
| C15:0 | 0.12 | 0.10 | 0.11 | 0.12 |
| C16:0 | 12.6 | 12.6 | 11.6 | 12.6 |
| C17:0 | 0.17 | 0.16 | 0.18 | 0.13 |
| C18:0 | 3.63 | 3.28 | 3.09 | 3.23 |
| C20:0 | 0.10 | 0.06 | 0.06 | 0.06 |
| C22:0 | 0.00 | 0.00 | 0.00 | 0.08 |
| C23:0 | 0.00 | 0.00 | 0.08 | 0.00 |
| Total SFA | 17.0 | 16.4 | 15.4 | 16.5 |
| C14:1 | 0.01 | 0.04 | 0.02 | 0.05 |
| C15:1 | 0.02 | 0.00 | 0.05 | 0.00 |
| C16:1 | 0.19 | 0.49 | 0.08 | 0.44 |
| C17:1 | 0.05 | 0.10 | 0.05 | 0.12 |
| C18:1 *n*-9 (Oleic) | 20.9 | 21.3 | 21.4 | 20.4 |
| C18:1 *n*-11 (trans Vaccenic) | 0.50 | 0.45 | 0.73 | 0.79 |
| C20:1 *n*-9 | 0.10 | 0.08 | 0.07 | 0.08 |
| Total MUFA | 21.8 | 22.4 | 22.3 | 21.9 |
| C18:2 *ct n*-6 (Linoleic) | 48.5 | 47.3 | 49.5 | 47.4 |
| C18:2 c9-t11 | 0.00 | 0.00 | 0.02 | 0.00 |
| C18:3 *n*-3 | 7.17 | 6.73 | 6.84 | 7.20 |
| C18:3 *n*-6 (γ-Linolenic) | 0.00 | 1.16 | 0.00 | 1.15 |
| C20:2 | 0.05 | 0.05 | 0.05 | 0.09 |
| C20:3 *n*-3 | 0.00 | 0.00 | 0.00 | 0.00 |
| C20:4 *n*-6 | 0.00 | 0.00 | 0.00 | 0.00 |
| C20:5 *n*-3 (EPA) | 0.77 | 0.85 | 0.92 | 0.85 |
| C22:6 *n*-3 (DHA) | 0.00 | 0.00 | 0.00 | 0.00 |
| Total PUFA | 56.5 | 56.1 | 57.3 | 56.7 |
| *n*-6 | 48.5 | 48.5 | 49.5 | 48.5 |
| *n*-3 | 7.94 | 7.58 | 7.76 | 8.05 |
| *n*-6/*n*-3 | 6.1 | 6.4 | 6.4 | 6.0 |

Fatty acid profile of the experimental diets (Table 3) evidenced a low SFA (average value: 16.3 % total FAME) and high PUFA (average value: 56.7 % total FAME) percentages. The latter was mostly attributable to the presence of linoleic acid and linolenic acid which showed average values of 48.2 % and 7.0 %, respectively.

As expected, the inclusion of 5% Spirulina and 3% Thyme slightly influenced the presence of single FA in the experimental diets, however without affecting the proportion of SFA, MUFA and PUFA classes. Specifically, the FA profile of Spirulina (Table 2) evidenced a quite high level of palmitoleic acid (C16:1 *n*-9: 6.93 % total FAME) leading to a high level of this MUFA in S-supplemented diets. The high content of γ-linolenic acid (GLA) in S and ST diets was a direct consequence of its high amount in the Spirulina supplement as well. Furthermore, as a result of FA profile, the supplementation with Spirulina and/or Thyme numerically increased the eicosapentaenoic acid (EPA) content of the diets.

### *3.3. Raw and cookwd rabbit LTL and HL proximate composition, heme-iron and cholesterol content and nutrient TR*

Spirulina and/or Thyme dietary supplementation did not affect rabbit LTL and HL proximate composition, cholesterol and heme-iron contents, or nutrient true retention in either raw or cooked meat in either the shorter or longer period (Tables 4 and 5). Our findings confirmed those of Peiretti & Meineri (2011), the only other study that has evaluated the effect of dietary Spirulina supplementation on rabbit meat quality, with the one exception that Peiretti & Meineri (2011) reported significantly higher meat lipid content in the rabbits fed Spirulina. Their result, however, was likely attributable to the lower ether extract content of the control group diet than to the Spirulina inclusion level. Rabbit meat confirmed to be a lean meat source characterized by high protein and moderately low cholesterol levels. The average protein content of raw LTL muscle was 22.9 g/100 g meat, which rose to 30.4 g/100 g in cooked meat; lipid content was 0.8 g/100 g in raw and 1.4 g/100 g in cooked meat, whereas average cholesterol content amounted to 51.3 mg/100 g in raw and 78.2 mg/100 g in cooked meat.

**Table 4.** Effect of dietary Spirulina (*Arthrospira platensis*) and Thyme (*Thymus vulgaris*) supplementation on proximate composition (g/100 g meat), Heme iron (mg/kg meat) and cholesterol (mg/ 100 g meat) contents and true retention (TR, %) of raw and cooked rabbit LTL meat

| | | Experimental groups | | | | | | | Significance | RSD[1] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | C-C | C-S | C-T | C-ST | S-S | T-T | ST-ST | | |
| No. | | 15 | 15 | 15 | 15 | 15 | 15 | 15 | | |
| | Raw | 75.0 | 75.0 | 75.3 | 75.1 | 75.1 | 74.9 | 75.0 | ns | 0.3 |
| Moisture | Cooked | 66.0 | 66.2 | 66.6 | 66.0 | 67.1 | 65.6 | 66.3 | ns | 1.3 |
| | TR | 67 | 68 | 68 | 68 | 69 | 68 | 68 | ns | 3.0 |
| | Raw | 22.9 | 22.8 | 22.6 | 23.0 | 23.1 | 22.9 | 23.0 | ns | 0.3 |
| Protein | Cooked | 30.5 | 30.1 | 29.9 | 30.9 | 29.9 | 31.4 | 30.1 | ns | 1.4 |
| | TR | 102 | 104 | 104 | 103 | 99 | 104 | 102 | ns | 5.1 |
| | Raw | 0.9 | 0.8 | 0.7 | 0.7 | 0.7 | 1.0 | 0.8 | ns | 0.2 |
| Lipids | Cooked | 1.5 | 1.5 | 1.2 | 1.3 | 1.4 | 1.4 | 1.5 | ns | 0.3 |
| | TR | 136 | 155 | 125 | 152 | 165 | 122 | 155 | ns | 41.7 |
| | Raw | 1.35 | 1.38 | 1.40 | 1.35 | 1.35 | 1.36 | 1.37 | ns | 0.14 |
| Ash | Cooked | 1.61 | 1.54 | 1.60 | 1.61 | 1.50 | 1.72 | 1.47 | ns | 0.17 |
| | TR | 93 | 88 | 90 | 93 | 87 | 96 | 85 | ns | 15.0 |
| | Raw | 51.5 | 51.4 | 51.2 | 51.8 | 49.8 | 52.4 | 51.1 | ns | 3.4 |
| Cholesterol | Cooked | 78.6 | 78.8 | 76.7 | 78.3 | 76.0 | 81.2 | 77.8 | ns | 4.7 |
| | TR | 116 | 120 | 116 | 116 | 119 | 118 | 117 | ns | 10.3 |
| Heme iron | Raw | 1.3 | 1.7 | 1.8 | 1.82 | 1.7 | 1.3 | 1.64 | ns | 0.4 |

[1]Residual Standard Deviation

The HL presented a slightly different chemical composition with higher fat (2.8 g/100 g raw meat) and consequently higher cholesterol (65.1 mg/100 g raw meat), even if these values remain moderate when compared to those of the other most common animal meat species. Being a muscle with a balanced oxidative and glycolytic fibre proportion, the HL showed also a higher heme-iron content than the LTL (3.12 *vs* 1.62 mg/100 g raw meat for HL and LTL, respectively), being the latter a muscle with prevalent fast glycolytic metabolism.

**Table 5.** Effect of dietary Spirulina (*Arthrospira platensis*) and Thyme (*Thymus vulgaris*) supplementation on proximate composition (g/100 g meat), Heme iron (mg/kg meat) and cholesterol (mg/ 100 g meat) contents and true retention (TR, %) of raw and cooked rabbit HL meat

| | | Experimental groups | | | | | | | Significance | RSD[1] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | C-C | C-S | C-T | C-ST | S-S | T-T | ST-ST | | |
| No. | | 10 | 10 | 10 | 10 | 10 | 10 | 10 | | |
| Moisture | Raw | 73.6 | 74.0 | 74.0 | 73.6 | 74.0 | 74.0 | 73.6 | ns | 0.6 |
| | Cooked | 67.7 | 68.0 | 68.0 | 67.4 | 68.0 | 67.0 | 67.1 | ns | 0.9 |
| | TR | 76 | 76 | 75 | 74 | 74 | 74 | 74 | ns | 3.0 |
| Protein | Raw | 22 | 22 | 22 | 22.4 | 22 | 22 | 22 | ns | 0.7 |
| | Cooked | 27 | 27 | 27 | 27.6 | 28 | 28 | 27.4 | ns | 0.7 |
| | TR | 100 | 100 | 101 | 100 | 102 | 100 | 101 | ns | 2.9 |
| Lipids | Raw | 2.9 | 2.8 | 2.6 | 2.7 | 2.8 | 2.8 | 3.1 | ns | 0.6 |
| | Cooked | 3.9 | 3.7 | 3.3 | 3.9 | 3.5 | 3.9 | 4.2 | ns | 0.6 |
| | TR | 109 | 105 | 105 | 117 | 102 | 118 | 112 | ns | 14.6 |
| Ash | Raw | 1.3 | 1.3 | 1.2 | 1.23 | 1.2 | 1.3 | 1.23 | ns | 0.06 |
| | Cooked | 1.2 | 1.2 | 1.2 | 1.18 | 1.2 | 1.2 | 1.16 | ns | 0.06 |
| | TR | 76 | 76 | 77 | 78 | 78 | 75 | 77 | ns | 5.7 |
| Cholesterol | Raw | 65.3 | 65.0 | 66.0 | 64.4 | 66.0 | 65.0 | 63.8 | ns | 2.95 |
| | Cooked | 86.3 | 87.0 | 89.0 | 87.6 | 89.0 | 88.0 | 88.1 | ns | 3.73 |
| | TR | 108 | 110 | 110 | 110 | 110 | 106 | 112 | ns | 6.0 |
| Heme iron | Raw | 3.09 | 3.20 | 3.20 | 3.00 | 3.10 | 3.10 | 3.12 | ns | 0.5 |
| | Cooked | 4.08 | 3.80 | 3.80 | 3.67 | 3.40 | 4.10 | 3.73 | ns | 0.8 |
| | TR | 111 | 99 | 98 | 96 | 93 | 106 | 97 | ns | 21.8 |

[1]Residual Standard Deviation

Unlike proximate composition, true retention (TR) of rabbit meat nutrients has not been widely studied until now (Dal Bosco, Castellini, & Bernardini, 2001). TR values of LTL meat nutrients were higher than those of HL due to the more intense moisture loss that led to a high nutrient concentration. In regard to the high average lipid true retention values that we observed in both LTL and HL (144 and 110 % for LTL and HL, respectively) that were higher than those previously provided in literature (Dal Bosco et al., 2001), some other factor than the simple loss of moisture might provide the explanation: the cooking procedure might have favored the concentration of lipids. The LTL and HL meat samples were vacuum-sealed in PVC bags , and after melting on heating, the fats might have diffused along the concentration gradient into the meat (Kumar & Aalbersberg, 2006).

Moreover, as Sheard, Nute, & Chappell (1998) observed in a work considering the effect of cooking on the chemical composition of beef meat products, the different lipid TR measured in the two cuts of our study might be attributed to the initial different fat content of LTL and HL (average values: 0.8 *vs* 2.8 g/100 g for LTL and HL, respectively). Regardless however of cooking procedure, meat cut or product, as fat content rises, the probability of fat coalescing and then leaking from the product increases in proportion as the mean free distance between fat cells decreases.

### *3.4. FA profile of raw and cooked rabbit LTL and HL, and TR values*

As observed with proximate composition, cholesterol, and heme-iron content, also the main FA groups of raw and cooked LTL were unaffected by dietary treatment (Table 6). On the contrary, however, Spirulina and/or Thyme dietary supplementation was observed to significantly influence SFA and the *n*-3 contents of cooked HL. Consequently also the *n*-6/*n*-3 ratio of cooked HL was affected by dietary treatments (Table 7). Differently, the significant effect of experimental diets on the *n*-6/*n*-3 ratio of raw HL was not generated by differences in *n*-3 PUFA, as in this case no statistical difference among dietary groups was observed. In particular, cooked HL meat of C-T animals had lower SFA content than that of ST-ST rabbits (1033 *vs* 1347 mg/100 g meat, respectively), whereas other dietary treatments provided intermediate results ($P<0.05$). Total *n*-3 content was higher in the cooked HL of rabbits in the ST-ST group than those in the S-S group (115.5 *vs* 87.6 mg/100 g meat, respectively). Consequently, the *n*-6/*n*-3 ratio of cooked HL meat was the worst in the S-S group (7.7 *vs* 8.7 for ST-ST and S-S groups, respectively). However, the *n*-6/*n*-3 ratio was kept between the recommended range of 5 to 10. For all the different feeding groups, the high average *n*-6/*n*-3 ratios of both raw meat cuts studied (12.7 and 8.7 for LTL and HL, respectively) were in line with literature because rabbit meat is known to be normally rich in Linoleic acid (Dalle Zotte, 2002).

**Table 6.** Effect of dietary Spirulina (*Arthrospira platensis*) and Thyme (*Thymus vulgaris*) supplementation on main FA groups (mg/100 g meat) and TR (%) of raw and cooked rabbit LTL meat

| | | Experimental groups | | | | | | | Significance | RSD[1] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | C-C | C-S | C-T | C-ST | S-S | T-T | ST-ST | | |
| No. | | 7 | 7 | 7 | 7 | 7 | 7 | 7 | | |
| Total SFA | Raw | 294 | 256 | 273 | 230 | 221 | 309 | 251 | ns | 83 |
| | Cooked | 544 | 539 | 438 | 492 | 479 | 633 | 520 | ns | 100 |
| | TR | 167 | 173 | 152 | 166 | 170 | 168 | 163 | ns | 43 |
| Total MUFA | Raw | 237 | 197 | 196 | 180 | 168 | 239 | 196 | ns | 73 |
| | Cooked | 422 | 405 | 303 | 386 | 358 | 466 | 360 | ns | 95 |
| | TR | 160 | 172 | 148 | 166 | 167 | 158 | 144 | ns | 42 |
| Total PUFA | Raw | 229 | 195 | 234 | 181 | 173 | 251 | 190 | ns | 63 |
| | Cooked | 427 | 445 | 364 | 370 | 370 | 492 | 398 | ns | 71 |
| | TR | 172 | 185 | 151 | 158 | 169 | 159 | 164 | ns | 47 |
| Σ *n*-6 | Raw | 207 | 179 | 213 | 165 | 158 | 227 | 173 | ns | 57 |
| | Cooked | 386 | 406 | 331 | 337 | 339 | 448 | 364 | ns | 64 |
| | TR | 172 | 184 | 151 | 158 | 168 | 160 | 165 | ns | 47 |
| Σ *n*-3 | Raw | 18.2 | 13.2 | 16.7 | 13.1 | 11.9 | 20.3 | 14.2 | ns | 5.4 |
| | Cooked | 33.3 | 31.5 | 25.7 | 26.2 | 25.3 | 35.4 | 27.1 | ns | 7.0 |
| | TR | 168 | 203 | 153 | 155 | 171 | 144 | 151 | ns | 54 |
| Σ *n*-6/Σ *n*-3 | Raw | 11.6 | 14.0 | 13.2 | 12.8 | 13.5 | 11.4 | 12.3 | ns | 1.7 |
| | Cooked | 11.9 | 13.4 | 13.2 | 13.2 | 13.4 | 12.7 | 13.6 | ns | 1.8 |

[1]Residual Standard Deviation

**Table 7.** Effect of dietary Spirulina (*Arthrospira platensis*) and Thyme (*Thymus vulgaris*) supplementation on main FA groups (mg/100 g) and TR (%) of raw and cooked rabbit HL meat

| | | Experimental groups | | | | | | | Significance | RSD[1] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | C-C | C-S | C-T | C-ST | S-S | T-T | ST-ST | | |
| No. | | 10 | 10 | 10 | 10 | 10 | 10 | 10 | | |
| Total SFA | Raw | 927 | 886 | 815 | 873 | 899 | 894 | 991 | ns | 178 |
| | Cooked | 1184$^{ab}$ | 1167$^{ab}$ | 1033$^{b}$ | 1208$^{ab}$ | 1074$^{ab}$ | 1190$^{ab}$ | 1347$^{a}$ | * | 196 |
| | TR | 105 | 109 | 99 | 114 | 102 | 111 | 111 | ns | 15 |
| Total MUFA | Raw | 732 | 698 | 623 | 683 | 702 | 693 | 792 | ns | 179 |
| | Cooked | 908 | 886 | 754 | 928 | 833 | 899 | 1036 | ns | 197 |
| | TR | 103 | 106 | 94 | 112 | 99 | 108 | 108 | ns | 15 |
| Total PUFA | Raw | 703 | 672 | 672 | 630 | 649 | 675 | 713 | ns | 121 |
| | Cooked | 977 | 923 | 905 | 955 | 840 | 980 | 1022 | ns | 128 |
| | TR | 114 | 115 | 106 | 125 | 109 | 119 | 119 | ns | 17 |
| Σ *n*-6 | Raw | 621 | 597 | 595 | 628 | 577 | 600 | 628 | ns | 107 |
| | Cooked | 854 | 814 | 799 | 838 | 743 | 858 | 893 | ns | 109 |
| | TR | 113 | 114 | 106 | 124 | 108 | 118 | 118 | ns | 17 |
| Σ *n*-3 | Raw | 75.0 | 66.7 | 68.1 | 63.7 | 64.5 | 68.5 | 73.6 | ns | 14.0 |
| | Cooked | 109.9$^{ab}$ | 95.6$^{ab}$ | 95.0$^{ab}$ | 105.0$^{ab}$ | 87.6$^{b}$ | 104.4$^{ab}$ | 115.5$^{a}$ | * | 17.1 |
| | TR | 121 | 120 | 110 | 136 | 114 | 124 | 125 | ns | 19.8 |
| Σ *n*-6/ Σ *n*-3 | Raw | 8.4 | 9.0 | 8.8 | 8.9 | 9.0 | 8.8 | 8.3 | * | 0.6 |
| | Cooked | 7.8$^{BC}$ | 8.6$^{AB}$ | 8.5$^{ABC}$ | 8.1$^{ABC}$ | 8.7$^{A}$ | 8.3$^{ABC}$ | 7.7$^{C}$ | ** | 0.5 |

Level of significance: *, $P < 0.05$; **, $P < 0.01$; $^{A, B}$ Means in the same row having different superscripts are significant at $P \leq 0.01$ level; $^{a, b}$ Means in the same row having different superscripts are significant at $P \leq 0.05$ level; [1]Residual Standard Deviation

Given that the rabbit is a monogastric hindgut fermenting animal, however, the diet's FA profile should be directly incorporated into adipose and intramuscular tissue lipids with only slight variation, in this way compensating for consequent modifications in meat FA composition.

Tables 8 and 9, in fact, show that diet influenced the content of many single fatty acids in both LTL and HL cuts, but except for GLA and dihomo-GLA (DGLA), differences among diets became significant only in cooked meat. The link between lipid membrane integrity and dietary treatment initially hypothesized was not confirmed by results regarding water holding capacity, cooking loss (Dalle Zotte, Sartori, Cullere, et al., 2012), and the TR values of main

FA groups (Tables 6 and 7). Moreover as previously described, proximate composition was unaffected by dietary treatment. The phenomenon therefore remains unexplained and requires further investigation. As mentioned previously, Spirulina is a rich source of GLA and this was confirmed in both LTL and HL cuts, in which GLA levels rose higher and higher the longer S supplementation continued. For this reason, S-S and ST-ST animals had similar GLA contents, which were always greater than those of all the other feeding groups. C-S and C-ST animals, whose GLA content was the same, presented lower values than S-S and ST-ST groups but higher values than C-C, C-T and T-T ($P$<0.001). GLA can be converted from essential linoleic acid through delta-6 desaturase activity, but the rate is limited. This might explain why the GLA content of the meat in C-T and T-T animals was similar to that of C-C group, even if Thyme has a higher content of linoleic acid than Spirulina (Table 1). We also observed great differences between LTL and HL in GLA content, with HL being far richer (11.5 *vs* 2.3 mg/100 g of raw meat for HL and LTL in long supplemented groups, respectively).

In the only work that has studied the effect of Spirulina supplementation on the FA profile of meat taken from growing rabbits, Peiretti & Meineri (2011) found that after increasing the percentage of dietary Spirulina, both the GLA content of *Longissimus dorsi* meat and perirenal fat increased. Although the DGLA content was not shown in their results, they observed that the levels of arachidonic acid, which becomes the second metabolite of GLA through the activity of elongase enzyme, were higher in the meat of Spirulina-supplemented rabbits than in the control group. The same study also showed that increasing Spirulina inclusion levels augmented both the meat's GLA content and perirenal fat, the latter to greater degree. The deposition rate progressively diminished however, more in the *Longissimus dorsi* muscle than in the perirenal fat. Similarly, in our study, although the GLA content of long supplemented animals (S-S and ST-ST) was higher than those receiving supplementation for shorter time (C-S and C-ST), longer supplementation reduced deposition efficiency in both meat portions. In raw LTL, the GLA content of long and short supplemented animals was 2.27 *vs* 1.51 mg/100 g meat, respectively, whereas raw HL GLA was 11.45 *vs* 6.90 mg/100 g meat for long and short supplemented animals, respectively. We also observed GLA to be predominantly deposited in the fattest muscle of the two, the HL.

Dietary treatment affected also other single FA, but different ones in LTL and HL cuts. As a result of the ether extract content (Table 1) and FA profile (Table 2) of Thyme dried leaves used in this trial, T-T supplemented animals showed the highest values of single FA in LTL cooked meat. Interestingly, for some single FA (C15:0, C20:0, C20:1 *n*-9 and

C20:3 *n*-3), the ST-ST group had lower values than the T-T group, suggesting a possible contrasting effect created by the simultaneous supplementation of S and T. This hypothesis was not confirmed by HL meat results, however (Table 9). In fact, whenever diets significantly affected single FA, the ST-ST group always presented the highest values. The only other study that considered both S and T supplementation in rabbit diets was conducted on dwarf rabbits, however (Dalle Zotte, Sartori, Bohatir, Rémignon, & Ricci, 2013), and no meat quality aspects were considered. Distilled thyme leaves have been tested as a dietary supplement in ewes with the aim of improving lamb meat FA profile (Martínez, 2013). In this case, thyme leaves increased PUFA and UFA levels at the expense of SFA, but higher Thyme inclusion levels than those we adopted were used. Moreover, a different species with a different digestive physiology was considered, and therefore comparisons with rabbits are difficult to draw.

A minimum average intake of 500 mg/day of combined EPA and docosahexaenoic (DHA) PUFAs is recommended for human cardiovascular health (Kris-Etherton, Grieger, & Etherton, 2009). In order to increase the content of EPA and DHA in rabbit meat, two effective strategies exist: dietary inclusion of linolenic acid (linseed and rapeseed oils), which provides biosynthesis of EPA and DHA through elongation and desaturation (Dal Bosco, Castellini, Bianchi, & Mugnai, 2004), or dietary supplementation of fish sources which are naturally rich in these long-chain *n*-3 FA (Tres, Bou, Codony, & Guardiola, 2008). Even if Spirulina and/or Thyme supplementation seemed to increase the dietary content of EPA compared to the control diet (Table 3), EPA and DHA contents of both LTL and HL meat (raw and cooked) were similar in all groups.

**Table 8.** Effect of dietary Spirulina (*Arthrospira platensis*) and Thyme (*Thymus vulgaris*) supplementation on single FA (mg/100 g meat) of raw and cooked rabbit LTL meat

| | | Experimental groups | | | | | | | Significance | RSD[1] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | C-C | C-S | C-T | C-ST | S-S | T-T | ST-ST | | |
| No. | | 7 | 7 | 7 | 7 | 7 | 7 | 7 | | |
| C15:0 | Raw | 5.24 | 4.64 | 5.27 | 3.88 | 3.68 | 5.35 | 4.15 | ns | 1.38 |
| | Cooked | 9.53 | 9.72 | 8.84 | 7.64 | 8.49 | 11.31 | 7.60 | * | 1.88 |
| C20:0 | Raw | 0.98 | 0.90 | 1.00 | 0.76 | 0.88 | 1.05 | 1.08 | ns | 0.28 |
| | Cooked | $2.10^{A}$ | $1.93^{AB}$ | $2.02^{AB}$ | $1.71^{AB}$ | $1.76^{AB}$ | $2.42^{AB}$ | $1.28^{B}$ | ** | 0.45 |
| C18:2 *n*-6 (Linoleic) | Raw | 173.2 | 141.2 | 174.7 | 132.1 | 123.8 | 192.3 | 135.8 | ns | 47.2 |
| | Cooked | 323.0 | 322.3 | 275.9 | 273.6 | 270.0 | 374.6 | 288.8 | * | 53.4 |
| C18:3 *n*-6 (γ-Linolenic) | Raw | $0.46^{C}$ | $1.54^{B}$ | $0.41^{C}$ | $1.47^{B}$ | $2.17^{A}$ | $0.39^{C}$ | $2.36^{A}$ | *** | 0.35 |
| | Cooked | $0.98^{C}$ | $3.89^{AB}$ | $1.10^{C}$ | $3.26^{B}$ | $4.62^{A}$ | $0.99^{C}$ | $4.61^{A}$ | *** | 0.69 |
| C20:1 *n*-9 | Raw | 1.92 | 1.31 | 1.64 | 1.26 | 1.23 | 1.36 | 1.24 | ns | 0.71 |
| | Cooked | 3.32 | 3.37 | 2.44 | 2.10 | 2.31 | 3.51 | 1.66 | * | 1.05 |
| C20:2 | Raw | 3.20 | 2.65 | 3.35 | 2.41 | 2.24 | 3.55 | 2.99 | ns | 1.09 |
| | Cooked | 7.26 | 6.43 | 6.17 | 5.64 | 5.68 | 8.42 | 5.95 | * | 1.31 |
| C20:3 *n*-3 | Raw | 0.46 | 0.29 | 0.45 | 0.28 | 0.44 | 0.43 | 0.52 | ns | 0.17 |
| | Cooked | $1.22^{A}$ | $0.88^{AB}$ | $1.08^{AB}$ | $0.78^{AB}$ | $0.81^{AB}$ | $1.25^{A}$ | $0.74^{B}$ | ** | 0.23 |
| C20:5 *n*-3 (EPA) | Raw | 1.30 | 1.20 | 1.30 | 1.08 | 0.99 | 1.27 | 1.17 | ns | 0.40 |
| | Cooked | 2.53 | 2.73 | 2.02 | 2.21 | 2.17 | 2.76 | 2.43 | ns | 0.56 |
| C22:6 n-3 (DHA) | Raw | 0.22 | 0.16 | 0.53 | 0.28 | 0.26 | 0.22 | 0.39 | ns | 0.33 |
| | Cooked | 0.38 | 0.6 | 0.51 | 0.79 | 0.73 | 1.0 | 0.83 | ns | 0.49 |
| C20:3 *n*-6 | Raw | $3.62^{B}$ | $4.81^{AB}$ | $3.85^{B}$ | $4.10^{B}$ | $5.53^{AB}$ | $3.61^{B}$ | $6.28^{A}$ | ** | 1.23 |
| | Cooked | $7.05^{C}$ | $10.63^{AB}$ | $6.03^{C}$ | $8.55^{BC}$ | $10.68^{AB}$ | $8.07^{BC}$ | $12.09^{A}$ | *** | 1.69 |

Level of significance: *, $P < 0.05$; **, $P < 0.01$; ***, $P<0.001$; [A, B] Means in the same row having different superscripts are significant at $P \leq 0.01$ level; [1]Residual Standard Deviation

**Table 9.** Effect of the dietary Spirulina (*Arthrospira platensis*) and Thyme (*Thymus vulgaris*) supplementation on single FA (mg/100 g meat) of raw and cooked rabbit HL meat

| | | Experimental groups | | | | | | | Significance | RSD[1] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | C-C | C-S | C-T | C-ST | S-S | T-T | ST-ST | | |
| No. | | 10 | 10 | 10 | 10 | 10 | 10 | 10 | | |
| C16:0 | Raw | 623 | 592 | 536 | 591 | 617 | 604 | 671 | ns | 125 |
| | Cooked | $792^{ab}$ | $778^{ab}$ | $680^{b}$ | $816^{ab}$ | $738^{ab}$ | $800^{ab}$ | $911^{a}$ | * | 143 |
| C17:0 | Raw | 19.0 | 19.3 | 18.1 | 18.0 | 18.4 | 17.1 | 20.5 | ns | 3.6 |
| | Cooked | 23.8 | 25.3 | 22.4 | 24.6 | 22.1 | 23.0 | 27.6 | * | 3.5 |
| C18:0 | Raw | 181.7 | 180.6 | 171.1 | 175.5 | 168.7 | 180.1 | 195.6 | ns | 36.9 |
| | Cooked | 231.3 | 230.6 | 213.5 | 237.7 | 196.7 | 236.4 | 261.5 | ** | 33.4 |
| C17:1 | Raw | 10.3 | 10.1 | 9.2 | 9.8 | 11.0 | 9.2 | 12.1 | ns | 2.4 |
| | Cooked | $13.6^{ab}$ | $14.0^{ab}$ | $11.3^{b}$ | $13.7^{ab}$ | $13.3^{ab}$ | $12.6^{ab}$ | $16.6^{a}$ | * | 2.8 |
| C18:3 *n*-3 (α-Linolenic) | Raw | 70.9 | 62.8 | 63.9 | 60.3 | 60.7 | 64.8 | 72.3 | ns | 13.5 |
| | Cooked | $102.6^{ab}$ | $89.2^{ab}$ | $88.0^{ab}$ | $97.7^{ab}$ | $82.1^{b}$ | $97.9^{ab}$ | $109.0^{a}$ | * | 16.2 |
| C18:3 *n*-6 (γ-Linolenic) | Raw | $1.83^{C}$ | $7.13^{B}$ | $2.52^{C}$ | $6.67^{B}$ | $11.44^{A}$ | $1.81^{C}$ | $11.45^{A}$ | *** | 1.99 |
| | Cooked | $2.32^{C}$ | $10.42^{B}$ | $2.34^{C}$ | $11.15^{B}$ | $15.42^{A}$ | $2.47^{C}$ | $18.50^{A}$ | *** | 2.51 |
| C20:5 *n*-3 (EPA) | Raw | 1.49 | 1.20 | 1.45 | 1.24 | 1.33 | 1.31 | 1.40 | ns | 0.53 |
| | Cooked | 2.19 | 1.99 | 2.12 | 2.24 | 1.93 | 2.56 | 2.30 | ns | 0.61 |
| C22:6 n-3 (DHA) | Raw | 0.32 | 0.35 | 0.55 | 0.08 | 0.25 | 0.55 | 0.25 | ns | 0.49 |
| | Cooked | 1.83 | 1.29 | 1.68 | 1.81 | 0.70 | 0.35 | 0.84 | ns | 1.35 |
| C20:3 *n*-6 | Raw | $5.41^{D}$ | $9.14^{AB}$ | $5.94^{CD}$ | $8.37^{BC}$ | $11.38^{A}$ | $6.08^{CD}$ | $11.67^{A}$ | *** | 1.98 |
| | Cooked | $8.34^{D}$ | $12.25^{BC}$ | $9.32^{CD}$ | $11.52^{BC}$ | $14.83^{AB}$ | $9.65^{CD}$ | $16.72^{A}$ | *** | 2.58 |

Level of significance: *, $P < 0.05$; **, $P < 0.01$; ***, $P<0.001$; [A, B] Means in the same row having different superscripts are significant at $P \leq 0.001$ level; [a, b] Means in the same row having different superscripts are significant at $P \leq 0.05$ level; [1]Residual Standard Deviation

*3.5. Raw and cooked rabbit LTL and HL TBARS*

Results concerning the oxidative stability of rabbit meat are reported in Table 10 and 11. The TBARS content of LTL meat, regardless of state (raw or cooked) or storage type (fresh or FD) was unaffected by dietary treatment ($P>0.05$). Unlike diet, state significantly influenced the amount of TBARS in each dietary group ($P<0.05$) except for T-T and ST-ST. Also storage influenced the oxidative status of each treatment, except for the meat coming from C-C animals. This result came as no surprise because freeze-drying is known to increase the amount in meat of 4-hydroxynonenal, which is a secondary product of *n*-6 fatty acid oxidation (Faustman, Sun, Mancini, & Suman, 2010). In addition, it is also known that the degree of lipid oxidation is higher in cooked than in raw meat as a result of accelerated oxidative reactions caused by heating. We observed increasing TBARS content from raw fresh to cooked freeze-dried LTL meat in most groups, in fact, with differences becoming significant when cooked freeze-dried samples were compared to raw fresh samples (average TBARS content: 0.432 *vs* 0.936 mg MDA/kg meat, for raw fresh and cooked FD LTL meat, respectively). A partly different situation was observed for C-T, T-T and ST-ST groups in which also cooked fresh samples had lower TBARS content than cooked FD samples and were comparable to those found in raw fresh meat. This suggest a possible positive effect of thyme supplementation in protecting membrane lipids from oxidative stress due to heating.

The oxidative status of HL meat (Table 11) markedly differed from that of LTL. In general, raw fresh HL samples presented an average higher oxidation degree (0.650 mg MDA/kg meat) than LTL samples (0.432 mg MDA/kg meat). This was probably due to the higher absolute fat content, particularly PUFA, of the HL compared to the LTL. Moreover, LTL is a prevalent glycolytic muscle, whereas HL has an intermediate metabolism suggesting a different susceptibility to oxidation. In particular, muscle fibre type and other factors, such as myoglobin content, carnosine content, amount of phospholipids, phospholipid fatty acid composition, and antioxidant accumulation in the tissue have been reported to affect oxidation (Andrés, Cava, Mayoral, Tejeda, Morcuende, & Ruiz, 2001; Botsoglu, Christaki, Fletouris, Florou-Paneri, & Spais, 2002). As HL is richer in fat and more vascularised than LTL, a higher deposition of antioxidant probably also occurred. In this case, in fact, diet affected the oxidative stability of raw FD meat, with C-T and T-T groups showing lower TBARS content than S-S meat (0.550 and 0.556 *vs* 1.029 mg MDA/kg meat for C-T, T-T, and S-S groups, respectively), whereas the other groups presented intermediate values. Similarly to as observed for LTL meat, although state and storage affected HL meat oxidative status ($P<0.05$), a different trend was noticed in this case. In particular, cooking had a great impact

on meat oxidative status, and therefore cooked, fresh meat always had higher TBARS content (average value: 1.742 mg MDA/kg meat) than raw fresh, raw FD, and cooked FD meat, regardless of dietary group ($P<0.001$). For this reason, the hypothesized protective effect against oxidative stress caused by heating exerted by thyme was disproved, at least as far as HL meat is concerned.

The antioxidant activity of Spirulina has been reported in many *in vitro* and *in vivo* studies on mice and rats (Deng & Chow, 2010) with phycocyanin, a phycobiliprotein, acting as oxygen free radical scavenger (Romay, González, Ledón, Remirez, & Rimbau, 2003). This effect was not confirmed however by Dal Bosco et al. (2014), who found dietary supplementation with Spirulina to be ineffective in protecting fresh rabbit meat from lipid oxidation during retail display, thus supporting the results presented in our study. Similarly, Kovács, Tuboly, Mézes, et al. (unpublished results) observed no effect of Spirulina supplementation on rabbit serum biochemistry, immune response, and antioxidant status.

We hypothesized that either the Spirulina level in the diet was inappropriate to providing protection against lipid oxidation or that some interference in antioxidant absorption had occurred, thus requiring further research.

On the contrary, thyme's antioxidant properties, even if their effectiveness may vary depending on lipid substrate and temperature, are well known and mainly due to the phenols thymol and carvacrol, which inhibit the peroxidation of liposome phospholipids (Yanishlieva, Marinova, Gordon, & Raneva, 1999). Meat taken from *Longissimusm dorsi* muscles of rabbits fed with thyme dried leaves and stored at +4 °C for 9 days, in fact, proved less susceptible to oxidation than both Spirulina-supplemented and control group animals (Dal Bosco et al., 2014). Even if the isomers thymol and carvacrol are volatile molecules, thyme essential oil still demonstrated good antioxidant properties when heated up to 180 °C (Tomaino, Cimino, Zimbalatti, et al., 2005). The lack of effectiveness in lowering lipid oxidation in LTL and HL meat subjected to intense oxidative stress, especially cooking, however, suggested that thyme antioxidants might have been partly or totally inactivated by heating. This hypothesis was corroborated by considering the cooking procedure adopted in this trial: boiling for 1h and 1.30h at 80 °C for LTL and HL, respectively. The accumulation of TBARS in boiled meat taken from *Longissimus dorsi* of rabbits supplemented with α-tocopheryl acetate, in fact, was more than twice that of roasted and fried samples (Dal Bosco et al., 2001).

Moreover, cooking method has been reported to affect also the antioxidant activity of vegetables to different extent depending on source, and therefore a certain effect can be expected also with thyme (Jiménez-Monreal, García-Diz, Martínez-Tomé, Mariscal, & Murcia., 2009).

In the only other trial that studied the effect of dietary supplementation of thyme dried leaves on cooked meat oxidation, Nieto et al. (2011) found that *Thymus zygis*, supplemented at 3.75% and 7.5% to ewe diets was effective in delaying the accumulation of MDA in cooked (heating plates reaching 72°C internal temperature) lamb meat during display. The oxidation degree of cooked lamb meat at day 0 was the same in all treatments, however, thus supporting the results of our study.

**Table 10.** Effect of dietary Spirulina (*Arthrospira platensis*) and Thyme (*Thymus vulgaris*) supplementation, State and Storage condition on TBARS values (mg MDA/kg meat) of raw and cooked rabbit LTL meat

| State | Storage | Experimental groups | | | | | | | Significance diets | RSD[1] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | C-C | C-S | C-T | C-ST | S-S | T-T | ST-ST | | |
| raw | fresh | 0.43 | 0.47 $^{Y}$ | 0.41 $^{Y}$ | 0.38 $^{Y}$ | 0.49 $^{Y}$ | 0.39 $^{Y}$ | 0.45 $^{Y}$ | ns | 0.10 |
| raw | FD | 0.61 | 0.64 $^{XY}$ | 0.58 $^{Y}$ | 0.71 $^{Y}$ | 0.71 $^{XY}$ | 0.66 $^{XY}$ | 0.84 $^{XY}$ | ns | 0.21 |
| cooked | fresh | 0.75 | 0.71 $^{XY}$ | 0.61 $^{Y}$ | 0.78 $^{XY}$ | 0.86 $^{XY}$ | 0.37 $^{Y}$ | 0.70 $^{Y}$ | ns | 0.25 |
| cooked | FD | 0.76 | 0.95 $^{X}$ | 1.04 $^{X}$ | 0.98 $^{X}$ | 1.13 $^{X}$ | 0.76 $^{X}$ | 0.93 $^{X}$ | ns | 0.24 |
| Significance-State | | * | ** | *** | ** | ** | ns | ns | | |
| Significance-Storage | | ns | * | ** | ** | * | ** | ** | | |
| RSD[1] | | 0.23 | 0.20 | 0.18 | 0.19 | 0.26 | 0.19 | 0.20 | | |

Level of significance: *, $P < 0.05$; **, $P < 0.01$; ***, $P<0.001$; $^{X, Y}$Means in the same column with no common superscript differ significantly ($P< 0.05$); [1]Residual Standard Deviation

**Table 11.** Effect of dietary Spirulina (*Arthrospira platensis*) and Thyme (*Thymus vulgaris*) supplementation, State and Storage condition on TBARS values (mg MDA/kg meat) of raw and cooked rabbit HL meat

| State | Storage | Experimental groups | | | | | | | Signif. diets | RSD[1] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | C-C | C-S | C-T | C-ST | S-S | T-T | ST-ST | | |
| raw | fresh | 0.65 $^{Y}$ | 0.61 $^{Y}$ | 0.63 $^{Y}$ | 0.69 $^{Y}$ | 0.63 $^{Y}$ | 0.54 $^{Y}$ | 0.79 $^{Y}$ | ns | 0.15 |
| raw | FD[2] | 0.88 $^{ab\ Y}$ | 0.68 $^{ab\ Y}$ | 0.55 $^{b\ Y}$ | 0.69 $^{ab\ Y}$ | 1.03 $^{a\ Y}$ | 0.56 $^{b\ Y}$ | 0.73 $^{ab\ Y}$ | * | 0.23 |
| cooked | fresh | 1.73 $^{X}$ | 2.11 $^{X}$ | 1.60 $^{X}$ | 1.70 $^{X}$ | 1.99 $^{X}$ | 1.25 $^{X}$ | 1.80 $^{X}$ | ns | 0.42 |
| cooked | FD[2] | 0.71 $^{Y}$ | 0.92 $^{Y}$ | 0.72 $^{Y}$ | 0.83 $^{Y}$ | 0.83 $^{Y}$ | 0.66 $^{Y}$ | 0.86 $^{Y}$ | ns | 0.15 |
| Significance-State | | *** | *** | *** | *** | ** | *** | ** | | |
| Significance-Storage | | *** | *** | *** | ** | * | *** | ** | | |
| RSD[1] | | 0.19 | 0.25 | 0.15 | 0.27 | 0.38 | 0.11 | 0.37 | | |

Level of significance: *, $P < 0.05$; **, $P < 0.01$; ***, $P<0.001$; $^{a,\ b}$Means in the same row with no common superscript differ significantly ($P< 0.05$); $^{X,\ Y}$Means in the same column with no common superscript differ significantly ($P< 0.05$); [1]Residual Standard Deviation; [2]Freeze-dried

## 4. Conclusions

Separate and simultaneous supplementation of 5% Spirulina and 3% Thyme in diets fed to growing rabbits provided raw and cooked rabbit LTL and HL meat with nutritional compositions similar to those of the C diet with comparable nutrient TR among treatments. The inclusion of 5% Spirulina diet improved the FA profile of the LTL and HL meat by significantly increasing GLA content.

Spirulina was not observed to improve the oxidative stability of rabbit meat subjected to severe oxidative stress, thus supporting the results of a previous shelf-life trial on raw rabbit meat. This engendered the hypothesis that either the Spirulina level in the diet had been too low or that Spirulina antioxidants had not been absorbed from the gut for some reason, and that further research is required. Thyme was shown effective in reducing lipid oxidation in raw and FD HL but not in cooked meat. Additional experiments are necessary to define whether and to what extent thyme's antioxidant activity is affected by the most common cooking procedures. This would be useful from both the scientific and the consumers' point of view.

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# CHAPTER 4

Running Head: Dietary Oregano, Rosemary, Vitamin E and *Saccaromyces cerevisiae* to growing rabbits

# Oregano, Rosemary, Vitamin E and *Saccaromyces cerevisiae* dietary supplementation to growing rabbits: Effect on growth performance, carcass traits, bone development and meat chemical composition

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## ABSTRACT

The study aimed to evaluate the effect of the dietary supplementation with different natural additives on the performance of growing rabbits, the nutritional composition and oxidative stability of their meat and on their hind leg bone traits. For this experiment a total of 320 weaned (at 30 days of age) New Zealand White rabbits were used. They were randomly allocated to 8 dietary groups (n=40 rabbits/group) until 80 days of age, when they were sacrificed. The control group (S) received a standard diet, without any supplementation, whereas the other groups received the S diet supplemented with 150 ppm of vitamin E (E), 0.2 % of oregano extract (O), 0.2 % of rosemary extract (R), 0.15 % of a product made of *Saccharomyces cerevisiae* bio-active cell (Thepax®, T), a combination of 0.1% O and 0.1% R (OR), a combination of 0.15% T and 0.2% O (TO) and a combination of 0.15% T and 150 ppm of vitamin E (TE). Rabbits belonging to O and OR groups showed the highest final live weight, carcass weight and, together with R rabbits, carcass yield ($P$<0.001). Oregano supplemented animals presented also the best feed conversion ratio. *Longissimus dorsi* (LD) meat of E and R rabbits had the higher protein content, whereas that of T, TE and TO was the fattest ($P$<0.001). All dietary treatments improved the oxidative stability of the LD meat compared to the S group. Treatments O, E and TE were the most effective in delaying the lipid oxidation of LD meat, followed by OR, R, T and TO diets ($P$<0.01). Considering the chemical composition of hind leg (HL) meat, T and TE diets lowered its protein content compared to the other groups, whereas all T-supplemented diets tended to increase its lipid content. Iron content of T group was higher than that of E and R groups, whereas E, O and OR groups were the richest in Na ($P$<0.001). The dietary inclusion of *Saccaromyces cerevisiae* increased the meatiness of the HL without affecting the femur fracture strength. The study showed that an adequate supplementation with natural antioxidants can also have a positive effect on productive performance and meat quality.



**Keywords:** Rabbit meat, Meat quality, Vitamin E, Oregano, Rosemary, *Saccaromyces cerevisiae*

## 1. Introduction

Rabbit meat represents a typical food for many Mediterranean countries like Algeria, Cyprus, Egypt, France, Italy, Spain, and some other European countries such as Belgium, Czech Republic, Luxembourg and Portugal (Dalle Zotte & Szendrő, 2011). From the nutritional point of view, it is ideal for all kinds of consumers, also those of Western countries whose diet is generally rich in fats and sodium, exposing them to health problems such as obesity, cardiovascular diseases and hypertension (Karppanen & Mervaala, 2006). In fact rabbit meat is rich in protein, B vitamins and minerals but it is also poor in sodium and characterized by a low fat and cholesterol contents which makes its energy content (789 kJ/100 g meat, average carcass value) mostly attributable to proteins. The fact that rabbit meat is particularly rich in unsaturated fatty acids, renders it susceptible to oxidation which causes loss of nutritive value and worsens meat physical characteristics and sensory quality. In addition, lipid oxidation negatively impacts meat healthfulness as it causes the formation of toxic compounds like malondialdehyde and cholesterol oxidation products which demonstrated to negatively affect human's health (Wood, Richardson, Nute, Fisher, & Campo, 2003).

For this reason, especially in the past, the meat industry extensively used synthetic antioxidants. However, in the last decade consumer's opinion towards this kind of additives has become more and more negative due to toxic and safety issues. This forced the meat industry to take into consideration and test antioxidants coming from natural sources, supplemented to animal's diets (Ao, Yoo,, Zhou, Wang, Meng, et al., 2011; Dal Bosco, Gerencsér, Szendrő, Mugnai, Cullere, et al., 2014) or directly added to the final product (Kanatt, Chander, Radhakrishna, & Sharma, 2005; McCarthy, Kerry, Kerry, Lynch, & Buckley, 2001; Cullere, Hoffman, & Dalle Zotte, 2013). In addition to their antioxidant potential, natural sources have been increasingly studied for their effects on the performance of farmed animal, especially after the European ban of growth promoters (1$^{st}$ January 2006) and the increasing restrictions also in the United States (Landy, Ghalamkari, & Toghyani, 2011).

In this context, plants of the *Labiatae* family are among the most widely studied both as antioxidants and as natural substitutes to growth promoting antibiotics in farmed animals. Oregano (*Origanum vulgare*) has attracted great interest as its essential oil is rich in the monoterpenes thymol and carvacrol, which exhibited good antioxidant and antimicrobial activities *in vitro* and *in vivo*, together with exerting a stimulating effect on animal digestion (Ertas, Güler, Çiftçi, DalkIlIç, & Simsek, 2005; Seydim & Sarikus, 2006; Soultos, Tzikas, Christaki, Papageorgiou, & Steris, 2009; Tomaino, Cimino, Zimbalatti, Venuti, Sulfaro, et al.,

2005). Similarly, rosemary (*Rosmarinus officinalis*) possesses antioxidant and antimicrobial properties due to its phenolic terpenes, such as rosmarinic acid and rosmarol (Cuppett & Hall, 1998). Another series of potential feed additives for animal production in alternative to growth promoters are probiotics. Specifically, in the few studies which have been conducted until now, the dietary inclusion of the yeast *Saccaromyces cerevisiae* significantly lowered mortality of growing rabbits, and in the majority of them it exerted a positive effect also on average daily gain and feed conversion ratio (Falcão-e-Cunha, Castro-Solla, Maertens, Marounek, Pinheiro, et al., 2007). For these reasons this yeast could represent an effective natural additive in rabbit nutrition, even if further research on this topic is definitively required.

On the basis of the above mentioned considerations, taking into account that it is still unclear whether phytogenic antioxidants are effective in replacing the antioxidants usually added to the feeds (Windisch,Schedle, Plitzner, & Kroismayr, 2007), and given that research on rabbit species with regards to phytogenics is still limited, in the current study growing rabbits will be supplemented with different natural additives: oregano, rosemary, Saccaromyces cerevisiae, and vitamin E as well as an effective reference for its antioxidant property and essential body functions (Ebeid, Zeweil, Basyony, Dosoky, & Badry, 2013). Their single and combined effects on rabbit performance, mortality rate, carcass yield, meat chemical composition and oxidative stability will be studied.

## 2. Materials and methods

### *2.1 Animals and diets*

The trial was carried out at the experimental rabbitry of the Department of Applied Biology of the University of Perugia. For this study a total of 320 weaned New Zealand White rabbits were used. At weaning (30 days of age), rabbits were randomly allocated to 8 dietary groups (40 rabbits/group) and housed in single wire net cages (600 x 250 x 330 mm) until 80 days of age, when they were slaughtered. The control group (S) received a standard diet, without any supplementation, whereas the other groups received the S diet supplemented with 150 ppm of vitamin E (E), 0.2 % of an Oregano (*Origanum vulgare*) extract (O), 0.2 % of a Rosemary (*Rosmarinus officinalis*) extract (R), 0.15 % of a product made of *Saccharomyces cerevisiae* bio-active cell (Thepax®, T), a combination of 0.1% O and 0.1% R (OR), a combination of T and 0.2% O (TO) and a combination of T and E (TE). Each diet contained also 50 mg/kg feed of vitamin E, 1 % of Conjugated Linoleic Acid (CLA-L,

LodeStar™) which was extracted by soy oil, 3 % of Omegalin® (Mignini&Petrini) and 0.5 % of a vitamin-mineral premix. All diets were isonitrogenous and isoenergetic (Table 1).

**Table 1.** Chemical composition (g/kg) of the experimental diets

| | Experimental diets | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **S** | **E** | **O** | **R** | **OR** | **T** | **TE** | **TO** |
| Moisture | 125 | 125 | 125 | 125 | 125 | 125 | 125 | 125 |
| Crude protein | 170 | 170 | 170 | 170 | 170 | 170 | 170 | 170 |
| Ether extract | 28 | 28 | 28 | 28 | 28 | 30 | 30 | 30 |
| Crude fibre | 190 | 190 | 190 | 190 | 190 | 190 | 190 | 190 |
| Ash | 95 | 95 | 95 | 95 | 95 | 90 | 90 | 90 |
| Ca | 11.5 | 11.5 | 11.5 | 11.5 | 11.5 | 11.5 | 11.5 | 11.5 |
| Na | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 |
| P | 5.5 | 5.5 | 5.5 | 5.5 | 5.5 | 5.5 | 5.5 | 5.5 |

Groups were homogeneous for live weight and gender, water was available *ad libitum* and the feeding program was adjusted based on the results obtained from previous *ad libitum* feeding tests. The temperature and lighting schedule in the rabbitry were 15-18 °C and 16L:8D, respectively.

In order to calculate the Feed Conversion Ratio (FCR) and Average Daily Gain (ADG), feed consumption and rabbits weight were recorded weekly. Dead animals were recorded and subjected to necropsy examination in order to determine the cause of death.

*2.2. Slaughter and analytical determinations*

At the end of the experiment (80 days of age) and after 12 hours of feed withdrawal the rabbits were weighed, electrically stunned and slaughtered by cutting the carotid artery and jugular vein. Subsequently, the carcasses were chilled for 24 h at +3 °C and 8 rabbits/group were dissected and their *Longissimus dorsi* muscles (LD) and hind legs (HL) were excised according to the procedures of the World Rabbit Science Association (Blasco & Ouhayoun, 1996).

After weighing, HL were deboned in order to determine the meat/bones ratio (Blasco and Ouhayoun, 1996). Femur and tibia were separately weighed, then length and minor diameter were measured with a digital caliper (JUWEL *Digital-Schieblehre Rostfrei* H4215/5X A12).

Femur fracture toughness (FT) was calculated at the average bone length point by using a dynamometer Texture TA-HD (SMS- *Stable Micro System*) with a 6 cm wide cell and a load rate of 0.5 mm/sec.

The meat of LD and HL were vacuum packaged and stored at -20 °C until analyses.

*2.3 Chemical analysis*

Chemical analyses were conducted on meat samples of 8 rabbits/group. Proximate composition (AOAC, 1995) was determined on raw LD and HL meat, with protein content calculated by difference. Cholesterol content was determined on raw LD and HL meat, following the procedure described by Casiraghi, Lucisano, Pompei, & Dellea (1994). Analysis of minerals Fe and Na was performed on raw HL meat by ICP-OES (Spectro Ciros Vision EOP) after microwave digestion (AOAC, 2000, 999.10). The extent of muscle lipid peroxidation of raw LD rabbit meat was evaluated by a spectrophotometer (set at 532 nm, Hitachi U-2000, Theodor - Heuss - Anlage 12, Mannheim, F.R. Germany), which measured the absorbance of thio-barbituric acid-reactive substances (TBARS), and a tetraethoxypropane calibration curve in sodium acetate buffer (pH=3.5, Dal Bosco, Mugnai, Mourvaki, Cardinali, Moscati, et al., 2009). Oxidation products were quantified as malondialdehyde (MDA) equivalents (mg MDA/kg muscle).

*2.3 Statistical Analysis*

Data were analyzed using the General Linear Model procedures of the statistical software STATA (StataCorp, 2005). A One-way ANOVA tested the dietary treatment as fixed effect on the considered variables and the significance level was calculated at the 5% confidence level. The significance of differences in the mortality rate was evaluated by the $\chi^2$ value.

## 3. Results and discussion

### *3.1. Growth performance, slaughter traits and mortality*

Dietary treatments significantly affected the productive performance and slaughter traits of growing rabbits (Table 2). Rabbits live weight (LW) showed consistent differences among groups at the end of the trial ($P$<0.0001). Particularly, the inclusion of 0.2 % of oregano extract (O) and the combination between 0.1 % oregano and 0.1 % rosemary extracts (OR) led animals to a higher LW compared to all other groups. Rosemary extract (R), vitamin E inclusion (E) and control diet (S) provided better performance than T, TE and TO supplemented diets. Diets TE and TO showed similar final LW which was better that T supplemented rabbits (OR>O=E=S=R>TE=TO>T). The trend above mentioned for LW was basically the same also considering the average daily gain (ADG), with O and OR dietary groups showing the best results and T animals being the less performing ($P$<0.0001). Accordingly, oregano supplemented animals showed also the best FCR, whereas T rabbits exhibited the worst index and the other dietary treatments provided intermediate results. Rabbits belonging to O and OR groups showed also the heaviest carcasses ($P$<0. 0001) and, together with R group, the highest carcass yield (P<0.0001).

During the feeding trial almost no mortality was observed, and when present (one animal in O and one in T groups) the necropsy indicated that it was not attributable to the diet.

**Table 2.** Effect of the dietary treatment on rabbits' productive performance, slaughter traits and mortality

| | Experimental diets | | | | | | | | *P*-value | RSD[1] |
|---|---|---|---|---|---|---|---|---|---|---|
| | **S** | **E** | **O** | **R** | **OR** | **T** | **TE** | **TO** | | |
| No. obs | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 | | |
| Live weight (30 days), g | 818.9 | 809.6 | 810.3 | 841.5 | 831.5 | 758.0 | 761.0 | 755.0 | 0.758 | 85.5 |
| Live weight (80 days), g | $2277^{C}$ | $2296^{C}$ | $2344^{C}$ | $2239^{C}$ | $2368^{D}$ | $2076^{A}$ | $2160^{B}$ | $2129^{B}$ | <0.0001 | 101 |
| ADG, g/d | $23.7^{C}$ | $23.6^{C}$ | $24.3^{D}$ | $23.9^{C}$ | $25.0^{D}$ | $21.0^{A}$ | $21.7^{AB}$ | $22.3^{B}$ | <0.0001 | 2.97 |
| FCR | $3.70^{B}$ | $3.75^{B}$ | $2.59^{A}$ | $3.68^{B}$ | $3.52^{B}$ | $4.02^{C}$ | $3.89^{B}$ | $3.78^{B}$ | <0.0001 | 0.46 |
| Carcass weight, g | $1338^{B}$ | $1341^{B}$ | $1425^{C}$ | $1367^{B}$ | $1418^{C}$ | $1212^{A}$ | $1246^{A}$ | $1285^{A}$ | <0.0001 | 102 |
| Carcass yield, % | $58.8^{A}$ | $58.4^{A}$ | $60.8^{B}$ | $61.1^{B}$ | $59.9^{B}$ | $58.6^{A}$ | $58.5^{A}$ | $59.5^{AB}$ | <0.0001 | 0.56 |
| Mortality*, % | 0 | 0 | 2.5 | 0 | 0 | 2.5 | 0 | 0 | - | |

[A, B] Means in the same row having different superscripts are significant at $P$<0.01;

[1]Residual Standard Deviation

*=$X^2$

Most of the existing research on dietary supplementation with oregano and rosemary as phytogenic additives is addressed to pigs and poultry and in both species it generally gave positive results promoting feed intake, increasing body weight and, as a consequence, improving FCR (Franz, Baser, & Windisch, 2010). Results were particularly positive when practical conditions of large-scale animal production were considered than studies under controlled experimental conditions, as in the latter a higher level of hygiene has generally been guaranteed. The majority of the few studies testing oregano dietary supplementation to rabbits found positive results on growth performance. A research on weaned New Zealand White rabbits (Ibrahim,El-Ghamry, & El-Mallah, 2000) found that a dietary supplementation with oregano increased LW and ADG significantly, compared to a control group. Later, also Chrastionvá, Chrenková, Lauková, Rafay, Simonová, et al. (2007) observed a significant improvement in FCR with oregano dietary supplementation. More recently, on growing Hy-plus hybrid rabbits the oregano-supplemented diet led to higher final LW, advantageous FCR and also to lower mortality (Szabóová et al., 2012). Differently, Botsoglou, Florou-Paneri, Christaki, Giannenas, & Spais (2004) observed that a dietary inclusion of 100 and 200 mg/kg diet with an essential oil from oregano exerted no growth-promoting effect on rabbits.

For what concerns rosemary, dietary supplementation with 0.15 % essential oil provided no growth promoting effect on Pannon White rabbits (Erdelyi, Matics, Gerencsér, Princz, Szendrő, et al., 2008). Phytogenic additives are reported to affect the amount and growth of bacterial groups in the rabbit gut (Vàntus, Bónai, Zsolnai, Dal Bosco, Szendrő, et al., 2012) and this might explain, together with the antimicrobial activity, their positive effect on the growth performance of farmed animals.

Results of T supplemented groups were surprising as the few existing studies regarding the probiotic effect of *Saccharomyces cerevisiae* on rabbit growth performance showed positive results (Onifade, Obiyan, Onipede, Adejumo, Abu, et al., 1999; Falcão-e-Cunha et al., 2007), thus requiring further investigations. The vitamin E supplementation confirmed (see Corino, Lo Fiego, Macchioni, Pastorelli, Di Giancamillo, et al., 2007; Szendrő, Gerencsér, Szabó, Fébel, Szín, et al., 2012; Ebeid et al., 2013) to act positively on rabbits growth performance and health, giving better results than the control diet and that with T inclusion.

*3.2. Proximate composition, cholesterol content and oxidative stability of LD meat*

Results presented in Table 3 showed that LD meat presented, on average, high protein (about 24 g/100 g meat) and low lipids (0.54 g/100 g meat) and cholesterol (49.8 mg/100 g meat) contents, thus corresponding to the health image that it is usually attributed to this meat species (Dalle Zotte & Szendrő, 2011). Dietary treatments significantly affected proximate composition and oxidative stability of raw LD meat, whereas cholesterol content remained unaffected. Moisture content was higher in meats of groups O and TE compared to the meat of E group (74.8 and 74.8 vs 73.2 g/100 g meat for O, TE and E groups, respectively), whereas other dietary treatments produced intermediate results.

Vitamin E and rosemary supplementations seemed to promote the deposition of muscular tissue to the detriment of lipids. In fact, E and R meat exhibited a higher protein content compared to O, T, TE, and OR groups (25.0 and 25.0 *vs* 23.5, 23.4, 23.2, and 23.6 g/100 g meat for R, E, O, T, TE and OR groups, respectively), difference being significant ($P<0.0001$) between E, R *vs* OR, T, TE. Also oregano essential oil was reported to increase muscle protein sedimentation (Alarcon-Rojo, Peña-Gonzalez, Janacua-Vidales, Santana et al., 2013), but this feature wasn't observed in our study. Interestingly, the addition of *Saccharomyces cerevisiae*, alone or in combination with other supplements, increased the lipid content of LD meat (0.70, 0.73 and 0.76 g/100 g meat for groups T, TE, TO, respectively) which was statistically different from that of the other groups. This situation negatively affected the protein content of T, TE and TO as described above. Also ash content of LD meat differed among groups, with TO meat showing a higher value than O and OR ($P<0.001$) with other treatments being intermediate.

Experimental diets contained the same amount of Ca, Na and P but T-supplemented diets were slightly lower in ash content, therefore there has been reason to suppose that also no ash difference in meat would have been observed. It seems that *Saccharomyces cerevisiae* was able to improve the gut absorption of some minerals, such as Fe, and so their deposition in meat (Table 4).

The LD meat chemical composition differed from that presented by Dal Bosco, Castellini, Bianchi, & Mugnai (2004). On overall, the LD meat of that experiment had lower protein and higher lipid contents compared to LD meat of the present study; when animals were supplemented with 200 mg/kg diet of α-tocopheryl acetate no effect on meat proximate composition was found. In another trial using the same dietary level of α-tocopheryl acetate of the previous cited work, Castellini, Dal Bosco, Bernardini, & Cyril (1998) observed that fresh

LD meat of the supplemented animals was richer in moisture than that of the control group. Unfortunately, in the latter study the complete proximate composition wasn't analysed.

Regarding the oxidative stability of the LD meat, all supplemented diets led to a lower TBARs content compared to the S group ($P<0.01$). The inclusion of an extra quote of vitamin E increased the oxidative stability of LD meat, thus both E and TE groups were positively affected. A partly different situation regarded oregano supplemented animals: O treatment showed the same oxidative stability of E and TE groups, but neither TO nor OR combination didn't provide the same positive result which was obtained with the simultaneous dietary inclusion of T and E. Specifically, E, TE and O groups showed a lower oxidation degree than all other treatments (0.17, 0.18 and 0.18 *vs* 0.24, 0.20, 0.21, 0.20 and 0.21 mg MDA/kg meat for E, TE, O, S, T, TO, OR and R groups, respectively).

Dietary vitamin E is known to accumulate within cell membranes, thus preserving their integrity by preventing the oxidation of membrane phospholipids and consequently improving meat oxidative stability (Corino, Pastorelli, Pantaleo, Oriani, & Salvatori, 1999; Dalle Zotte, Cossu, & Parigi Bini, 2000; Descalzo & Sancho, 2008; Ebeid et al., 2013). Similarly, oregano essential oil is rich in thymol and carvacrol thus protecting meat lipids from oxidative damage. Even if this is documented mainly in pigs and poultry, in the few scientific works on rabbits the active compounds present in oregano essential oil were absorbed by the animals, and this increased the antioxidant capacity of tissues. This was observed in the present study and also in the work by Botsoglou et al. (2004), in which a dietary supplementation with 200 mg/kg diet of oregano essential oil significantly delayed oxidation of meat lipids. However, a recent study of Rotolo, Gai, Nicola, Zoccarato, Brugiapaglia, et al. (2013) did not find differences in meat quality traits and oxidative lipid stability of LD meat from rabbits fattened with a diet supplemented with 1% (w/w) of oregano and sage dried leaves, likely due to the low concentration of active compounds.

Rosemary extract was less effective in counteracting meat lipid oxidation than oregano (0.21 *vs* 0.18 mg MDA/kg meat; $P<0.05$) but the difference with the control diet was still significant (0.21 vs 0.24 mg MDA/kg meat; $P<0.05$). In another trial on poultry (Lopez-Bote, Gray, Gomaa, & Flegal, 1998), the dietary supplementation with rosemary and sage extracts (500 mg/kg diet) significantly reduced lipid oxidation of meat, even if it was less effective than dietary α-tocopheryl acetate (200 mg/kg diet). A further study on poultry (Yesilbag, Eren, Agel, Kovanlikaya, & Balci, 2011) confirmed results of the previous cited work and those of the present study. Dietary supplementation with *Saccharomyces cerevisiae* was not widely studied in rabbit and the few existing experiments haven't considered its effect on

meat oxidative stability, focusing almost exclusively on growth performance and digestive efficiency of the animals (Falcão-e-Cunha et al., 2007).

**Table 3.** Effect of the dietary treatment on the proximate composition (g/100 g meat), cholesterol (mg/100 g meat) and TBARs (mg MDA/kg meat) contents of rabbits' *Longissimus dorsi* (LD) muscle

| | Experimental diets | | | | | | | | *P*-value | RSD[1] |
|---|---|---|---|---|---|---|---|---|---|---|
| | **S** | **E** | **O** | **R** | **OR** | **T** | **TE** | **TO** | | |
| No. obs | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | | |
| Moisture | $74.0^{ABC}$ | $73.2^{A}$ | $74.8^{C}$ | $73.3^{AB}$ | $74.7^{BC}$ | $74.6^{ABC}$ | $74.8^{C}$ | $74.1^{ABC}$ | 0.0006 | 0.85 |
| Protein | $24.3^{AB}$ | $25.0^{B}$ | $23.5^{A}$ | $25.0^{B}$ | $23.6^{A}$ | $23.4^{A}$ | $23.2^{A}$ | $23.8^{AB}$ | <0.0001 | 0.76 |
| Lipids | $0.42^{AB}$ | $0.40^{A}$ | $0.41^{AB}$ | $0.41^{AB}$ | $0.50^{ABC}$ | $0.70^{ABC}$ | $0.73^{BC}$ | $0.76^{C}$ | 0.0001 | 0.20 |
| Ash | $1.30^{AB}$ | $1.36^{AB}$ | $1.25^{A}$ | $1.29^{AB}$ | $1.24^{A}$ | $1.37^{AB}$ | $1.33^{AB}$ | $1.44^{B}$ | 0.0009 | 0.09 |
| Cholesterol | 49.5 | 51.9 | 48.4 | 51.5 | 51.2 | 47.7 | 48.4 | 50.1 | 0.0692 | 3.18 |
| TBARs | $0.24^{C}$ | $0.17^{A}$ | $0.18^{A}$ | $0.21^{B}$ | $0.20^{B}$ | $0.20^{B}$ | $0.18^{A}$ | $0.21^{B}$ | <0.005 | 0.10 |

$^{A, B}$ Means in the same row having different superscripts are significant at $P<0.01$;

[1]Residual Standard Deviation

*3.3. Proximate composition, cholesterol and mineral contents of HL meat*

Dietary treatments significantly affected also the proximate composition and mineral content of raw HL meat, whereas cholesterol content was similar in all groups (Table 4). As it was mentioned for LD, also HL meat showed a healthy chemical composition thanks to an average high protein (21.8 g/100 g meat) and moderate lipids (2.6 g/100 g meat) contents. The HL of T and TE animals had a significantly (P<0.0001) lower protein content compared to all other groups (21.2 and 21.1 *vs* 22.0, 22.2, 22.1, 22.2 and 22.1 g/100 g meat for T, TE, S, E, O, O and R groups, respectively), whereas the simultaneous supplementation of oregano and *Saccharomyces cerevisiae* (TO) provided intermediate results (21.7 g/100 g meat). Lipids content differed only between TO and OR groups, being lower in the latter (3.18 *vs* 2.19 g/100 g meat; P<0.05). As for LD meat, also in HL meat the T-supplemented diets led to numerically higher lipids content. Similarly to lipids, ash content of HL meat in T, TE and TO groups was higher compared to the others (*P*<0.0001). Animals receiving the T diets exhibited HL meat richer in Fe than that of animals fed the E and O diets (1.38 *vs* 0.84 and 0.85 mg/100 g meat for T, E and O groups, respectively), whereas the other treatments didn't differ each other. This trend was partly reversed when considering Na content of HL meat, where E, O and OR HL meats presented a higher Na content than those T, TE and TO (54.3, 53.7 and 55.2 *vs* 48.7, 49.3 and 47.4 mg/100 g HL meat for E, O, OR, T, TE and TO groups, respectively; P<0.0001).

**Table 4.** Effect of the dietary treatment on the proximate composition (g/100 g meat), cholesterol (mg/100 g meat), Fe and Na (mg/100 g meat) contents of rabbits' hind leg (HL) meat

| | Experimental diets | | | | | | | | *P*-value | RSD[1] |
|---|---|---|---|---|---|---|---|---|---|---|
| | **S** | **E** | **O** | **OR** | **R** | **T** | **TE** | **TO** | | |
| No. obs | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | | |
| Water | 74.3 | 74.3 | 74.1 | 74.3 | 74.2 | 74.5 | 74.7 | 73.7 | 0.2386 | 0.70 |
| Protein | $22.0^{B}$ | $22.2^{B}$ | $22.1^{B}$ | $22.2^{B}$ | $22.1^{B}$ | $21.2^{A}$ | $21.1^{A}$ | $21.7^{AB}$ | <0.0001 | 0.40 |
| Lipids | $2.41^{ab}$ | $2.31^{ab}$ | $2.57^{ab}$ | $2.19^{a}$ | $2.46^{ab}$ | $2.74^{ab}$ | $2.77^{ab}$ | $3.18^{b}$ | 0.0279 | 0.57 |
| Ash | $1.25^{A}$ | $1.21^{A}$ | $1.27^{A}$ | $1.27^{A}$ | $1.27^{A}$ | $1.57^{B}$ | $1.47^{B}$ | $1.44^{B}$ | <0.0001 | 0.10 |
| Cholesterol | 65.4 | 65.8 | 69.5 | 67.3 | 64.8 | 68.2 | 67.0 | 70.7 | 0.0978 | 4.30 |
| Fe | $0.92^{ab}$ | $0.84^{a}$ | $1.02^{ab}$ | $0.96^{ab}$ | $0.85^{a}$ | $1.38^{b}$ | $1.13^{ab}$ | $0.93^{ab}$ | 0.0214 | 2.80 |
| Na | $53.1^{BC}$ | $54.3^{C}$ | $53.7^{C}$ | $55.2^{C}$ | $53.0^{BC}$ | $48.7^{A}$ | $49.3^{AB}$ | $47.4^{A}$ | <0.0001 | 25.0 |

[A, B] Means in the same row having different superscripts are significant at $P<0.01$; [a, b] Means in the same row having different superscripts are significant at $P<0.05$;

[1]Residual Standard Deviation

In literature no studies considering the effect of dietary oregano and rosemary extracts on rabbits meat proximate composition were conducted until now. Only one study, reported in the previous paragraph (Rotolo et al., 2013), tested the combination of oregano and sage dried leaves on proximate composition of LD meat only. The E diet did not modify the proximate composition of HL meat confirming results of Ebeid et al. (2013). The mineral content of rabbit meat is rarely considered in literature, even if it would represent an important nutritional information for consumers. The average Fe content of HL meat was 1.0 mg/100 g meat and that of Na was 51.8 mg/100 g meat. The Fe value found in our study was in line with the range found in literature (1.1-1.3 mg/100 g edible fraction; Dalle Zotte & Szendrő, 2011) whereas that of Na slightly exceeded the range of the literature (37-47 mg/100 g edible fraction). Again, as above stated, further research on the effect of *Saccharomyces cerevisiae* on gut mineral absorption is requested to confirm our results.

### *3.4. Bone traits of the HL*

Bone traits of the raw HL (Table 5) were also significantly affected by dietary treatments. Interestingly, T and TO had heavier HL than S, E, O, OR and R groups, whereas TE showed an intermediate weight ($P<0.0001$). This result was partly unexpected as rabbits supplemented with *Saccharomyces cerevisiae* showed opposite trends in terms of LW at 80 days and carcass weight (Table 2).

**Table 5.** Effect of the dietary treatment on bone traits of rabbits' hind leg (HL)

| | Experimental diets | | | | | | | | *P*-value | RSD[1] |
|---|---|---|---|---|---|---|---|---|---|---|
| | **S** | **E** | **O** | **OR** | **R** | **T** | **TE** | **TO** | | |
| No. obs | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | | |
| HL weight, g | $225^{A}$ | $215^{A}$ | $232^{AB}$ | $225^{A}$ | $217^{A}$ | $262^{C}$ | $250^{BC}$ | $266^{C}$ | <0.0001 | 13.6 |
| Bones weight, g | $38.4^{AB}$ | $36.4^{A}$ | $41.3^{B}$ | $39.5^{AB}$ | $38.8^{AB}$ | $36.2^{A}$ | $38.7^{AB}$ | $36.6^{A}$ | 0.0013 | 2.42 |
| Bones, % HL | $17.1^{C}$ | $16.9^{BC}$ | $17.8^{C}$ | $17.6^{C}$ | $17.9^{C}$ | $13.8^{A}$ | $15.5^{B}$ | $13.8^{A}$ | <0.0001 | 0.98 |
| Meat, % HL | $82.9^{A}$ | $83.1^{AB}$ | $82.2^{A}$ | $82.4^{A}$ | $82.1^{A}$ | $86.2^{C}$ | $84.5^{B}$ | $86.2^{C}$ | <0.0001 | 12.8 |
| Meat/bones ratio | $4.84^{A}$ | $4.93^{AB}$ | $4.62^{A}$ | $4.70^{A}$ | $4.60^{A}$ | $6.27^{C}$ | $5.51^{B}$ | $6.25^{C}$ | <0.0001 | 1.28 |
| Femur weight, g | $15.1^{ABC}$ | $14.1^{A}$ | $16.8^{C}$ | $16.2^{BC}$ | $15.3^{ABC}$ | $14.6^{AB}$ | $15.2^{ABC}$ | $15.1^{ABC}$ | 0.0007 | 1.24 |
| Femur, % HL | $6.71^{BC}$ | $6.53^{BC}$ | $7.27^{C}$ | $7.22^{C}$ | $7.09^{C}$ | $5.56^{A}$ | $6.08^{AB}$ | $5.70^{A}$ | <0.0001 | 0.50 |
| Femur minor ∅, mm | 6.59 | 6.33 | 6.64 | 6.32 | 6.16 | 6.29 | 6.27 | 6.21 | 0.6837 | 0.59 |
| Femur length, mm | $94.7^{B}$ | $93.1^{AB}$ | $93.2^{AB}$ | $94.7^{B}$ | $92.5^{AB}$ | $90.7^{A}$ | $90.6^{A}$ | $90.0^{A}$ | <0.0001 | 2.10 |
| Femur fracture, kg | $45.0^{ab}$ | $46.9^{ab}$ | $49.1^{ab}$ | $44.3^{ab}$ | $42.8^{a}$ | $48.1^{ab}$ | $48.6^{ab}$ | $51.2^{b}$ | 0.0328 | 5.12 |

[A, B] Means in the same row having different superscripts are significant at $P<0.01$; [a, b] Means in the same row having different superscripts are significant at $P<0.05$;

[1]Residual Standard Deviation

Observing the results of bones weight and their incidence on the HL, we could state that the higher HL weight of T, TE and TO animals was attributable to a high meatiness of the cut rather than an improved bones development or weight. In fact, meat/bones ratio of T, TO and TE was higher than that of the other dietary treatments ($P<0.0001$). Despite this, femur stress fracture didn't differ among treatments with the exception of TO animals which showed a higher value than R supplemented rabbits (51.2 *vs* 42.8 kg for TO and R groups, respectively). Results of our study differed from those of Eiben et al. (2013) as concerns the vitamin E effect on HL meatiness, who supplementing rabbits with 60, 150 or 300 mg/kg diet of synthetic vitamin E they observed a lower meatiness than those of our study.

## 4. Conclusions

This study showed that an adequate supplementation with phytogenic additives and probiotics to growing rabbits can exert a positive effect on productive performance, meat quality and protection against lipid oxidation. Oregano was the most promising natural source in order to ensure optimal growth and feed efficiency of growing rabbits, whereas rosemary and vitamin E showed results comparable to those of the control group. Even if all the natural additives improved the oxidative stability of the meat compared to the standard diet, oregano was the most effective in this sense.

Interestingly, rosemary supplementation improved the nutritional composition of LD meat in terms of protein content, thus suggesting a possible positive effect on the muscle metabolism. However, in order to be confirmed, the latter would require further investigation. The dietary inclusion of *Saccharomyces cerevisiae* increased the meatiness of the HL, but surprisingly rabbits supplemented with this yeast showed the worst productive performance, thus requiring additional research. Finally, further studies to find the optimal inclusion level and taking also into account economical aspects, together with the evaluation of different plant extracts combinations should be performed.

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# CHAPTER 5

Running Head: unfermented and fermented rooibos to ostrich meat products

# First evaluation of unfermented and fermented rooibos (*Aspalathus linearis*) in preventing lipid oxidation in meat products

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## ABSTRACT

This study consisted of two trials aiming to evaluate, for the first time, the antioxidant potential of rooibos in meat products. With this purpose, the first trial evaluated three unfermented (green) rooibos forms (dried leaves, water extract, freeze-dried extract) added at 2% inclusion level to ostrich meat patties on an 8-day shelf-life trial. A Control group without green rooibos inclusion was also considered. The second trial evaluated the addition of different concentrations (0%, 0.25%, 0.5% and 1%) of a fermented rooibos extract to nitrite-free ostrich salami. The 2% green rooibos inclusion considerably lowered the TBARS content of ostrich patties, in this way extending their shelf-life. The fermented form (0.5% and 1%) was also effective in delaying lipid oxidation in ostrich salami until 15 days of ripening. The antioxidant potential of both green and fermented forms of Rooibos in meat products was confirmed, even if its effect on lipid oxidation requires further study and long-term effects are not yet fully understood.



**Keywords:** Rooibos, Ostrich meat, Salami, TBARS, Lipid oxidation

## 1. Introduction

In addition to causing deterioration of color, texture, flavor, and nutritive value, lipid oxidation in foods generates end-products which may be harmful to human health. Compounds such as malondialdehyde and cholesterol oxidation products are reported to have cytotoxic and genotoxic potential and have been linked to the promotion of atherosclerosis, cardiovascular disease, and cancer (Kanner, 2007; Muselík, García-Alons, Martín-López, Žemlička, & Rivas-Gonzalo, 2007; Soyer, Özalp, Dalmiş, & Bilgin, 2010). Several studies have focused on the correlation between the consumption of red meat and nitrate-containing or smoked cured meat and the incidence of colorectal cancer (Chao, Thun, Connell, McCullough, Jacobs, Flanders, Rodriguez, Sinha, & Calle, 2005; Santarelli, Pierre, & Corpet, 2008; Bastide, Pierre, & Corpet, 2011; Corpet, 2011). Colorectal cancer is the third most common type of cancer worldwide and the second most common cause of cancer death in affluent countries (Bastide et al., 2011). For these reasons, in 2007 the World Research Cancer Fund panel recommended that the intake of red meat should be reduced and that processed meat should be avoided (World Cancer Research Fund, 2007). Specifically, Corpet (2011) stated that the promotion of tumor formation induced by the consumption of fresh red meat was explained by the fat peroxidation pathway, whereas the N-nitroso pathway mainly explained the close relationship between cancer and nitrite-cured meat.

A way to prevent peroxidation could be represented by the inclusion of antioxidants or the removal of oxygen by vacuum packaging. Nitrosation can be avoided by eliminating the addition of nitrite to meat or removing it from the gastrointestinal tract (Santarelli et al., 2008). A possible alternative to the substitution of nitrite in preventing oxidative stress may be obtained by using natural antioxidants, which include phenolic compounds mainly derived from plants currently ranked among the most widely studied natural antioxidants (Tang, Kerry, Sheehan, Buckley, & Morrissey, 2000; Karpińska, Karlsson, Schinkel, Streller, Süss, Melzer, & Wingsle, 2001; Bozkurt, 2006; Georgantelis, Blekas, Katikou, Ambrosiadis, & Fletouris, 2007).

Rooibos tea (*Aspalathus linearis*) is made from a South African leguminous shrub that has been traditionally used as a beverage by the Khoi people since 1772 (Morton, 1983). The use of Rooibus as an alternative to *Camellia sinensis* in the production of Oriental tea has been gaining popularity since the early 1900s. Currently, only the Rocklands or red type vaunts commercial importance, and in 2010 it accounted for 23% of the entire South African tea market with an export volume of approx. 6,000 tons (Joubert, & De Beer, 2011). Such growing popularity has promoted many *in vitro* and *in vivo* studies focused primarily on the

health-promoting properties of unfermented (green) and fermented rooibos. Rooibos has been found effective in preventing chemically-induced liver damage (Uličná, Vančová, Waczulíková, Božek, Janega, Babál, Lišková, & Greksák, 2008), inflammation (Baba, Ohtsuka, Haruna, Lee, Nagata, Maeda, Yamashiro, & Shimizu, 2009), lipid oxidation (Fukasawa, Kanda, & Hara, 2009), hyperglycemia (Kawano, Nakamura, Hata, Minakawa, Miura, & Yagasaki, 2009), and oxidative stress (Marnewick, Rautenbach, Venter, Neethling, Blackhurst, Wolmarans, & Macharia, 2011). A study on plasma antioxidant status showed the consumption of rooibos tea to provide a source of dietary antioxidants in human volunteers (Villaño, Pecorari, Testa, Raguzzini, Stalmach, Crozier, Tubili, & Serafini, 2010). These antioxidant properties are due to its unusual polyphenolic compounds, which include aspalathin, a dihydrochalcone *C*-glucoside exclusive of rooibos; nothofagin, which is another rare *C*-*C* linked dihydrochalcone glucoside; and orientin and iso-orientin, which are both oxidation products of aspalathin characterized by a higher stability under heat processing and varying pH conditions than aspalathin, vitexin, iso-vitexin, luteolin, chrysoeriol, quercetin, isoquercitrin, or rutin (Joubert, & De Beer, 2011). The fermentation process that produces the distinctive reddish-brown colored tea leads to a consistent change in phenolic composition due to oxidation associated with apparently lower antioxidant and antimutagenic effects (McKay, & Blumberg, 2007; Joubert, Viljoen, De Beer, & Manley, 2009).

Bearing these considerations in mind, this study aimed to test for the first time the antioxidant activity induced by unfermented (green) and fermented rooibos in ostrich meat, which is particularly prone to oxidation due to its high polyunsaturated fatty acids and heme iron content (Cooper, & Horbañczuk, 2002; Lombardi-Boccia, Martinez-Dominguez, & Aguzzi, 2002). The first trial tested green rooibos in a shelf-life trial on ostrich meat patties; the second trial evaluated a fermented rooibos extract as a natural antioxidant additive in the production of ostrich salami.

## 2. Materials and Methods

### *2.1 Experimental design*

Two experimental trials were conducted.

The first trial considered ostrich meat patties: one batch with no green rooibos supplement (Control; C) was compared to 3 batches of 3 different green rooibos forms: dried leaves (T1), water extract (T2) and freeze-dried extract (T3).

The second trial considered ostrich salami: a control group (C; 0% rooibos) was compared to salami with 3 increasing levels (0.25% w/w, 0.5%, 1%) of fermented rooibos extract (Treatments T1, T2 and T3, respectively).
Both trials took place at the laboratory of Stellenbosch University (South Africa).

*2.2 Preparation of green and fermented rooibos extracts*

The water extract and the freeze-dried extract from green rooibos (first trial) were prepared as follows: 2 g of dried and crushed green rooibos leaves were steeped in 50 mL boiling distilled water for 10 minutes. After 20 minutes of cooling at room temperature and subsequent filtration through a Whatman N° 1 filter paper, 25 mL remained (T2: water extract). The water extract was then placed in a plastic cup, frozen at -80 °C for 30 minutes, and then dried in a Christ Loc-1m drier overnight (T3: freeze-dried extract).

The fermented rooibos extract incorporated in the salami (second trial) was a commercial powdered product subjected to the procedure reported by Joubert et al. (2009). Fermented rooibos was stored at room temperature in the dark in sealed transparent plastic tubes in a desiccator until use. The phenolic composition of the fermented rooibos extract (Table 1) was analyzed through a newly-optimized reversed phase liquid chromatographic separation technique and the method developed by Beelders, Sigge, Joubert, de Beer, & de Villiers (2012).

**Table 1**. Phenolic composition of fermented rooibos extract used in the manufacture of ostrich salami

| Flavonoid | Concentration (g compound/100 g extract) |
|---|---|
| Aspalathin | 0.482 |
| Nothofagin | 0.056 |
| Isoorientin | 0.956 |
| Orientin | 0.823 |
| Quercetin-3-*O*-robinobioside | 0.637 |
| Isoquercitrin | 0.138 |
| Vitexin | 0.179 |
| Hyperoside | 0.167 |
| Rutin | 0.179 |
| Isovitexin | 0.144 |
| Luteolin-7-*O*-glucoside | 0.196 |

Analyzed according to the method of Beelders, Sigge, Joubert, de Beer, & De Villiers (2012)

*2.3 First trial: Shelf-life of ostrich meat patties*

For this trial, 4 batches of ostrich meat patties were prepared. One meat batch without rooibos inclusion was considered as the Control (C), whereas the other 3 batches were supplemented with a 2% (w/w) of T1, T2 and T3 green rooibos forms.

Thirty-day frozen ostrich meat (*Iliofibularis,* or fan fillet, muscle) was defrosted overnight at ±4 °C and then ground with a meat grinder through 5 mm diameter holes. The meat was then divided into four equal parts of 200 g each and manually mixed with T1, T2 and T3 green rooibos. The meat patty of each treatment was then split into five sub-samples of ≈ 40 g each and individually wrapped in plastic film to minimize direct air contact.

In order to simulate retail display conditions, patties were stored at 4±1 °C under fluorescent light illumination (L58 W/ 20, Osram, Germany) at 870 lux (MT 940, Major) for an 8-day shelf-life trial. Each of the five sub-samples above represented a separate Day of analysis in the shelf-life trial. The Thiobarbituric acid reactive substance (TBARS) content of the meat samples was analysed at Day 0, 4, 6 and 8, whereas L*a*b* color values and pH measurements were determined at Day 0, 2, 4, 6 and 8 of storage (6 repetitions for each Day of analysis).

*2.4 Second trial: Oxidation stability of ostrich salami*

Four batches of 24 ostrich salamis each (n=96) were prepared. One batch represented the Control (C) without any fermented rooibos tea extract inclusion (0%), whereas the other 3 batches were provided with the three inclusion levels of fermented rooibos extract mentioned above (Treatments T1, T2 and T3). The other ingredients used in the salami were defrosted ostrich meat (fan fillet) and fresh pork belly in a 75:25 ratio, 2.4% NaCl, 0.5% ground black pepper, and a starter culture mix of *L. plantarum* 423 and *Micrococcus* sp. in order to ensure $1x10^5$ cfu/g meat. Ingredients were mixed using a Butcherequip grinder through 5 mm diameter holes and stuffed into permeable cellulose casings in order to obtain salami rolls of about 500 g/each. All the salami were nitrite-free and slow-fermented, and were ripened in a dedicated chamber for 30 days. After one day of fermentation (18 °C and 90% relative humidity, RH), the temperature was gradually reduced by one degree/day until reaching 12°C (75-85% RH), which was maintained for the rest of the ripening period. Salami weights were measured at Day 0, Day 7, Day 15, and Day 30, and cumulative weight losses were subsequently calculated. At Day 0, Day 15, and Day 30 of ripening, the TBARS content and the pH of the salami were determined, whereas L*a*b* color values were measured at Day 0

and Day 30. For each variable, 4 salami/Day/Treatments were dedicated to analysis (for pH and color values, measurements were repeated 6 times on each salami: n= 24/Day). Water activity ($a_w$) was measured at Day 30 on 16 salamis/Treatment.

*2.5 TBARS and water activity*

With the exception of $a_w$ determination, which was performed at the Animal Medicine, Production, and Health Department of the University of Padova (Italy), all analyses were performed at Stellenbosch University (South Africa). TBARS analysis of both meat patties and salami rolls was performed using the method reported by Lynch, & Frei (1993). Water activity was measured with a water activity meter (Aqualab Decagon series 4 TEV) at room temperature (21 °C). After the instrument was calibrated according to the manufacturer's instructions, six samples from each salami were taken (1 cm length and 3 mm width) and put in a Teflon® capsule for measurement.

*2.6 Statistical analysis*

Data were analyzed using the General Linear Model procedures of statistical analysis software SAS 9.1.3 for Windows (SAS, 2006). A one-way Anova was used to determine the effects of the green rooibos type of preparation (dried leaves, water extract, freeze-dried extract; Trial 1) and those of the fermented rooibos extract inclusion levels (0.25% 0.5%, 1%; Trial 2) on TBARS, L*, a*, b* color values, pH, weight loss, and $a_w$. Least square means were obtained using the Bonferroni test. The significance of the statistics was calculated at a 5% confidence level; normality of data was analysed with a Shapiro-Wilk confidence level of 85%. LSMeans of the estimated parameters were assessed for each Day of trial. Since the quantity of data available was insufficient to perceive the between-days variability, the L*, a*, b* color value data of First Trial were pooled, i.e., the Day of the trial was not included in the model.

## 3. Results and Discussion

### *3.1 First trial: Shelf-life of ostrich meat patties*

The TBARS results obtained for ostrich meat patties at Day 0, Day 4, Day 6, and Day 8 of shelf-life evaluation are reported in Table 2. At Day 0, all experimental groups showed the same Malondialdehyde (MDA) content (2.37 *vs* 2.41 *vs* 2.27 *vs* 2.40 mg MDA/kg meat for C, T1, T2 and T3, respectively), but from Day 4 until the end of the trial (Day 8), the 2% unfermented rooibos inclusion significantly (P<0.001) reduced TBARS values compared to the C group. Green rooibos therefore appeared to considerably lower the oxidation rate of ground/minced ostrich meat regardless of the form in which it was added. At the end of the shelf life trial (Day 8), T1, T2, and T3 showed TBARS values comparable to those found in the C group on Day 4 (4.17, 3.78, 3.98 and 4.33 mg MDA/kg meat for T1, T2 and T3 and C, respectively).

**Table 2.** TBARS values (mg MDA/kg meat) of ostrich meat patties prepared without (C) or with 2% dried leaves (T1), water extract (T2) or freeze-dried extract (T3) obtained from green rooibos

| | Treatments | | | | *P*-value | RSD[(1)] |
|---|---|---|---|---|---|---|
| | C | T1 | T2 | T3 | | |
| Samples, No. | 24 | 24 | 24 | 24 | | |
| Day 0 | 2.37 | 2.41 | 2.27 | 2.40 | 0.145 | 0.11 |
| Day 4 | $4.33^{A}$ | $2.44^{B}$ | $2.20^{B}$ | $2.42^{B}$ | <0.0001 | 0.16 |
| Day 6 | $7.25^{A}$ | $2.83^{B}$ | $2.70^{B}$ | $2.67^{B}$ | <0.0001 | 0.61 |
| Day 8 | $7.83^{A}$ | $4.17^{B}$ | $3.78^{B}$ | $3.98^{B}$ | <0.0001 | 0.27 |

Means in the same row with unlike superscripts differ (*P* <0.0001); [(1)] Residual Standard Deviation

Together with 4-hydroxynonenal, MDA is among the major aldehyde products of lipid peroxidation; both are formed by the oxidation of polyunsaturated fatty acids (PUFA; Bastide et al., 2011). The initial MDA content of ostrich meat patties measured at Day 0 was high, proving once again that ostrich meat is particularly prone to oxidation due to its high PUFA and heme iron content. Iron, both heme and non-heme forms, acts as a pro-oxidant, and PUFA are more sensitive to the oxidative action of free radicals (Carlsen, Møller, & Skibsted, 2005). This was confirmed when comparing the TBARS values measured in this study with those of Rhee, & Myers (2003), Allen, & Cornforth (2010), and Kong, Zhang, & Xiong, (2010) on goat (0.27 mg MDA/kg meat), beef (0.25 mg MDA/kg meat), and pork (< 1 mg/kg meat) meat, respectively.

In this study, TBARS values measured at Day 0 were relatively high (ranging from 2.27 to 2.40 mg MDA/kg meat) compared to those reported by Leygonie, Britz, & Hoffman (2011) in ground ostrich meat packaged under different modified atmosphere packaging (MAP) conditions (ranging from 1.2 to 2.21 mg MDA/kg meat). Despite this, from Day 0 to Day 6, green rooibos, in all three forms in which it was added to the meat, stabilized the lipid oxidation rate, and this made the TBARS values lower than those reported by Leygonie et al. (2011) on meat stored under MAP (70% oxygen, 30% carbon dioxide) for the same shelf life time. Differing from the study of Leygonie et al. (2011), the ostrich meat used in this investigation was previously frozen at -20 °C for 30 days, and this was most probably responsible for the meat's higher initial MDA content, as has been shown by Soyer et al. (2010) in chicken meat. Moreover, the grinding process increased the exposed surface area of the meat, making it more susceptible to oxidation.

Ostrich steaks which did not undergo freezing or grinding processes (Fernández-López, Sayas-Barberá, Muñoz, Sendra, Navarro, & Pérez-Alvarez; 2008), in fact, showed lower initial TBARS values (< 0.4 mg MDA/kg meat). Nevertheless, green rooibos provided better results than every combination of MAP used for ostrich meat storage (on Day 4: > 4 and > 3 mg MDA/kg meat for 1:1 and 3:1 headspace ratios, respectively; Bingol, & Ergun, 2011). In another study (Seydim, Acton, Hall, & Dawson, 2006) in which AIR (80% nitrogen, 20% oxygen) and $O_2$ (80% oxygen, 20% carbon dioxide) MAP conditions were compared for freshly ground ostrich meat in a shelf life evaluation, the MDA values measured at Day 6 were from 2 to 8 fold higher than those found in our rooibos-enriched ostrich meat patties (5 vs >20 vs 2.4 mg MDA/kg meat, for AIR, $O_2$ and rooibos-added ostrich meat patties, respectively).

The pH of ostrich meat patties had high and increasing values throughout the shelf life trial (Table 3). At Day 0, the average meat pH was around 6.3, corresponding to the value expected for ostrich meat (Viljoen, Hoffman, & Brand, 2005) and required for the desirable water binding capacity that makes the meat ideal for processing. In this trial, the T1 group (dried green rooibos leaves) already showed a significantly lower pH ($P<0.001$) compared to the other groups at the beginning of the trial (6.23 *vs* 6.27, 6.28 and 6.29 for T1 *vs* T2, T3 and C, respectively), and this difference remained statistically significant up to Day 6 of storage (6.31 *vs* 6.37, for T1 *vs* T2, T3, C, respectively). The pH-lowering effect of green rooibos dry leaves requires confirmation by further research, however. Contrary to observations in other ostrich meat shelf-life trials where pH values fell from an initial naturally high pH to values < 6.0 (Seydim et al., 2006; Bingol et al., 2011), the pH values of all groups in this study increased from Day 0 to Day 8 (Day 8 average pH=6.5).

Except for the initial pH value however, our findings were in accordance with the trend observed in the Control group reported by Leygonie et al. (2011) in which *Iliofibularis* muscle steaks placed in polystyrene trays wrapped in plastic film stored at ±4 °C exhibited an initial pH=6.18 (Day 0) that constantly increased until reaching values of 6.45 by the end of the shelf-life trial (Day 10). In our study, in which the meat was previously frozen for 30 days, thawed, and then used for patty preparation, the initial rise in pH was more probably a result of gradual protein breakdown than non-protein nitrogen compound formation.

**Table 3**. pH values of ostrich meat patties prepared without (C) or with 2% dried leaves (T1), water extract (T2) or freeze-dried extract (T3) obtained from green rooibos

| | Treatments | | | | *P*-value | RSD[(1)] |
|---|---|---|---|---|---|---|
| | C | T1 | T2 | T3 | | |
| Samples, No. | 30 | 30 | 30 | 30 | | |
| Day 0 | $6.29^{A}$ | $6.23^{B}$ | $6.27^{A}$ | $6.28^{A}$ | <0.0001 | 0.26 |
| Day 2 | $6.33^{Ab}$ | $6.30^{B}$ | $6.35^{Aa}$ | $6.35^{Aa}$ | <0.0001 | 0.01 |
| Day 4 | $6.35^{Ab}$ | $6.31^{B}$ | $6.36^{Aa}$ | $6.34^{Ab}$ | <0.0001 | 0.01 |
| Day 6 | $6.37^{A}$ | $6.31^{B}$ | $6.37^{A}$ | $6.37^{A}$ | <0.0001 | 0.02 |
| Day 8 | 6.51 | 6.41 | 6.46 | 6.43 | 0.1102 | 0.07 |

Means in the same row with unlike superscripts differ (*P* <0.0001); [(1)] Residual Standard Deviation

The L*a*b* color values (Table 4) showed that ostrich meat patties made with 2% green rooibos water extract (T2) had higher L* values (P<0.01) than those of C and T1, except for the patties made with 2% freeze-dried extract, which did not differ from the other experimental groups (L*=30.86 *vs* 29.58 and 29.64 for T2 *vs* C and T1, respectively). Group T2 also showed a significantly higher redness value (a*), (P<0.001) compared to T1 and T3 (a*=15.12 *vs* 11.29 and 12.62 for T2 *vs* T1 and T3, respectively). Lastly, T1 and T2 exhibited higher yellowness (b*) values than those of C and T3, which showed no differences between each other (8.61 vs 10.68 vs 10.88 vs 9.49 for C, T1, T2 and T3, respectively). These color changes are most probably linked to the red color of rooibos tea extracts.

**Table 4.** Colour values (lightness [L*], redness [a*] and yellowness [b*]) for ostrich meat patties prepared without (C) or with 2% dried leaves (T1), water extract (T2) and freeze-dried extract (T3) obtained from green rooibos

| | Treatments | | | | *P*-value | RSD[(1)] |
|---|---|---|---|---|---|---|
| | C | T1 | T2 | T3 | | |
| Samples, No. | 30 | 30 | 30 | 30 | | |
| L* | $29.58^{B}$ | $29.64^{B}$ | $30.86^{A}$ | $30.22^{AB}$ | 0.0058 | 1.56 |
| a* | $13.25^{AB}$ | $11.29^{B}$ | $15.12^{A}$ | $12.62^{B}$ | <0.0001 | 2.86 |
| b* | $8.61^{B}$ | $10.68^{A}$ | $10.88^{A}$ | $9.49^{B}$ | <0.0001 | 1.50 |

Means in the same row with unlike superscripts differ ($P < 0.01$); [(1)] Residual Standard Deviation

*3.2 Second trial: Oxidation stability of ostrich salami*

The results on ostrich salami obtained with increasing levels (0.25, 0.50 and 1%) of rooibos extract are shown in Tables 5, 6, 7, and 8. Salami weight losses during ripening were always significantly lower (P<0.001) at the two highest rooibos extract inclusion levels (0.5 and 1%) compared to T1 and C (Table 5). This result may be hypothesized as an effect of the freeze-dried form of the rooibos extract added to the salami. The freeze-dried rooibos extract probably rehydrated after it was mixed with meat and fat, and this delayed the salami water losses.

**Table 5.** Cumulative weight losses (%) of ostrich salami prepared without (C) or with increasing levels of fermented rooibos extract: 0.25% (T1), 0.5% (T2) and 1% (T3)

| | Treatments | | | | *P*-value | RSD[(1)] |
|---|---|---|---|---|---|---|
| | C | T1 | T2 | T3 | | |
| Samples, No. | 62 | 63 | 63 | 63 | | |
| Weight losses 0-7 days of ripening | $44.2^{A}$ | $41.7^{A}$ | $30.1^{B}$ | $30.7^{B}$ | <0.0001 | 4.6 |
| Weight losses 0-15 days of ripening | $49.2^{A}$ | $49.2^{A}$ | $44.9^{B}$ | $46.4^{B}$ | <0.0001 | 2.0 |
| Weight losses 0-30 days of ripening | $55.3^{A}$ | $56.3^{A}$ | $53.4^{B}$ | $54.3^{B}$ | <0.0001 | 1.1 |

Means in the same row with unlike superscripts differ ($P$ <0.0001) [(1)] Residual Standard Deviation

Regardless of the treatment, the initial TBARS values of the salami (Table 6) were two fold higher than those observed in the first trial on ostrich meat patties independently of the rooibos inclusion level (Table 2). Because the meat used for the salami consisted of the same meat portions and was stored in the same way as the ostrich meat patties studied in the first trial, the higher initial oxidation observed in the salami than in the patties may be attributed to the

more intense handling of ingredients required for satisfactory homogenization and stuffing into casings. In addition, the 30-day freezing period surely affected the initial salami oxidation process. When producing fermented goat meat sausage with different levels of rosemary extract and a very short freezing storage, in fact, Nassu, Gonçalves, Da Silva, & Beserra (2003) obtained lower initial MDA contents than ours. At Day 0, salami with 0.5% fermented rooibos extract inclusion level (T2) had higher TBARS values than groups C and T1 (5.63 *vs* 4.81 and 4.70 mg MDA/kg meat for T2 *vs* C and T1 groups, respectively; P<0.01) but no difference with those in T3 (5.04 mg MDA/kg meat). This was surprising because flavonoids are supposed to be effective in chelating iron and scavenging peroxyl radicals and therefore to inhibit lipid oxidation (Deng, Fang, & Wu, 1997), but perhaps longer time is required for such inhibition to become effective. This consideration was supported by the results at Day 15 of ripening, when a clearly decreasing trend in TBARS content was observed as fermented rooibos inclusion level increased, and the C and T1 groups showed significantly higher values than T2 and T3 (5.81 and 5.52 *vs* 3.96 and 3.93 mg MDA/kg meat, respectively; P<0.0001). This suggests that rooibos seems effective in delaying lipid oxidation in ostrich salami in the long-term from a 0.5% inclusion level upwards. At Day 30, the TBARS value of T2 was lower than that of the other groups, but the differences were only statistically significant at P<0.10. Further research with a higher number of replicates would be advisable in order to verify the antioxidant activity length of fermented rooibos extract.

**Table 6**. TBARS values (mg MDA/kg meat) for ostrich salami prepared without (C) or with ncreasing levels of fermented rooibos extract: 0.25% (T1), 0.5% (T2) and 1% (T3)

| | Treatments | | | | *P*-value | RSD[(1)] |
|---|---|---|---|---|---|---|
| | C | T1 | T2 | T3 | | |
| Samples, No. | 12 | 12 | 12 | 12 | | |
| Day 0 | $4.81^{B}$ | $4.70^{B}$ | $5.63^{A}$ | $5.04^{AB}$ | 0.0056 | 0.31 |
| Day 15 | $5.81^{A}$ | $5.52^{A}$ | $3.96^{B}$ | $3.93^{B}$ | 0.0001 | 0.49 |
| Day 30 | 9.55 | 12.43 | 6.63 | 10.80 | 0.0618 | 2.73 |

Means in the same row with unlike superscripts differ ($P < 0.01$); [(1)] Residual Standard Deviation

In the past decade, natural phytochemicals of plant extracts and tea have been widely investigated for their antioxidant effects of cell proliferation, radical scavenging activity, and lipid peroxidation inhibitory activity (Lee, Hwang, Ha, Jeong, & Kim, 2003; Katalinic, Milos, Kulisic, & Jukic, 2006; Sharangi, 2009). After evaluating the effect of natural antioxidants in Turkish dry-fermented sausages using lamb and beef meat, Bozkurt (2006) observed the positive effect of a green tea extract and *Thymbra spicata* oil on the oxidative status of the product.

Another study that considered ostrich meat in the preparation of Spanish salchichon (Soriano, García Ruiz, Gómez, Pardo, Galán, & González Viñas, 2007) found higher TBARS values in commercially ripened salchichon (27.63 mg MDA/kg meat) than those obtained in this work.

Rooibos extract began affecting salami L*a*b* color from the earliest readings on mixed products ($P<0.001$; Table 7). Lightness (L*) decreased linearly as rooibos inclusion levels increased (from 34.34 to 31.81 to 30.94 and to 29.36 for C, T1, T2, T3, respectively). This was explained by the fact that after absorbing moisture from the raw salami surface, the rooibos extract reverted to its dark brick red color, tending in this way to dull the natural brilliant color of fresh ostrich meat. The rooibos extract only significantly affected the redness value (a*) at the highest inclusion level ($P<0.0001$), whereas the yellowness (b*) value did not change with treatment.

At the end of the ripening period (Day 30; Table 7), the intensity of each color parameter was lower than the value measured at Day 0 as a result of the ripening process, which affected the oxidation state of the pigments in the meat, leading in this way to a change in color. As regards L* values, the trend described above was partially maintained up to 0.5% rooibos extract inclusion level (T2). Compared to Day 0, an interesting inverse trend was observed for a* values at Day 30, with T3 exhibiting significantly higher a* value than the other three experimental groups (14.42 *vs* 11.98, 11.09 and 12.53, respectively; $P<0.0001$). As fermented rooibos extract inclusion levels increased, b* values increased linearly as well (8.36, 9.07 *vs* 10.33 *vs* 11.56 for C, T1 *vs* T2 *vs* T3, respectively; $P<0.0001$). These results seem to favor the inclusion of rooibos extract, given that the salami containing this extract maintained a reddish color during ripening. Confirmation of the positive effect of rooibos supplements on salami appearance would require further research on consumer acceptance and sensory analysis, however.

**Table 7.** Colour parameters (lightness [L*], redness [a*] and yellowness [b*]), measured at Day 0 and Day 30 of ripening, of ostrich salami prepared without (C) or with increasing levels of fermented rooibos extract: 0.25% (T1), 0.5% (T2) and 1% (T3)

| | Treatments | | | | *P*-value | RSD[(1)] |
|---|---|---|---|---|---|---|
| | C | T1 | T2 | T3 | | |
| Samples, No. | 48 | 48 | 48 | 48 | | |
| Day 0 | | | | | | |
| L* | $34.34^{A}$ | $31.81^{B}$ | $30.94^{BC}$ | $29.36^{C}$ | <0.0001 | 2.35 |
| a* | $16.55^{A}$ | $17.38^{A}$ | $16.71^{A}$ | $15.47^{B}$ | <0.0001 | 1.25 |
| b* | 13.03 | 13.43 | 12.99 | 12.75 | 0.2677 | 1.19 |
| Day 30 | | | | | | |
| L* | $29.82^{A}$ | $26.95^{B}$ | $26.78^{B}$ | $28.01^{AB}$ | 0.0033 | 3.09 |
| a* | $11.98^{B}$ | $11.09^{B}$ | $12.53^{B}$ | $14.42^{A}$ | <0.0001 | 2.35 |
| b* | $8.36^{C}$ | $9.07^{C}$ | $10.33^{B}$ | $11.56^{A}$ | <0.0001 | 1.42 |

Means in the same row with unlike superscripts differ ($P$ <0.01); [(1)] Residual Standard Deviation

Table 8 presents the pH values of ostrich salami measured at Day 0, 15, and 30, and also the water activity ($a_w$) values measured at Day 30 of ripening. Despite the hygroscopic effect on moisture content mentioned above, increasing rooibos extract inclusion had no negative effect on product quality. Salami pH values were unaffected by the amounts of fermented rooibos extract added for up to 15 days of ripening, whereas at the end of the ripening period, the pH of T1 was significantly lower (P<0.01) than that of T2 salami, with C and T3 treatments exhibiting intermediate values. At Day 30, the $a_w$ was low for all experimental treatments, therefore ensuring the microbial safety of the product, with C and T1 groups presenting lower values than T2 salami, whereas T3 salami had an intermediate $a_w$ value (0.82, 0.82, 0.84, 0.83 for C, T1, T2 and T3, respectively).

**Table 8.** pH measured at Day 0, 15, 30 and water activity ($a_W$) measured at Day 30 of ostrich salami prepared without (C) or with increasing levels of fermented rooibos extract: 0.25% (T1), 0.5% (T2) and 1% (T3)

| | Treatments | | | | *P*-value | RSD[(1)] |
|---|---|---|---|---|---|---|
| | C | T1 | T2 | T3 | | |
| Samples, No. | 72 | 72 | 72 | 72 | | |
| pH: | | | | | | |
| Day 0 | 5.99 | 5.97 | 5.95 | 5.93 | 0.1307 | 0.09 |
| Day 15 | 5.80 | 5.78 | 5.80 | 5.80 | 0.1183 | 0.04 |
| Day 30 | $6.12^{AB}$ | $6.09^{B}$ | $6.18^{A}$ | $6.12^{AB}$ | 0.0015 | 0.07 |
| Samples, No | 16 | 16 | 16 | 16 | | |
| $a_w$: | | | | | | |
| Day 30 | $0.82^{B}$ | $0.82^{B}$ | $0.84^{A}$ | $0.83^{AB}$ | 0.0004 | 0.02 |

Means in the same row with unlike superscripts differ ($P < 0.01$); [(1)] Residual Standard Deviation

Studying the physicochemical characteristics of different types of commercial ostrich salchichon, Soriano et al. (2007) reported lower average $a_w$ (0.80) and pH (5.44) values than those measured in our study. Regarding pH, their results were probably due to the addition of sucrose, which stimulates lactic acid bacteria growth and thus promotes more intense acidification.

The average $a_w$ (0.83) value observed in our study, however, is comparable to values routinely measured in typical long-ripened Italian salami (Moretti, Madonia, Diaferia, Mentasti, Paleari, Panseri, Pirone, & Gandini, 2004).

## 4. Conclusion

This study provided interesting and promising results on the antioxidant potential of rooibos (*Apalathus linearis*) in meat products. Independently of the form in which it was added to the meat, the 2% green rooibos supplement prolonged the shelf-life of ostrich meat patties by delaying lipid oxidation. Similarly, salami supplemented with 0.5% and 1% fermented rooibos extract showed a slower oxidation rate until 15 days of ripening when compared to 0% and 0.15% inclusion levels. Despite these encouraging results, further studies with higher numbers of replicates are needed in order for these preliminary findings to be confirmed. Scientific advance in this direction could offer new concrete possibilities for South African industry to develop new products that add value to a local resource and for consumers to make healthier choices.

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# CHAPTER 6

Running Head: Fat and NaCl levels and microbial starter cultures on ostrich salami at two ripening times

# Two different fat inclusion levels, NaCl contents and two LAB starter cultures in the manufacturing of Italian-type ostrich salami ripened for 10 and 20 weeks: Part 1. Weight loss, proximate composition and cholesterol content

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## ABSTRACT

The aim of this experiment was to study the effect of two different fat inclusion levels (30% and 40%), NaCl contents (2.4 and 2.6%) and starter cultures (LAB 6 and LAB 8) on the weight loss, proximate composition and cholesterol content of Italian-type ostrich salami. With this purpose, 6 batches of 6 salami each (n=36) were prepared. Salami were dried for a 5 days long period: relative humidity (RH) was kept between 65-85 % and Temperature (T) started from 19 °C and decreased of 1 °C/day. Subsequently, salami were ripened for 10 and 20 weeks: RH was 70-80 % and T decreased of 1 °C/day until 12 °C, afterwards it remained constant. The lowest fat and highest salt inclusion levels provided the highest cumulative weight losses throughout the trial. At 10 weeks of ripening, salami with 40 % fat were the richest in terms of moisture and ether extract, whereas leanest ones had the highest protein, ash and cholesterol contents. The presence of LAB 6 provided salami richest in moisture and protein, whereas inoculation of LAB 8 increased ether extract and cholesterol contents. At 20 weeks of ripening, only fat significantly affected proximate composition of ostrich salami, with similar trends to those observed at 10 weeks.



**Keywords:** Italian-type salami, Ostrich meat, Sodium reduction, Fat reduction, Starter cultures

## 1. Introduction

Italian-type salami are intended as slow ripened sausages, rarely smoked, with pH not below 5 and generally between 5.3 and 6.2. They have been produced for centuries, starting from Roman times and traditionally they are made out of pork meat and fat in variable ratios, salt, and eventually sugar and nitrate/nitrite (Comi, Urso, Iacumin, Rantsiou, Cattaneo, Cantoni, & Coccolin, 2005). As no starter cultures are used, fermentation process is driven by autochthonous microflora and this originates a huge regional diversity which is typical of artisanal-made salami. Nowadays, starter cultures are being increasingly used in the salami manufacturing as they ensure product safety and acceptable quality, together with reducing ripening time (Rantsiou, Drosinos, Gialitaki, Urso, Krommer, Gasparik-Reichardt, et al., 2005). Lactic acid bacteria (LAB) mainly ferment sugars into lactic acid, being thus responsible for the acidification of the product, but they generally they lack main aroma production pathways (Talon, Leroy, & Lebert, 2007). Differently, coagulase negative cocci (CNC) like *Staphylococcus* degrade free amino acids and inhibit the oxidation of unsaturated free fatty acids, ultimately contributing to the colour and flavor formation in the salami. Among LAB species, *Lactobacillus (L.) sakei* is often the dominant one in traditional salami, followed by *L. curvatus* and *L. plantarum*. Considering CNC, *Staphylococcus xylosus* is reported to be the most common species in Italian-type salami (Talon et al., 2007). Overall, many studies demonstrated that the above mentioned microbial species are suitable and adapted to the meat environment and ripening process with specificities linked to different ripening conditions, ingredients and meat species (Dicks, Mellet, & Hoffman, 2004; Olesen, Meyer, & Stahnke, 2004; Leroy, Verluyten, & De Vuyst, 2006; Todorov, Koep, Van Reenen, Hoffman, Slinde, & Dicks, 2007).

Salt (NaCl) is a key ingredient for salami manufacturing because it affects the final taste of the product, being a flavor enhancer, and texture, together with ensuring microbiological stability mainly through water activity reduction. For these reasons, in order to provide a satisfactory fermentation process and quality of the final product, its level should always be >2 % (Ruusunen & Puolanne, 2005). Despite raw meat has generally a low NaCl content, meat products can provide 20-30 % of NaCl dietary intake (Zanardi, Ghidini, Conter, & Ianieri, 2010). In salami manufacturing, fat can account for 40-50 % of the final product and it has pivotal role as it affects flavor formation, texture and colour, thus guaranteeing an optimal final quality of the salami.

However, nowadays more and more concerns for products containing significant quantities of salt and fat have been risen by consumers and health organisations. In fact, the consumption of saturated fats and cholesterol is associated to the development of hypercholesterolemia (Severini, De Pilli, & Baiano, 2003), whereas salt (NaCl) overconsumption increases incidence of hypertension which is a major risk factor in the development of cardiovascular disease (Desmond, 2006). For this reason recent health recommendations have been pushing towards the reduction of total dietary fat and sodium intakes, thus into novel approaches to develop healthy food products, included fermented sausages, moves accordingly (Flores, Olivares, & Corral, 2013; Leroy, Geyzen, Janssens, De Vuyst, & Scholliers, 2013; Olmedilla-Alonso, Jiménez-Colmenero, & Sánchez-Muniz, 2013).

On the basis of the above mentioned considerations, this research work aimed to study the effects two different levels of fat and salt inclusions and two different LAB starter cultures on the quality of an Italian-type salami manufactured with ostrich meat and evaluated at two different ripening times. Ostrich meat was chosen because it is a healthy alternative to pork meat thanks to its low fat, mainly polyunsaturated fatty acids, content (Hoffman, Jones, Muller, Joubert, & Sadie, 2013). This paper, which is the first part of a wide research project considering also fatty acids profile, chemical composition and sensory profile, deals with weight loss, proximate composition and cholesterol content of the studied product.

## 2. Materials and Methods

### *2.1 Animal, diet and meat*

The present work was a collaboration between the Medicine, Production and Health Department of the University of Padua and the private farm "Azienda Struzzo 2000" (Volto, RO, Italy). For the experiment, a 90 kg male blue-neck ostrich was used. The ostrich was reared in a 100 $m^2$ outdoors paddock of the above mentioned private farm where it was fed with a crumbled mix of 60% alfalfa (*Medicago sativa*), 20% of maize and 20% of fresh carrots provided by the horticultural production of the farm. Chemical composition on the ostrich diet is shown in Table 1. The ostrich was delivered to the slaughterhouse where, according to the animal welfare dispositions (Council regulation N. 1099/2009, 2009), it was electrically stunned and bled. The carcass was then plucked, skinned and eviscerated. It was then carried through a pre-cooling tunnel and cut in two halves (thighs) which were cooled for 24 h at +4 °C.

Subsequently the carcass was dissected and, after bones, tendons and cartilage elimination, the obtained meat was used for salami preparation.

**Table 1.** Chemical composition (g $kg^{-1}$ on DM basis) of the ostrich diet

| Analyzed composition (g $kg^{-1}$) | on DM basis |
|---|---|
| Dry matter (DM) | 874 |
| Crude protein (CP) | 168 |
| Ether extract (EE) | 20.4 |
| Ash | 69.5 |
| Starch | 243 |
| Crude fibre (CF) | 163 |
| Neutral detergent fibre (NDF) | 264 |
| Acid detergent fibre (ADF) | 178 |
| Acid detergent lignin (ADL) | 26 |
| Acid Insoluble Ashes (AIA) | 5.4 |
| Ca | 12 |
| Fe | 0.13 |
| P | 2.86 |

*Estimated

*2.2 Experimental design and salami preparation*

The present experiment was planned as a 2 x 2 x 2 design: 6 batches of 6 ostrich salami each (n=36) using 2 different levels of fat (30 % and 40 %), NaCl (2.4 % and 2.6 %) and two different LAB starter cultures (LAB 6 and LAB 8) were produced. For the salami preparation 28.5 kg of ostrich meat and 14,4 kg of pork back-fat were used. Meat and fat that were ground separately through 6 and 7 mm diameter holes, respectively. Subsequently, following the experimental design, ground meat and fat were divided into two batches according to two different fat inclusion percentages (30 and 40 %) and then mixed. Subsequently, each batch was separated in two equal units and added with 2.4 or 2.6 % of NaCl, followed by a spices mix (0.78% black pepper, 0.009% cinnamon, 0.009% cloves, 0.009% nutmeg) and 3.55% red wine. Each unit was finally split into two equal parts which were inoculated and mixed with two different LAB starter cultures (1.6 g/kg): LAB 6 (*Lactobacillus curvatus/Staphylococcus xylosus* + dextrose) or LAB 8 (*Lactobacillus sakei*/*Staphylococcus xylosus* + dextrose).

Batches were then individually mixed and put in a refrigerated chamber at +4 °C for 12 h. The following day all the batters were stuffed into natural casings, labeled and put in a dedicated chamber using controlled ripening conditions. All the salami were nitrite-free and slow-fermented.

*2.3 Drying and ripening*

Initially, all the salami were dried for a 5 days long period with RH (Relative Humidity) ranging from 65 to 85 % and T (Temperature) starting from 19 °C and decreasing of 1 °C/day. When T was 14 °C, ripening phase started: RH was between 70 and 80 % and T decreased of 1 °C/day until 12 °C, afterwards it remained constant. Ripening of the first group of salami (1 salami/treatment) was stopped when the first of them lost up to 35 % of its initial weight, at 10 weeks, whereas the second group of salami (5 salami/treatment) was ripened for further 10 weeks.

*2.4 Weight loss determination and chemical analysis*

Once a week, for 20 weeks, salami were individually weighted using a commercial scale and cumulative weight loss for each treatment was subsequently calculated. After collection, at 10 and 20 weeks of ripening, salami were transported to the Animal Medicine, Production and Health Department of the University of Padova, individually freed from casings, frozen in liquid nitrogen and homogenized using a Retsch Grindomix GM 200 (15 seconds at 10000 rpm) and then analyzed. Proximate composition (AOAC, 1995) was determined at 10 and 20 weeks on n=1 salami/treatment (4 replications each) and n=5 salami/treatment, respectively. Protein content was then calculated by difference. Cholesterol content of the salami was determined at 10 and 20 weeks of ripening, as well as that of the batter (Fat 30 % and NaCl 2.6 %), following the method described by Casiraghi, Lucisano, Pompei, & Dellea (1994).

*2.5. Chemical composition of the diet*

Analyses of the ostrich diet was carried out in duplicate using AOAC (2000) methods in order to determine the concentrations of DM (934.01), crude protein (CP; 2001.11), crude fibre (CF; 978.10), ash (967.05) and starch (amyloglucosidase- α-amylase method, 996.11). Ether extract (EE) was determined after acid-hydrolysis (EC, 1998). Neutral detergent fibre (NDF, without sodium sulphite), acid detergent fibre (ADF), acid detergent lignin (ADL) and acid-

insoluble ash (AIA) were analyzed according to Mertens (2002), AOAC (2000, procedure 973.187) and Van Soest et al. (1991), respectively, using the sequential procedure and the filter bag system (Ankom Technology, New York). The gross energy calculated using the formula provided by . Mineral analyses of the diet (Ca, P, K, Na, Fe) was performed by ICP-OES (Spectro Ciros Vision EOP) after microwave digestion (AOAC 2000, 999.10).

### *2.6. Statistical Analysis*

Data were analyzed using SAS 9.1 statistical analysis software for Windows (SAS, 2008) General Linear Model (GLM) procedures. A three-way Anova, which was stratified by ripening time (2.5 and 5 months), tested fat, salt and LAB starter cultures as fixed effects on cumulative weight loss, proximate composition and cholesterol content of artisanal-made Italian-type ostrich salami. The statistical analysis considered also double (Fat x Salt; Fat x LAB; Salt x LAB) and triple (Fat x Salt x LAB) interactions. When no significant effects were found, only main effects were considered. Least square means were obtained using the Bonferroni test. *P* values were considered significant when <0.05.

## 3. Results and Discussion

### *3.1. Cumulative weight loss*

Weight loss of ostrich salami was significantly affected by fat (F) and salt (S) inclusion levels, from the beginning until the end of the ripening at 20 weeks, whereas LAB starter culture (L) resulted ineffective (Table 2). After drying phase, salami manufactured with 30 % fat showed a more intense weight loss compared to those with a 40 % fat content ($P$<0.0001). This difference tended to increase during the trial (Figure 1) and resulted in a 14 % discrepancy in cumulative weight loss, at the end of the ripening. This finding could be explained by the crucial role of fat for rheological and textural properties of meat products as, especially when the product is massaged, it binds water to form stable emulsions (Claus, Hunt, Kastner, & Kropf, 1990). As a consequence, a lower fat content leads to a lower emulsion stability which causes a higher moisture loss (Crehan, Hughes, Troy, & Buckley, 2000).

Similarly to our study, Muguerza, Fista, Ansorena, Astiasaran, & Bloukas (2002) evidenced that weight losses of fermented sausages were significantly affected by fat level and that the lower the fat level the higher the weight losses of sausages. Moreover, at 28 days of processing time, the average weight loss of sausages manufactured with 30 % pork backfat

was 38.5 % which is much higher than values observed in our study (28.1 % of cumulative weight loss for salami belonging to the 30 % fat inclusion level, at 4 weeks of ripening).

**Table 2.** Effect of two levels of fat, salt and LAB starters cultures and their interactions on cumulative weight loss (% of the initial weight) of ostrich salami ripened for 20 weeks

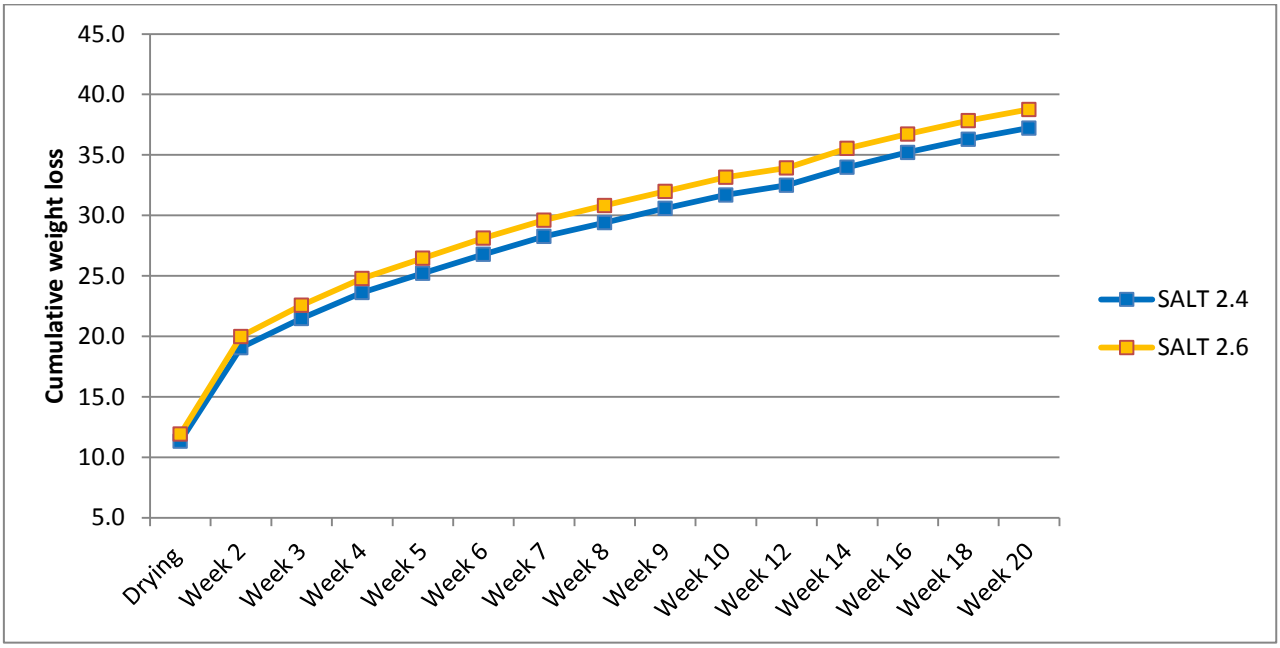
| | FAT (F) | | SALT (S) | | LAB (L) | | Significance | | | | | RSD[1] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 30 | 40 | 2.4 | 2.6 | 6 | 8 | F | S | L | F x S | F x L | |
| Drying | 13.2 | 10.1 | 11.3 | 11.9 | 11.5 | 11.7 | <0.0001 | <0.01 | ns | <0.05 | ns | 0.65 |
| Week 2 | 22.2 | 16.5 | 19.1 | 20.0 | 19.4 | 19.7 | <0.0001 | <0.001 | ns | <0.05 | ns | 0.86 |
| Week 3 | 25.6 | 18.5 | 21.5 | 22.6 | 21.9 | 22.2 | <0.0001 | <0.001 | ns | <0.01 | ns | 0.92 |
| Week 4 | 28.1 | 20.3 | 23.6 | 24.8 | 24.1 | 24.3 | <0.0001 | <0.001 | ns | <0.05 | <0.05 | 0.98 |
| Week 5 | 30.1 | 21.6 | 25.2 | 26.5 | 25.7 | 26.0 | <0.0001 | <0.0001 | ns | <0.05 | <0.05 | 0.98 |
| Week 6 | 31.9 | 23.0 | 26.8 | 28.1 | 27.3 | 27.6 | <0.0001 | <0.05 | ns | ns | ns | 1.95 |
| Week 7 | 33.5 | 24.4 | 28.3 | 29.6 | 28.9 | 29.0 | <0.0001 | <0.0001 | ns | <0.05 | <0.05 | 1.04 |
| Week 8 | 34.8 | 25.4 | 29.4 | 30.8 | 30.0 | 30.2 | <0.0001 | <0.0001 | ns | <0.05 | <0.05 | 1.04 |
| Week 9 | 36.1 | 26.5 | 30.6 | 32.0 | 31.2 | 31.4 | <0.0001 | <0.0001 | ns | <0.05 | <0.05 | 1.06 |
| Week 10 | 37.4 | 27.5 | 31.7 | 33.2 | 32.3 | 32.5 | <0.0001 | <0.0001 | ns | <0.05 | ns | 1.06 |
| Week 12 | 38.2 | 28.3 | 32.5 | 33.9 | 33.1 | 33.4 | <0.0001 | <0.0001 | ns | <0.01 | <0.05 | 0.95 |
| Week 14 | 39.8 | 29.8 | 34.0 | 35.6 | 34.6 | 35.0 | <0.0001 | <0.0001 | ns | <0.05 | <0.05 | 0.97 |
| Week 16 | 41.0 | 30.9 | 35.2 | 36.7 | 35.8 | 36.2 | <0.0001 | <0.0001 | ns | <0.05 | <0.05 | 0.97 |
| Week 18 | 42.1 | 32.0 | 36.3 | 37.8 | 36.8 | 37.3 | <0.0001 | <0.0001 | ns | <0.01 | ns | 0.99 |
| Week 20 | 43.1 | 32.9 | 37.2 | 38.8 | 37.8 | 38.2 | <0.0001 | <0.0001 | ns | <0.05 | ns | 1.01 |

[1]Residual Standard Deviation

As weight losses are known to depend on many processing factors such as temperature, relative humidity and air movement, this difference is not surprising as processing parameters differed between the two experiments (Baldini, Cantoni, Colla, Diaferia, Gabba, et al., 2000; Olivares, Navarro, Salvador, & Flores, 2010).

Salt (NaCl) is responsible for the solubilisation of myofibrillar proteins in meat, which activates the proteins to increase hydration and water-binding capacity resulting in an improved texture, tenderness and juiciness (Ruusunen & Puolanne, 2005). Despite this, a higher NaCl content favored weight loss which was higher in the 2.6 % inclusion level compared to 2.4 % ones ($P$<0.05). In this case, the divergence between the two groups of salami increased from the drying phase until the third week of ripening, but after that it remained constant until the end of the trial (Figure 2). This finding could be explained by the fact that salt decreases water activity, thus leading to the loss of free water (Guàrdia, Guerrero, Gelabert, Gou, & Arnau, 2006). Also the interaction F x S significantly affected cumulative weight loss of ostrich salami (Table 2).

**Figure 2.** Effect of salt level on cumulative weight loss (% of the initial weight) of ostrich salami ripened for 20 weeks




Interestingly, it was observed that within the group of salami with 30 % fat, those manufactured with 2.4 % and 2.6 % salt inclusions exhibited the same cumulative weight loss, whereas at 40 % fat inclusion level salami with a higher salt content showed a more intense weight loss compared to those belonging to the 2.4 % NaCl inclusion level (Figure 3). This happened probably because, at 30 % fat level, the 2.4 % salt was already enough to let the salami loose the free water, whereas at 40 % fat only a higher salt concentration could reduce water availability as a higher stability of the emulsion fat-water was reached. This hypothesis was supported by data presented in the companion paper by Novelli, Cullere, Sartori, & Dalle Zotte (same issue), in which salami manufactured with 2.6 % salt showed a lower $a_w$ compared to those prepared with 2.4 % salt inclusion, both at 10 and 20 weeks of ripening.

**Figure 3.** Effect of the double interaction FAT x SALT (F x S) on the cumulative weight loss (% of the initial weight) of ostrich salami at the end of the drying phase

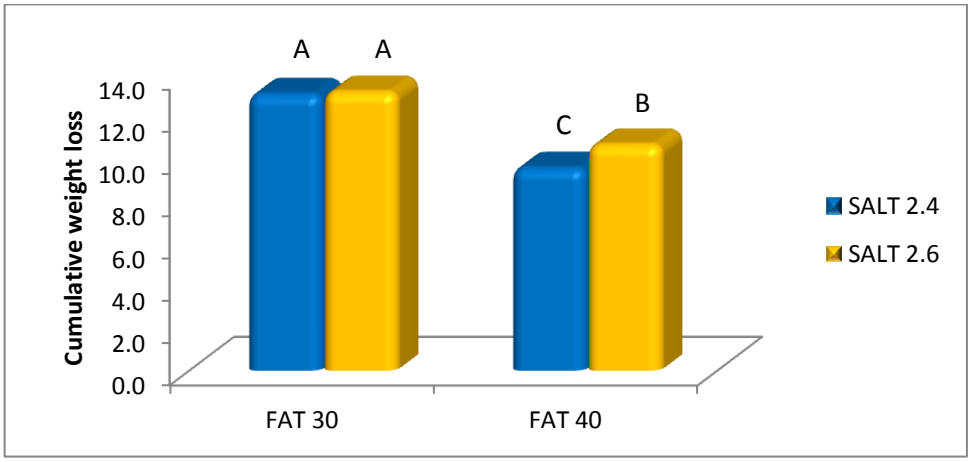
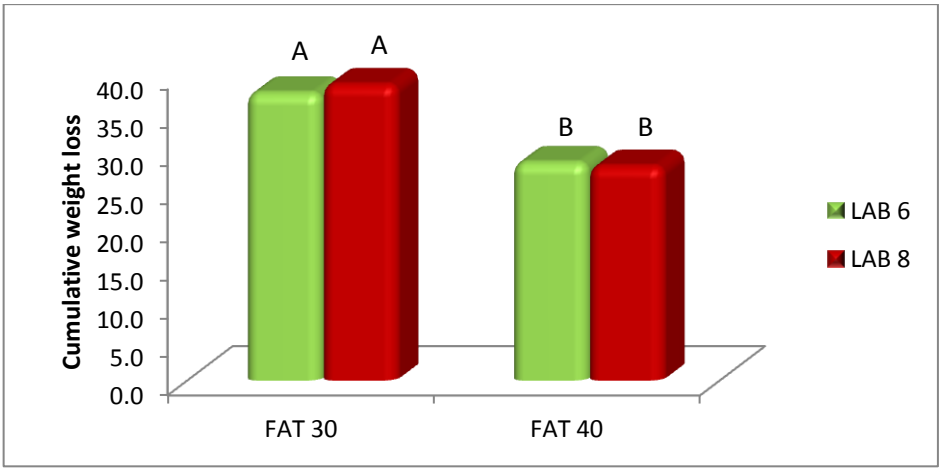



**Figure 4.** Effect of the double interaction FAT x LAB (F x L) on the cumulative weight loss (% of the initial weight) of ostrich salami ripened for 16 weeks




Different LAB starter cultures didn't affect cumulative weight loss of ostrich salami and this finding was in line with the study by Kenneally, Schwarz, Fransen, & Arendt (1998) on salami inoculated with different microbial starters. Sometimes, in our study the interaction F x L resulted significant (Table 2), but observing the histogram showing this interaction at 16 weeks of ripening, different LAB starter cultures didn't produce significant variations in terms of weight loss within the same fat inclusion level (Figure 4). For this reason, it was hypothesized that the significance of the interaction was mostly attributable to the different fat content rather than different LAB starter cultures.

*3.2 Proximate composition and cholesterol content of ostrich salami at 10 weeks of ripening*

The results presented in Table 3 on proximate composition and cholesterol content of Italian-style ostrich salami at 10 weeks of ripening, showed that fat level affected moisture, protein, ether extract, ash and cholesterol contents ($P$<0.0001). Particularly, salami incorporated with 30 % pork backfat showed lower moisture and fat, higher protein, ash and cholesterol contents compared to salami manufactured with 40 % backfat. This result could be explained by the fact that salami with the lowest fat content had lost more weight during ripening compared to the fattest ones (Table 2), thus a higher concentration of their constituents was expected. A higher presence of pork backfat directly increased the ether extract content of the salami, which was higher in the 40 % than the 30 % groups (35.6 *vs* 32.8 g/100 g, respectively). Interestingly, the leanest salami had a higher cholesterol content than the fattest ones (93.4 *vs* 83.1 mg cholesterol/100 g meat, for 30 and 40 % fat inclusion levels, respectively). This finding was partially explained by the lower moisture content of the salami with 30 % fat compared to 40 % ones, which caused a higher nutrients concentration (cholesterol content of the batter was 58.2 mg/100 g). Moreover, cholesterol is naturally present in membranes of muscle cells, thus a higher inclusion of lean meat in our salami, especially if considering that ostrich meat is characterized by muscle fibres with prevalent oxidative metabolism, was expected to compensate for cholesterol found in animal fats (Chizzolini, Zanardi, Dorigoni, & Ghidini, 1999).

Different salt percentages affected only the ash content of ostrich salami, being the highest in the 2.6 % salt group ($P$<0.0001), as a direct consequence of the highest presence of salt, whereas other variables remained unaffected. This finding was supported by results presented in a study evaluating the effect of NaCl partial substitution on proximate composition of Italian salami (Zanardi et al., 2010), where salami manufactured with 2.7 % NaCl had the same proximate composition compared to salami prepared with low NaCl formulations.

Tissue lipase are primarily responsible for lipolysis during the fermentation process, but it's now accepted that staphylococci can act as lipolytic bacteria, thus playing an important role in aroma formation (Bedia, Méndez, & Bañón, 2011). Similarly, In the first stages of ripening proteolysis in meat is due to endogenous enzymes such as calpains and cathepsins which break sarcoplasmic and myofibrillar proteins, whereas it's in the last stages of ripening that microbial enzymes play a predominant role in the secondary hydrolysis of oligopeptides and small peptides. (Casaburi, Di Monaco, Cavella, Toldrá, Ercolini, & Villani, 2008).

However, an appropriate choice of a combination of strains in the formulation of a starter culture is fundamental for a successful fermentation and ripening processes, as different strains and microbial species are known to act differently according to different meat type, technological characteristics of the fermentation and ripening processes (Todorov et al., 2007; Baka, Papavergou, Pragalaki, Bloukas, & Kotzekidou, 2011; Casquete, Benito, Martín, Ruiz-Moyano, Hernández, & Córdoba, 2011). In fact, in our trial the use of different starter cultures significantly affected the proximate composition of ostrich salami (Table 3). Specifically, the combination of *Lb. sakei* and *S. xylosus* seemed to promote lipolysis as salami inoculated with LAB 8 showed lower ether extract (35.3 *vs* 33.1 g/100 g for LAB 6 and LAB 8, respectively) and cholesterol (90.8 *vs* 85.7 mg/100 g, for LAB 6 and LAB 8, respectively) contents compared to those inoculated with LAB 6. Differently, the presence of *Lb. curvatus* and *S. xylosus* lowered protein and moisture contents compared to salami inoculated with LAB 8, thus suggesting a high contribution to proteolytic activity.

In a study on the effect of starter culture on proteolytic changes during processing of fermented sausages, Candogan, Wardlaw, & Acton (2009) observed that sausages inoculated with *Lb. sakei* and *Lb. carnosus* differed in terms of some individual amino acids. However, differently from processing stage which was the main factor affecting proteolytic process, type of starter culture was not a factor affecting total free amino acids concentration. Another study on traditional Croatian fermented sausages, found that the inclusion of *Lb. sakei* increased the proteolytic index compared to a control group, whereas a higher free fatty acids content was observed in the control sausages compared to those inoculated with starter culture (Zdolec, Hadžiosmanović, Kozačinski, Cvrtila, Filipović, Škrivanko, et al., 2008). A research on the proteolytic activity of lactic acid bacteria (Fadda, Sanz, Vignolo, Aristoy, Oliver, & Toldrá, 1999) on muscle proteins showed that different enzyme combinations from *Lb. sakei* and *Lb. curvatus* provided different results, the first exhibiting exopeptidase activity and the second modifying the peptide profile.

**Table 3.** Effect of two levels of fat, salt, LAB starters cultures and their interactions on proximate composition g/100 g) and cholesterol content (mg/100 g) of ostrich salami ripened for 10 weeks

| | FAT (F) | | SALT (S) | | LAB (L) | | Significance | | | | | RSD[1] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 30 | 40 | 2.4 | 2.6 | 6 | 8 | F | S | L | F x S | F x L | |
| Moisture | 33.2 | 36.4 | 34.9 | 34.6 | 33.8 | 35.7 | <0.0001 | ns | <0.0001 | <0.0001 | <0.0001 | 0.36 |
| Protein | 25.8 | 20.8 | 23.2 | 23.4 | 23.1 | 23.5 | <0.0001 | ns | <0.05 | <0.05 | ns | 0.55 |
| Ether extract | 32.8 | 35.6 | 34.4 | 34.0 | 35.3 | 33.1 | <0.0001 | ns | <0.001 | <0.05 | <0.01 | 1.37 |
| Ash | 4.91 | 4.28 | 4.34 | 4.86 | 4.57 | 4.63 | <0.0001 | <0.0001 | ns | <0.05 | <0.01 | 0.07 |
| Cholesterol | 93.4 | 83.1 | 88.6 | 87.9 | 90.8 | 85.7 | <0.0001 | ns | <0.001 | ns | ns | 3.83 |

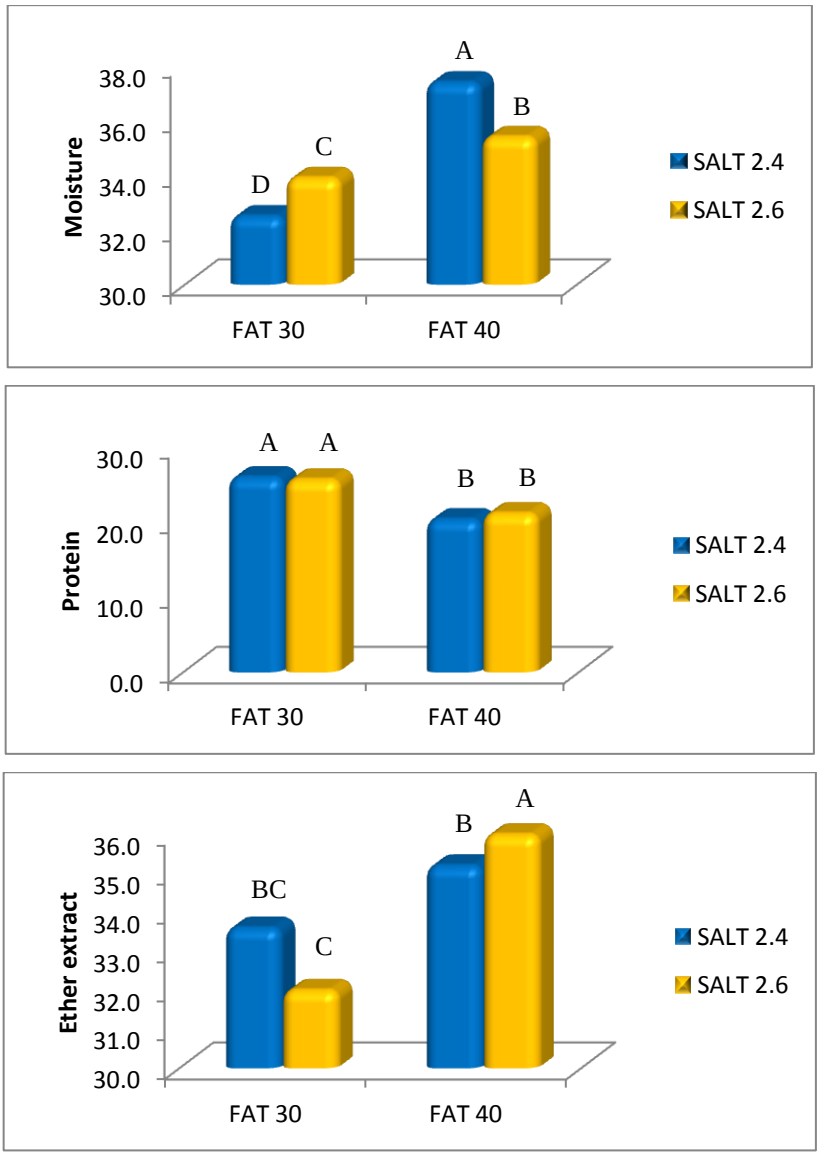
Cholesterol content of the batter was 58.2 mg/100 g

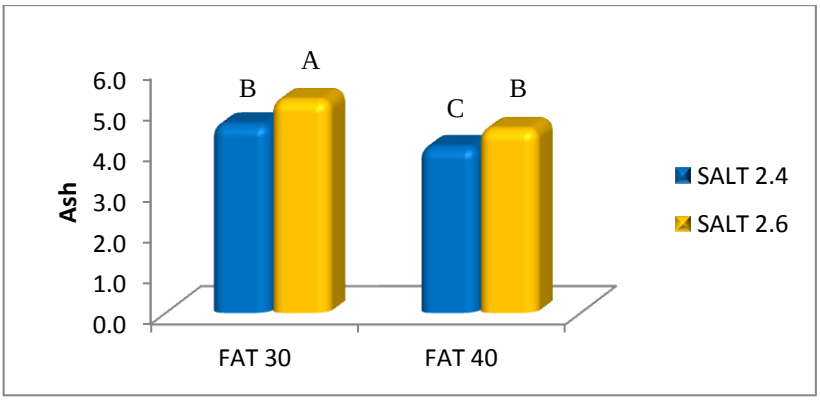
[1]Residual Standard Deviation

The double interaction F x S significantly affected moisture ($P$<0.0001), protein ($P$<0.05), ether extract ($P$<0.05) and ash ($P$<0.05) contents of ostrich salami ripened for 10 weeks. When 2.4 % salt was added, moisture content of salami manufactured with 40 % fat (Figure 5a) was higher compared to salami prepared with 2.6 % salt (37.4 *vs* 35.4 %, for 2.4 and 2.6 % salt groups, respectively), thus confirming the considerations of the above mentioned results on cumulative weight loss. However, when the fat content of salami decreased to 30 %, the situation was exactly the opposite with 2.4 % salt group exhibiting a lower moisture content than the 2.6 % group (32.5 *vs* 33.9 %, for 2.4 and 2.6 % salt groups, respectively). The amount of water and location of that water in meat can change depending on numerous factors related to the tissue itself and how the product is handled (Huff-Lonergan & Lonergan, 2005). Even if increasing levels of salt should increase water holding capacity in meat (Puolanne, Ruusunen, & Vainionpää, 2001), our finding wasn't expected. In fact, previous results showing the effect of the double interaction F x S on cumulative weight loss (Figure 3) showed a different situation. On one hand, a higher presence of NaCl should be able to extract more free water than a lower presence, as it was observed for salami with 40 % fat inclusion. On the other hand, however, NaCl solubilises myofibrillar proteins in meat, thus increasing water-binding capacity of protein due to their gelation which would result in a reduction of moisture loss (Ruusunen & Puolanne, 2005). In our study it seemed likely that, at 30 % fat content, the need of myofirbillar proteins to hydrate themselves overcame the ability of fat to form stable emulsions with water; as a consequence, a higher presence of salt would determine a higher moisture content of ostrich salami. Differently, increasing the presence of fat to 40 %, water formed stable emulsions with fat and this mechanism overcame the force of the meat proteins to attract water and hydrate themselves, thus explaining the higher moisture content of low-salt salami (2.4 %) compared to high-salt (2.6 %) ones at 30 % fat level.

As a consequence of the effect of the double interactions F x S on moisture content of ostrich salami ripened for 10 weeks, protein content was the highest in 30 % fat salami, with no differences between the two levels of salt within the same fat group (Figure 5b). Differently, ether extract content of ostrich salami in the two fat groups manufactured with two different levels of salt, presented the exact inverse trend observed for moisture content (Figure 5a). Similarly, ash content was higher in low fat compared to high fat salami as a result of a more intense moisture loss and, within each fat group, a higher presence of salt increased the ash content compared to salami manufactured with the lowest salt level (Figure 5c).

**Figure 5 (a, b, c, d).** Effect of the double interactions FAT x SALT (F x S) on the moisture (a. *P*<0.0001), protein (b. *P*<0.05), ether extract (c. *P*<0.05) and ash (d. *P*<0.05) contents (g/100 g) of ostrich salami ripened for 10 weeks





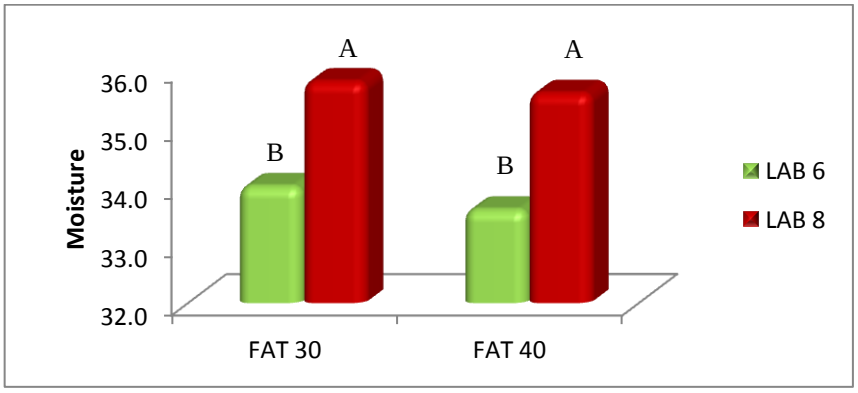

The double interaction F x L significantly affected moisture, ether extract and ash contents of ostrich salami ripened for 10 weeks (Figure 6 a, b, c). Specifically, salami inoculated with LAB 6 starter culture showed a lower moisture content compared to those inoculated with LAB 8, both at 30 and 40 % fat inclusion levels ($P$<0.0001). However, these differences weren't due to the results of the double interaction F x L on the cumulative weight loss of ostrich salami (Figure 4). In this case, it is likely that the two starter cultures showed different growth intensities, with LAB 6 using more free water than LAB 8.

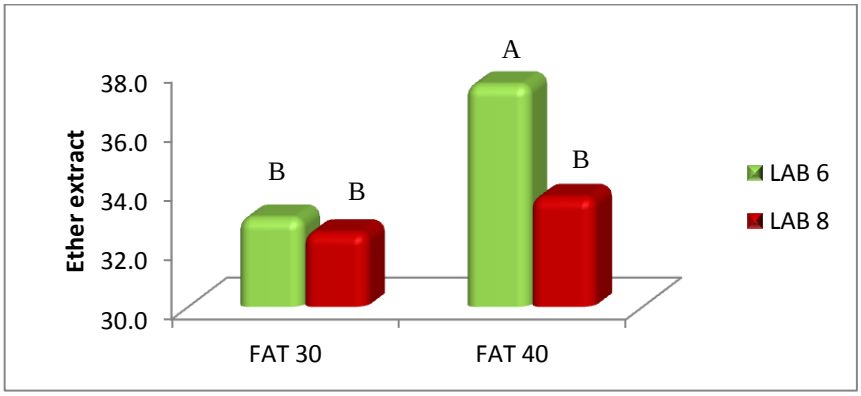
This hypothesis found confirmation in the companion paper (Novelli et al. 2014) where results showed that LAB 6 salami had lower $a_w$ compared to LAB 8 salami. Results of a study evaluating possible starter cultures for the production of fermented sausages in Southern Italy (Casaburi, Aristoy, Cavella, Di Monaco, Ercolini, Toldrá, et al., 2007), showed that the use of different strains of *Staphylococcus xylosus* determined different moisture contents in salami ripened for 38 days, thus further supporting our hypothesis. Salami with 30 % pork backfat and inoculated with different starter cultures showed the same ether extract content, whereas at 40 % fat inclusion level, the combination *Lactobacillus curvatus*/*Staphylococcus xylosus* provided salami with a higher ether extract content compared to those containing *Lactobacillus sakei*/*Staphylococcus xylosus*. ($P$<0.01).

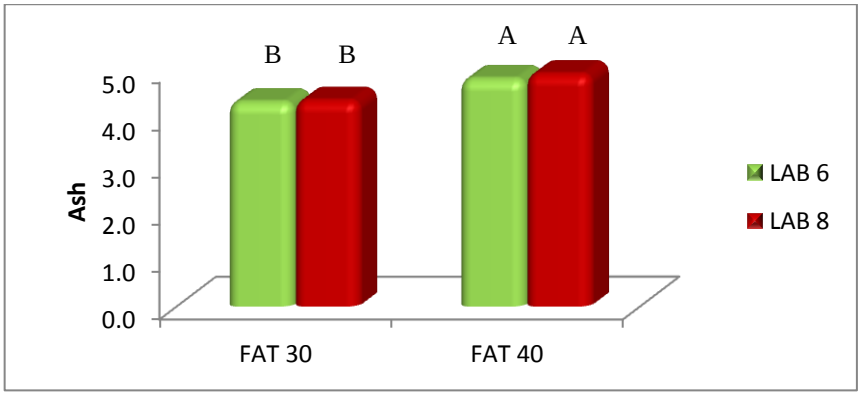
This finding suggested that the significance of the main effect of the starter culture on the ether extract content of ostrich salami (Table 3) was particularly true when the fat level of the salami was 40 %, whereas at 30 % inclusion percentage only a tendency was observed. Ash content was different and greater in high fat salami compared to low fat ones, with different starter cultures showing similar results within the same fat inclusion level ($P$<0.01).

As no specific studies evaluating the effect of different fat contents on the growth of starter cultures for the production of fermented sausages have been conducted until now and given that the choice of the appropriate starter mix for each specific fermented meat products is of fundamental importance for satisfactory product quality (Leroy & De Vuyst, 2004), further research on this topic is required.

**Figure 6 (a, b, c).** Effect of the double interaction FAT x LAB (F x L) on the moisture (a. $P<0.0001$), ether extract (b. $P<0.01$) and ash (c. $P<0.01$) contents (g/100 g) of ostrich salami ripened for 10 weeks



*3.3 Proximate composition and cholesterol content of ostrich salami at 20 weeks of ripening*

Interestingly, at 20 weeks of ripening, only fat content significantly affected the proximate composition of ostrich salami, whereas salt and LAB starter cultures were ineffective (Table 4). This result was expected as initial different fat contents changed the proportion of nutrients as well as the moisture content of ostrich salami, thus affecting the proximate composition of the product throughout the ripening process. Differently, as a result of their metabolic activity LAB starter cultures generated differences until 10 weeks of ripening, whereas at 20 weeks of ripening salt concentration was probably too high for their survival (Ordóñez, Hierro, Bruna, & de la Hoz, 1999), thus their activity was expected to be reduced or absent. In fact, growth and survival of staphylococci, but especially lactic acid bacteria, are known to be significantly affected by the NaCl concentration Olesen et al. (2004). Moisture content was higher in salami manufactured with 40 % fat than those made with 30 % fat ($P$<0.0001), thus leaner salami, as a result of a higher nutrients concentration, had higher protein (27.5 *vs* 21.5 g/100 g for 30 and 40 % fat inclusion levels, respectively), ash (5.54 *vs* 4.79 g/100 g for 30 and 40 % fat inclusion levels, respectively) and cholesterol (104.3 *vs* 86 mg/100 g for 30 and 40 % fat inclusion levels, respectively) contents.

**Table 4.** Effect of two levels of fat, salt, LAB starters cultures and their interactions on proximate composition g/100 g) and cholesterol content (mg/100 g) of ostrich salami ripened for 20 weeks

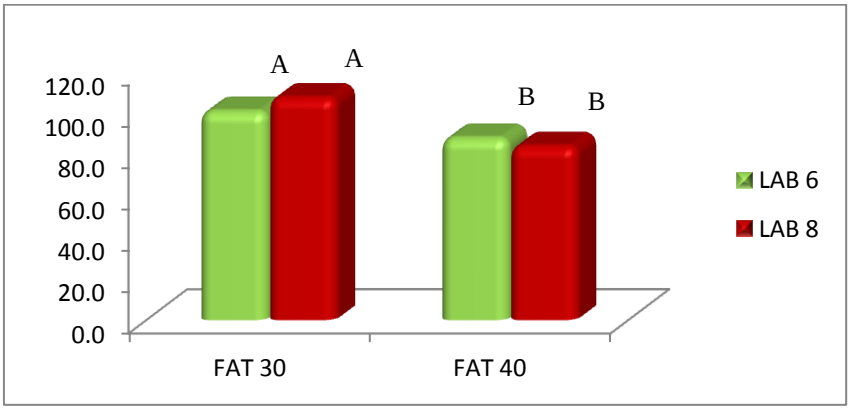
| | FAT (F) | | SALT (S) | | LAB (L) | | Significance | | | | RSD[1] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 30 | 40 | 2.4 | 2.6 | 6 | 8 | F | S | L | F x L | |
| Moisture | 26.4 | 31.1 | 29.0 | 28.5 | 28.0 | 29.5 | <0.0001 | ns | ns | ns | 2.61 |
| Protein | 27.5 | 21.5 | 24.3 | 24.7 | 24.2 | 24.8 | <0.0001 | ns | ns | ns | 1.18 |
| Ether extract | 36.5 | 36.5 | 36.4 | 36.6 | 37.1 | 35.9 | ns | ns | ns | ns | 3.58 |
| Ash | 5.55 | 4.79 | 5.07 | 5.28 | 5.22 | 5.13 | <0.0001 | ns | ns | ns | 0.42 |
| Cholesterol | 104.3 | 86.0 | 95.7 | 94.7 | 94.5 | 95.9 | <0.0001 | ns | ns | <0.05 | 6.54 |

[1]Residual Standard Deviation

The only significant interaction was exerted by F x L on the cholesterol content. Even if this difference regarded high and low fat groups, independently to the starter culture used within group, the histogram (Figure 7) suggested a numerical difference between LAB 6 and LAB 8 within the same fat level.

Specifically, at 30 % fat LAB 6 had a lower cholesterol content than LAB 8 salami (101.0 *vs* 107.6 mg /100 g for LAB 6 and LAB 8, respectively), whereas at 40 % fat the opposite situation was observed (88.0 *vs* 84.1 mg/100 g for LAB 6 and LAB 8, respectively). These inverted trends, as it was mentioned previously, could indicate different sensitivities of the starter cultures to different fat inclusion levels, which would require further investigation.

**Figure 7**. Effect of the double interaction FAT x LAB (F x L) on the cholesterol content (mg/100 g) of ostrich salami ripened for 20 weeks



## 4. Conclusions

Fat inclusion level had a great impact on the weight loss and proximate composition of Italian-style ostrich salami, independently to ripening phase. A lower fat content consistently shortened ripening time, thus being a positive aspect in terms of productivity, and it determined a higher nutrients concentration compared to high fat salami, with the only drawback of a higher cholesterol content compared to high fat salami. Reducing the NaCl inclusion from 2.6 % to 2.4 % in the manufacturing of ostrich salami, retarded the weight loss of ostrich salami of about 1 week, without affecting proximate composition and cholesterol content of the final product. At 10 weeks of ripening, *Lb. sakei* provided salami with healthier nutritional composition compared to salami inoculated with *Lb. curvatus*. The metabolic activity of tested LAB starter cultures seemed to be affected by the fat inclusion level, even if further investigations to clarify this point is necessary. Understanding the latter could help to ensure a high quality product.

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# CHAPTER 7

## General conclusions

Appropriate dietary strategies can successfully provide healthier meat without negatively affecting productive performance nor health status of the animals. Specifically:

- Spirulina effectively fortified vitamin $B_{12}$ and γ-Lionolenic acid contents in rabbit meat. However, the negative impact on nutrient digestibility and lack of effectiveness in counteracting lipid oxidation of the meat, together with its high cost, render it unsuitable for practical application in rabbit farming.
- Thyme didn't impair productive performance of growing rabbits and protected fresh meat from oxidation, also with a 3 weeks supplementation period, making it a possible natural antioxidant to preserve fresh meat qualty.
- Oregano implemented growth performance of rabbits, improved the proximate composition of their meat, increased its oxidative stability and, overall, it provided similar results compared to vitamin E. For this reason it represents an interesting natural supplement for rabbit farming.
- Rosemary, even if it exerted positive effects on the studied traits, overall it provided lower benefits compared to oregano and vitamin E.
- *Saccaromyces cerevisiae* worsened productive performance of growing rabbits and didn't improve qualitative characteristics of the meat. Moreover, even if it provided a certain protection to meat lipids against oxidation, results were less convinvincing than those observed with oregano and vitamin E.

An appropriate product reformulation, as well as the choice of the right natural additive, can provide interesting results and promising perspectives in producing healthy and safe high quality meat products. Specifically:

- Rooibos, in both its unfermented and fermented forms, improved the colour of ostrich meat patties and Italian-type salami and was effective in retarding their lipid oxidation. For this reason, further studies should focus on the optimal inclusion level which would improve the shelf-life of the product, ensuring also acceptable sensory attributes.

Scientific advance in this direction could offer new concrete possibilities for South African industry to develop new products that add value to a local resource and for consumers to make healthier choices.

~ Even if to ensure a healthier ostrich salami it would be necessary to reformulate the product (fat and NaCl substitutions), a simple fat and NaCl reduction seemed sufficient to improve the nutritional composition of nitrite-free Italian-style ostrich salami without negatively affecting processing parameters. At the same time, this approach would protect the traditional recepy and production process. However, as the metabolic activity of the microbial starter cultures seemed to be affected by the presence of fat, attention should be paid for the choice of the appropriate species and strains.

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