Effects of extrusion process variables on the nutritional quality of dry dog feeds

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Riassunto

La qualità delle materie prime è un requisito fondamentale per ottenere alimenti per animali da compagnia sani e altamente digeribili. Soprattutto nel caso di alimenti "completi", cioè studiati per soddisfare l'intero fabbisogno degli animali da compagnia, la digeribilità è un importante indice della qualità degli ingredienti ed un parametro di assoluto rilievo per l'industria del pet food. Essa può essere valutata attraverso l'uso di equazioni, metodiche in vivo che prevedono l'utilizzo di animali sperimentali e metodiche in vitro, che simulano il passaggio gastrointestinale degli alimenti mediante l'uso di enzimi digestivi. La quasi totalità degli alimenti destinati agli animali da compagnia è ottenuta attraverso l'estrusione, processo produttivo che consente in pochi secondi di passare dalla materia prima (farina o residui freschi dell'industria alimentare umana) al prodotto estruso, sanitizzato e pronto per essere stabilizzato tramite essiccazione. Il processo di estrusione prevede l'applicazione di alte temperature (fino a 150°C) e pressioni (fino a 40 atm), che possono sia migliorare la digeribilità (gelatinizzazione dell'amido, denaturazione proteica) degli alimenti, sia comprometterne la qualità nutrizionale (reazione di Maillard, retrogradazione dell'amido, ossidazione, perdita di vitamine). L'attento monitoraggio del processo di estrusione viene ad essere quindi un elemento basilare per poter garantire un prodotto nutrizionalmente corretto.

Il presente lavoro di tesi ha avuto lo scopo di approfondire il processo di estrusione di alimenti per cani, monitorando il processo produttivo per verificarne l'impatto sulla digeribilità e le risposte metaboliche degli animali alle diete prodotte.

Il primo contributo ha avuto come oggetto l'esecuzione di un processo estrusivo ai fini di valutare le ripercussioni dello stesso sulla materia prima aggiunta di due alfa amilasi differenti. Si è

rilevato come l'aggiunta di tali enzimi al prodotto consente di raggiungere un aumento della produttività dell'estrusore ed una sensibile riduzione del costo energetico.

Nel secondo contributo sperimentale è stata approfondita l'influenza delle dimensioni delle materie prime e della temperatura di estrusione sul processo produttivo e sulla qualità nutrizionale degli alimenti prodotti, espressa in termini di digeribilità e fermentazione intestinale. Nel presente esperimento è stata formulata una dieta di mantenimento per cani adulti, in seguito macinata utilizzando quattro setacci con granulometrie differenti. Le diete così ottenute sono state successivamente estruse utilizzando due diverse conformazioni dell'estrusore. Il piano sperimentale ha quindi previsto il test *in vivo* delle otto diete sperimentali su , 48 animali Le temperature di estrusione non hanno influito su nessun parametro. Al contrario, si è evidenziato una correlazione positiva tra granulometria e produttività. Considerando che l'energia impiegata per la macinazione aumenta di pari passo con la diminuzione della granulometria desiderata, si evince come si possa ottenere un'ulteriore diminuzione dei costi produttivi. L'esperimento in vivo ha dimostrato che ad un aumento delle dimensioni delle materie prime corrisponde una diminuzione della digeribilità di materia secca, proteine e grassi, ma anche una maggiore proliferazione microbica (batteri aerobi totali, anaerobi totali e bifido batteri), una diminuzione del pH e della concentrazione di ammoniaca fecale, ed una maggiore produzione di acidi grassi a catena corta, tutti fattori che migliorano la salute degli animali.

Il lavoro descritto nel terzo contributo ha avuto come finalità l'implementazione di una metodica *in vitro* che prevede la simulazione del processo digestivo con enzimi quali pepsina e pancreatina. Attraverso l'introduzione di uno passaggio aggiuntivo di precipitazione con etanolo si è cercato di recuperare la componente solubile indigerita, che altrimenti verrebbe persa in fase di filtrazione. Si è inoltre cercato di rendere il metodo facilmente riproducibile anche con le strumentazioni più comuni; Infatti, in sostituzione della camera termostatata dotata di un agitatore magnetico utilizzata nel metodo originale, si è proceduto utilizzando un bagno termostatato in cui i

campioni venivano agitati manualmente. Per tale lavoro, sono stati impiegati campioni di mangimi su cui erano già state eseguite precedenti analisi di digeribilità *in vivo*.

L'introduzione della fase di precipitazione, ha favorito l'avvicinamento della stima di digeriblità *in vitro* al valore ottenuto *in vivo*. Inoltre, l'utilizzo del bagno termostatato come sistema di incubazione non sembra interferire con la stima della digeribilità *in vitro* e ha dimostrato una riproducibilità e ripetibilità del tutto paragonabili a quelle del metodo di incubazione originale. Grazie a questi incoraggianti primi risultati, sarebbe lecito pianificare in futuro l'applicazione dello stesso protocollo su una maggiore numerosità campionaria, al fine di affinare e irrobustire i risultati fin qui ottenuti.

Abstract

In order to obtain safe and highly digestible foods destined to copanion animals the quality of the raw materials is essential. Food digestibility is a fundamental aspect in the pet foood industries, and particularly for the production of "complete foods", that are opportunely formulated for the satisfaction of the entire animals nutritional needs.

Dog food digestibility can be evalutated thorugh specific equations, through *in vivo* methods in which are employed animals, or *in vitro* methods, that simulate the passage of the food through the gastrointestinal tract, by using digestive enzymes.

The majority of the pet foods sold nowadays are obtained by using the extrusion, a fast process that in few seconds can mix and heat raw materials (both dry and/or fresh) and turn into kibbles, that are already sanitized, and ready to be stabilizd through a drying phase.

The extrusion process applies high temperatures (until 150°C) and pressure (40 atm), and in those conditions the digestibility of the material can be increased (starch gelatinisation, protein denaturation), but also the nutritional quality can be compormised by a decrease of available aminoacids (Maillard reaction) or available starch (starch retrogradation), fats oxydation and vitamin losses. A careful monitoring of the extrusion process is a key point in the assurance of the feed nutritional quality.

In the present work the extrusion process for the dog foods production, the monitoring of the process parameters and the following evaluation of the digestibility, and the metabolic resoponses to the diets produced were studied.

A further study regarded also the *in vitro* methods for the estimation of the food digestibility.

This thesis is composed of an introductive bibliographic review and 3 experiments.

The objective of the first contribution was the evaluation of the use of alpha amylases during the extrusion process, as possible mean for increasing the process procutivity. Two experiments were conducted, in each one the addiction of a different alpha amylase was tested. The productive parameters, along with the diets digestibility were evaluated. Resuld demonstrated an increase in extrusion productivity (until 40%) and a reduction of the energetic costs when the alpha amylase is added, without altering the food digestibility.

The second contribution focused on the influence of the raw materials particle size and extrusion temperature on prodtive process and nutritional quality (digestibility, glycemic response and intestinal fermentations). To this purpose the same diet was ground using 4 different sieves mesh (0.5,0.8, 1.4 e 2 mm), and further extuded using two different extruder conformations,that allowed to reach two different temperatures (110 or 135°C). The 8 experimental diets obtained were then tested on 48 adult healthy dogs. Extrusion temperatures did not affect any parameter considered, while the raw material particle sze modified the productibity, that increased linearly to a particle size increase.

If it is considered that in order to obtain finer particle sizes the energetic expense increases, the increase in productivity, along with a lower energetic demand for the grinding can be traduced in lower porductive costs. The *in vivo* tests demonstrated that to an increase in particle size corresponds a decrease in dry matter and protein digestibility, but also a higher microbial proliferation (significant linear increase of total aerobes, anaerobes and bifidobacteria). Moreover a lower fecal pH and ammonia, along with a higher SCFA production were registered in animals fed diets with higher particle size. Those factors can contribute in keeping animals in healthy intestinal conditions.

In the third contribute an *in vitro* 2 steps method (digestion with pepsin and pancreatin) was studied and some possible improvements were tested. A precipitation step with ethanol, before the recovery of the digested samples throug filtration, was introduced with the objective of increasing the soluble but not digested feed fraction, that otherwise wolud be lost during the filtration phase.

Another objective of the study was to make the method easily reproducible in any simply equipped laboratory, to this purpose the in vitro procedure was carried out in a waterbath with manual or automatic agitation, instead of the equipment used in the original method, a thermostated camera with a magnetic stirrer . Samples used were previously tested for their *in vivo* digestibility.

The *in vitro* estimation of the dog feed digestibility improved with ethanol precipitation, but a higher number of samples should be tested for the confirmation of an effective improvement.

The use of a waterbath with manual agitation does not seem to interfere with the *in vitro* digestibility estimation; the reproducibility and repeatibility of the test conducted in waterbath is similar to that of the incubation equipment employed in the original method.

Chapter 1: Bibliographic review

The estimation of dog pet food digestibility can be performed by different techniques: by estimating the energy content of the feed through chemical analyses and then applying specific equations, by *in vivo* trials or by *in vitro* digestibility simulations. In the following paragraphs the three methods will be widely described, and pros and cons will be discussed.

1.1.1 Equations for the estimation of digestibility

The NRC in 1985 proposed the following equation for the estimation of the digestible energy (DE) of dog petfoods:

$$DE (Kj/kg) = CP \times 13.9 + EE \times 35.6 + NFE \times 14.6$$

Several years later some authors (Kienzle et al., 1998; Castrillo et al., 2001) proposed equations taking into account the crude fibre (CF) content of the diets as a negative contribution to the digestibility of the dry dog feeds. These equations showed a high correlation with *in vivo* determined digestible energy (Table 1).

Table 1: Relationships between *in vivo* determined DE (MJ/kg DM) and DE (MJ/kg DM) values estimated through different proposed methods

Equation	N	r	Reference
DE=21.4 x CP+37.4 x EE+14.6 x NFE-12x CF	128	0.979	Kienzle et al., 1998
DE=14.96 + 30.15 ± 2.28 x EE-4.63 ± 13.5 x CF	38	0.966	Castrillo et al., 2001

These studies demonstrated that the CF content of the diets reduces the digestibility of the feed. Recently the NRC (2006) accepted this concept and revised the formula for the digestibility estimation:

Energy digestibility (%)= 91.2 - (1.43xCF%DM);

the DE value is obtained by : DE (KJ/g)= GE (KJ/g) x Ed (%)/100.

Kienzle et al. (2006) tried to predict the energy content of the complete pet dry food through the fiber content of the diets, considering fibre content either as CF or as total dietary fibre (TDF). Fiber was considered the independent variable, while the *in vivo* measured energy digestibility being the dependent variable. Authors demonstrated that TDF content in DM gives a little more accuracy than the CF (r=0.94 and r=0.87 respectively), but further investigations are needed to clarify if the introduction of TDF instead of CF is necessary, considering that TDF is a more complex technique than the CF method for the estimation of fiber content. The estimation of digestibility through equations is faster than *in vivo* or *in vitro* trials. Nevertheless digestibility overestimations of low-density feeds with high fiber content or underestimations of feeds made with highly digestible ingredients can occur (Castrillo et al., 2009).

1.1.2 *In vivo* evaluation of the apparent digestibility of feeds

The Association of American Feeds Control Officials (AAFCO) published specific protocols for the estimation of dogs and cats feeds digestibility: it is recommended to subject animals to a 5-days period for the adaptation to the new diet, followed by 5-days of feces collection. The period of adaptation can be however reduced at 4 days for the adaptation period and 3 days for the collection period without compromising the digestibility estimation (Nott et al. 1994).

Specific equations are used for the estimation of the caloric needs of the animals, in function of age, sex and life stage of the animals. The last NRC guidelines (2006) indicated the following equation to calculate the Metabolic Energy (ME) needed for adult dogs:

$$ME=130 \times BW^{0.75} \, kg$$

The digestible energy (DE) digestibility is calculated as:

DE (per g food) = (GE of food consumed - GE of faeces collected) / amount of food consumed.

The same formula is uesd also for the determination of digestibile nutrients, above is reported the formula for thedigestibile protein (DP):

DP (% of food) = (CP of food consumed - CP of faeces collected) x100/ amount of food consumed.

This is called "apparent" digestibility, since the endogenous components that are excreted with the feces (bacteria, mucus, blood, urea) are not considered, whereas are accounted in the "true" digestibility. The variability between apparent and true digestibility is considerable, previous studies have for example verified that half of the nitrogen compounds found in the feces actually are produced by intestinal microflora (Karr-Lilienthal et al., 2004).

Higher accuracy in the estimation of the digestibility could be reached by sampling the fecal material from the ileum, avoiding the interference due to the fermentative phenomena that take place in the large intestine (Harmon 2007). The samples collection in the ileum however requires a chirurgical cannulation of the animals. One of the negative points of the *in vivo* digestibility evaluation of the feeds is that the collection of the totality of the feces is hard to perform; in order to avoid this problem and improve the preciseness of the method, digestibility markers can be used (AAFCO 2004). The marker is a substance that is not absorbed by the animal,that does not interfere with the digestibility process or with the bacterial fermentation and flows out with the digesta.

Other fundamental characteristics of the digestibility markers are the intimate association with the feed material and ease quantification. Many external digestibility markers, such as acid insoluble ash, titanium oxide, chromic oxide, rare earths have been adopted in various species, but the most commonly used marker in companion animals is the chromic oxide (Carciofi et al., 2007).

The digestibility test using a marker is conducted including a defined amount of the marker, approximately 0.25% of a high quality chromic oxide (FEDIAF 2011) in the diet. The amount of that substance found in the feces will be an index of the digestibility of the feed.

The marker will be quantified through atomic absorption spectrophotometry (Arthur, 1970) in both test feed and feces. When using the marker, the formula to calculate DE will be:

DE (kcal or kJ/g) = {1 - (GE of faeces x % Cr2O3 in food) } x GE of food
(GE of food x % Cr2O3 in faeces).

1.1.3 *In vitro* estimation of pet food digestibility

Many researches focused on the *in vitro* trials as useful methods for the estimation of pet food digestibility, proposing simple, rapid, reproducible methods which give a reliable estimation of the *in vivo* digestibility. The *in vitro* trials could be a good alternative for the estimation of digestibility in pet foods as *in vivo* experimentation on companion animals may bring to both managing and ethic problems. Adequate structures, such as individual boxes, and labor force are not easily available; the recruitment of pet animals is difficult, and housing conditions cannot be standardized; furthermore, the public opinion is definitely not favorable to animal experimentation. For these reasons *in vitro* trials could be a good option for substituting the *in vivo* studies in the assessment of the energy and nutrients digestibility of pet foods, being more reproducible and easy to perform.

The methods for the *in vitro* estimation of food digestibility in monogastrics were first introduced by Sheffner et al., (1956), who simulated the protein digestion in rats and humans by an *in vitro* hydrolysis step with pepsin. Further studies tried to simulate the pre-cecal digestion with a two steps of incubation, samples were first incubated in a buffer solution with pepsin and secondly with pancreatin in rats (Büchmann 1979) and pigs (Babinsky et al., 1990, Boisen 1991). The *in vitro* digestibility method proposed by Boisen (1991) for pig digestibility simulation has been used for the simulation of other monogastric species (in humans: Dikeman et al., 2006; in dogs: Sunvold et al., 1995; Gajda et al., 2005). Recently the same method has

been specifically adapted to canine gastrointestinal tract by Hervera et al. (2007), finding a higher correlation with the *in vivo* DE, compared to the equations proposed by the NRC (2006). The detailed steps of the *in vitro* method adopted by Hervera et al., (2007) and some improvements on this method (Palagiano et al., 2011) will be reported in a further chapter.

1.2.1 The pet food

The first typology of dog food, an especially formulated biscuit sold in UK, was introduced in the market in 1860. In 1930 appeared in the market the complete diets, as canned food and dry meat meals for dogs, and only 30 years later were developed the dry expanded diets (Barnes 2005). The worldwide market of petfood is nowadays an extremely flourishing sector, the turnover for the year 2007 for example was estimated around $45 billion, (Euromonitor International), and the market growth registered an increase of 43% from 2002.

The categories of dog petfoods sold in U.S., expressed in millions of $, along with the % of change between years 2003-2004 are reported in table 1; the most common type of pet food used for canine nutrition is dry food, and its popularity could be probably due to its complete and balanced formula as well as its practicalness for the pet owners (Lankhorst et al., 2007).

Table 2: US retail petfood sales (US$ Million) by category of food destined to dogs

Category of petfood	Year			% change
	2002	2003	2004	
Dry dog food	5210	5485	5882	+6.1
Wet dog food	1380	1416	1432.7	+1.1
Dog treats	1480	1520	1630	+7.2
Semi-moist dog food	65	45	48	+6.7
Total dog food	8135	8466	8932	+5.5

Source: Krestel-Rickert, 2005

1.2.2 Sources of starch in the production of pet foods

Dry pet foods can contain up to 60% of starch, which represents for animals an excellent energy source (Carciofi et al., 2008). The digestibility of starch in dogs is higher than 95% (Bednar et al., 2001), but some variations can be observed in relation to the source of starch provided and the processing to which the material undergoes. Starch, being hydrolyzed in glucose, is the nutrient implicated in the glucose metabolism and in the post-prandial insulin response. Moore et al., (1980) evaluated the dog digestibility of air-dried feeds containing rice, corn and oat, but did not observe any difference among the types of starch. Murray et al. (1999) evaluated *in vivo* the digestibility of dog diets containing the same amount of barley, corn, potato, rice, sorghum and wheat flours, finding similar dry matter and organic matter digestibility values for all the flours tested, except for sorghum, for which the digestibility resulted significantly lower. This difference was explained by the presence of strong bonds between proteins and starch in the sorghum grain, which make the substrate less accessible for the digestion. Twomey et al. (2002) tested *in vivo* the digestibility of extruded dog diets differing for the source of starch included (corn, sorghum and rice), finding lower digestibility values for the diets containing sorghum and rice compared with corn (85, 87 and 90% respectively). Carciofi et al., (2008) evaluated the *in vivo* digestibility and glycemic response to 6 different starchy sources (cassava flour, brewer's rice, corn, sorghum, peas or lentils), identifying faster post prandial glucose and insulin responses for brewer's rice, corn and cassava flour diets, probably due to different starch granule chemical structures.

1.2.3 Extrusion for the production of dry pet foods

The extrusion technology has been applied since 1960s for the production of petfoods, (Barnes 2005) and currently 95% of petfood worldwide is produced with this method (Spears and Fahey, 2004). Figure 2 shows a flowsheet of the production of extruded dry diets for pet animals.

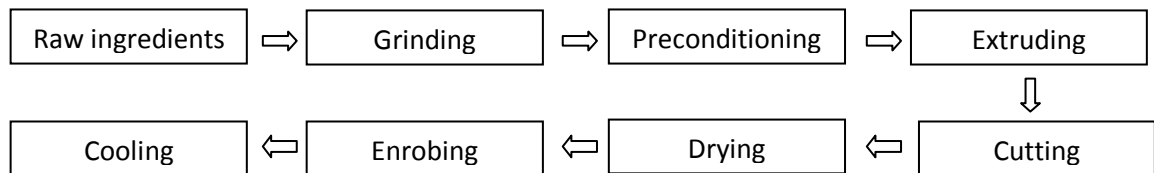| Raw ingredients	⇒	Grinding	⇒	Preconditioning	⇒	Extruding
Cooling	⇐	Enrobing	⇐	Drying	⇐	Cutting

Figure 1. Flowsheet of the process for dry pet food production

Raw materials are first grinded and mixed properly, and then carried in the pre-conditioner apparatus, a horizontal blender equipped with steam and liquid injectors. During the pre-conditioning step, liquid ingredients and steam are injected in the feed material until reaching the appropriate moisture content (15-30%), at which the material can be subjected to a first partial cook. At the end of the pre-conditioning phase the material is moved into the extruder. The extruder is a horizontal jacketed barrel tightly fitted with a single or twin flighted screw. The type of extruder and the geometry of the screw are chosen in relation to the type of material. In 90% of the pet foods manufacturers a single screw extruder is adopted, but in specific cases, for example when the feed material is very wet, sticky or when the formulation includes fresh meat (Cowell et al, 2000) or contains more than 25% of fats, and the consequent lubricating effect do not allow a correct mixing, the twin screw extruder is preferred (Riaz 2000). The twin screw extruder can mix the feed more effectively, and it allows a more uniform cooking. The single screw can have different geometries, for example a decreasing diameter of the barrel and a constant root, or an increasing diameter of the root, decreasing pitch, to the purpose of increasing the pressure to which the feed material is subjected at the end of the barrel. The section of the extruder can be divided in 3 parts, depending on the action on the feed mix: the feed section, the compression and the metering section (figure 3). In the first portion the granular feed mix is compacted in a plasticized dough thank to the shear forces applied, in the second section the mass is further compressed, the dough

loses its granular definition and the pressure in the barrel builds up. In this extruder zone steam at high pressure can also be injected, with the double purpose to increase both temperature and moisture content of the dough. In the final portion of the barrel the mass passes through the melting phase, in which temperature and pressure of the dough are increased.
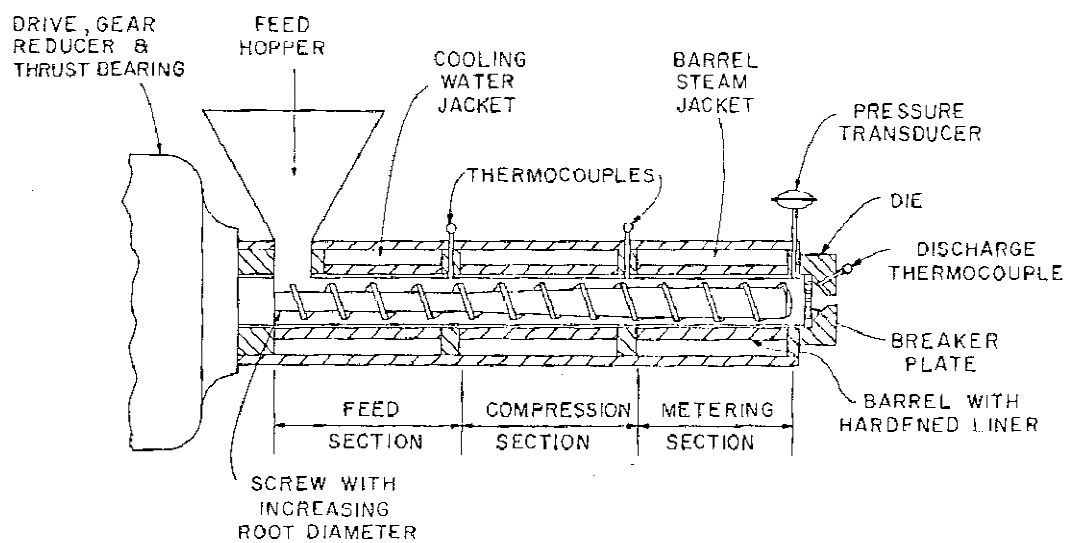
Figure 2: Scheme of a typical extruder for pet foods production

In the final part of the extruder the pressure can reach 20-40 atm, and temperature can reach the maximum point, around 145°C (Riaz 2000). At the end of the extruder the material passes through a die, that gives the opportune shape to the product. The sudden change of pressure and temperature when the product exits from the extruder causes the instantaneous evaporation of water, the expansion and the hardening of the feed material. At the end of the extruder a rotating knife is positioned, which cuts the material at the desired size. The moisture content of the material at the exit of the die is around 25-27%, and the further step in the production process is the drying phase, conducted by a hot air flux, during which the product is stabilized at a moisture content of 3-5% (Dziezak 1989). The last step in the pet food production is the

covering phase, during which the exuded kibbles are sprayed on with fats and/or flavors in the form of liquid or powder, with the aim of increasing the palatability of the product. After the covering phase dog food is ready to be weighted and packaged.

1.2.4 Effect of extrusion on nutrients quality

The wide diffusion of the extrusion technology for the production of pet foods is due to the versatility of the productive process, during which raw materials are mixed, pasteurized, sanitized, deprived of anti-nutritional factors, cooked and texturized in a short time (Riaz 2000). Depending on length and intensity of the thermal treatment and shear forces applied during extrusion, feed constituents can undergo to many changes (Tran et al., 2008); some of them are desired, such as the increase of digestibility of the material, the starch gelatinization, the decrease of anti-nutritional factors, as for example (trypsin inhibitors, haemagglutinins, tannins and phytates (Singh et al., 2007). In some cases high temperatures applied during extrusion can however damage the nutritional quality of the products, reducing the bioavailability of essential aminoacids and vitamins (Bjorck and Asp, 1983). Parameters that can be monitored during the extrusion process and that can influence the quality of the extrudates are: particle size of the raw materials, moisture and temperature in the extruder barrel, rotation speed, retention time of the material and pressure. A detailed description of the phenomena that involve the nutrients will be given in the next paragraphs.

1.2.5 Influence of raw materials particle size

Different studies have been conducted on the effects of particle size on the quality of extruded products, and all agreed on the fact that a reduction of the particle size of the raw materials corresponds to an increase of the starch gelatinization, of the expansion ratio, and of the extrudate water absorption. More in detail, Chauhan and Bains in 1985 evaluated the starch

gelatinization, the expansion ratio, the water absorption index and the water solubility index of rice flour during extrusion in a single screw extruder. They concluded that with decreasing particle size of the flour (from 0.542mm to 0.175mm) all the parameters monitored increased. Desrumaux and colleagues (1998) conducted an experiment on extrusion of corn grits of different particle size (from 0.1 to 0.6 mm), and similarly concluded that at increasing particle size, the extrudate resulted in harder texture and reduced in longitudinal expansion ratio. Lue et al., (1991) considered the starch gelatinization degree and the modifications in fibre content in extrudates composed by a mix of corn meal and sugar beet fibre of different size (from 2mm to 0.074mm), and concluded that a decreasing size of the fibrous material corresponded to a higher radial expansion and a lower elongation of the products, while no differences in the total dietary fibre and the soluble and insoluble fractions content were observed. Mathew et al., (1999) investigated the effect of particle size of corn, that varied between 1,5mm and 0,75mm, on the characteristics of pet food extrudates, concluding that the finer the particles are, the higher the expansion ratio and the water absorption index of the extrudates. A recent work (Bazolli et al., 2011, unpublished) investigated the nutritional effects of 3 extruded dry diets differing for the starchy source (corn, rice and sorghum), by using raw materials ground at 3 different sizes (0,8, 1,5 and 3 mm). Results indicate that at increasing particle size the digestibility of nutrients decreases quadratically for the diet containing corn, and linearly for the diet containing sorghum, while no effects were observed for the diet containing rice.

Increasing the particle size of the diets corresponded to a decrease in the fecal pH and a higher production of short chain fatty acids.

However no specific studies on pet food have evaluated the extent of starch gelatinization and retrogradation in relation to the particle size of the starchy raw material used for extrusion.

1.2.6 Effect of extrusion on Proteins

During the extrusion process, proteins are modified by the shear forces, the pressure and the high temperatures applied. Proteins can lose their tertiary and quaternary structure and can align with the flow of the material (figure 4), hydrogen and disulphur bonds can be broken and reformed, and residues that were first hidden, are then exposed and free to react with reducing sugars and other components of the feed material (Camire, 1991).

Flow of the material
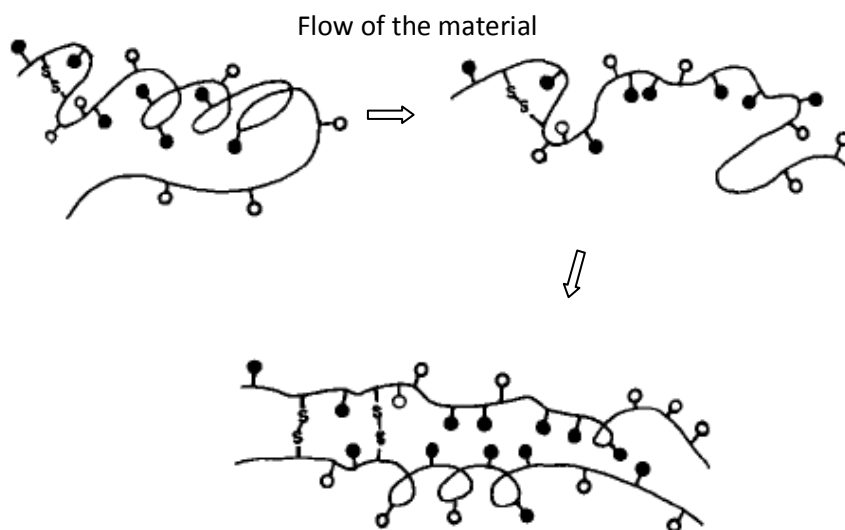
Figure 3: Schematic diagram of a protein molecule unfolding, aligning with the flow in the extruder barrel, and forming new bonds with another molecule (based on Camire, 1991).

The main chemical reaction that involves proteins during the extrusion process is the Maillard reaction, responsible of the browning and the flavoring of the material during the cooking phase, but also liable of decreasing the nutritional quality of the petfood due to the loss of bioavailable lysine, a limiting aminoacid (Camire 1991).

The Maillard reaction can be resumed in 3 principle steps:

1. condensation between an amino group and a reducing sugar and formation of an N glycoside

2. rearrangements and formation of Amadori compounds

3. dehydration, fragmentation, cyclization and polymerisation reactions in which amino groups participate again.

Factors that influence the speed of this reaction are type and amount of aminoacids and reducing sugars, temperature and time of heat exposure, pH, moisture content and interactions in the food matrix (van Boekel 2006).

Following the AAFCO guidelines (2002), the lysine requirements for dogs are 0.48% and 0.59% of the diet for growing and adult animals, respectively, calculated for foods containing 3500kcal ME/kg.

Williams and colleagues (2006) published a first study on the lysine content of 33 dry canine diets available on the market, concluding that the lysine content was sufficient to cover the dog needs, but a large proportion of the lysine in the diets (15% of the total lysine in maintenance diets and 25% of total lysine in diets addressed to growing animals) appeared to have been damaged and so unavailable for the dogs. A recent study (Lankhorst et al., 2007) analyzed the influence of some extrusion parameters, such as temperature (110, 130 or 150°C) and moisture content (200 or 300 g/kg), on the lysine reactivity, concluding that extrusion temperatures employed during the experiment did not influence the presence of lysine, maybe because temperatures at which proteins can be damaged are higher (180°C) (Cheftel 1986). More studies are needed to identify the best productive conditions to minimize the heat damage of essentials aminoacids, specifically lysine on extruded pet foods.

1.2.7 Effect of extrusion on starch

A brief description of the starch structure is necessary for a better understanding of the effects of extrusion on this polymer. Starch is the most abundant molecule in nature after cellulose, it is composed by glucose residues that are organized in 2 polymers: amylose and amylopectin. Amylose is a linear molecule, composed by 100-10000 D-glucose residues linked with α 1-4 linkage; the base of the amyopectin structure is the same of the amylose, but in addition to α 1-4

bonds, β 1-6 bond are also found (Figure 4A), which confer an extremely branched structure to the polymer (Haralampu 2000). Amylopectin is organized in clusters, and it is composed by three types of chain, named A- B- and C-chains (Figure 4B), that differ for their level of branching. A-chains are unbranched and attached to the molecule by a single linkage, B-chains are branched and connected to two or more other chains, while only one C-chain is found for each amylopectin molecule, and its peculiarity is the presence of the sole reducing group. The arrangement of amylopectin chains in the starch granules originate a compact radial structure, in which amorphous and crystalline structures are alternated (figure 4C). Amylose molecules are randomly interposed among the amylopectin clusters, both in the crystalline and in the amorphous region. Large amylose molecules are able to link with amylopectin in double helices, while smaller amylose molecules are able to leach from the granule (Oates, 1997). It is generally reported that the starch granule is formed for a 20-30% by amylose, while amylopectin content is around 70-80% (Jane et al., 1999), but these percentages can vary depending on the botanical origin of the starch, some varieties of maize for example have been recently selected for obtaining a starch granule containing 99% of amylopectin, called waxy maize.

A	B	C
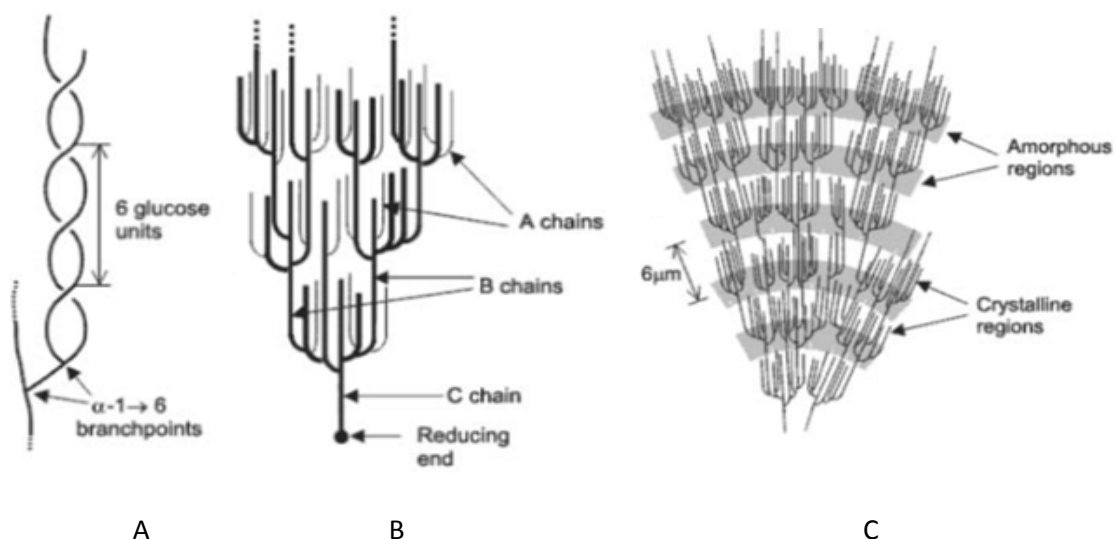
Figure 4: Structure of amylopectin (A, B) and organization of amylopectin clusters in a starch granule (Modified from Sajilata et al 2007)

In presence of abundant water and 60-70°C heat (Hermansson and Svegmark, 1996), starch granules undergo to the phenomenon called gelatinization: starch granules swell and imbibe, hydrogen bonds are disrupted and the typical crystalline structure is lost (Zeng et al., 1997); a schematic view of the process is shown in figure 5. During gelatinization the amylose chains diffuse out of the granule and form a continuous gel phase. In the following cooling phase starch undergoes to a relatively slow re-association process called retrogradation, during which amylose molecules tend to re-associate in double helices structures, stabilized by hydrogen bonds (Haralampu 2000).
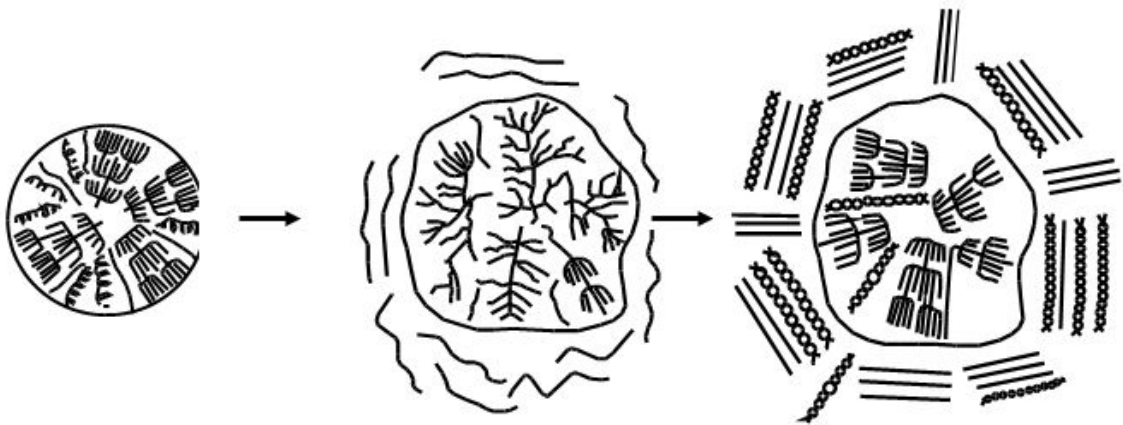
Figure 5: Schematic representation of a native starch granule that undergoes to gelatinization and retrogradation processes. Source: http://www.food-info.net/uk/carbs/starch.htm.

The quality of the starchy extrudates is evaluated through the extent of the gelatinization of starch and the expansion ratio of the final products. It is generally reported that the complete gelatinization of the starch can be obtained when the water:starch ratio is 1,5:1. However, shear forces to which the starch granules are subjected during the extrusion process can damage the starch integrity, favoring a faster water absorption, and consequently a faster gelatinization (Lai et al., 1991).

Owusu-ansah et al. (1983) studied the effects of some extrusion parameters (temperature, moisture content and screw speed) on the gelatinization of cornstarch, finding moisture and extrusion temperatures as the most relevant factors. Bhattacharya and Hanna (1987) also studied

the effects of extrusion on corn starch gelatinization, concluding that an increase of moisture content is associated to a lower degree of gelatinization, while higher temperatures bring to a higher degree of starch gelatinization. Pet foods are however composed not only by cereal products, but also animal origin ingredients, containing proteins and fats. The presence of nutrients other than starch influence the starch gelatinization process: it has been demonstrated that the addition of lipids on a pet food formulation during extrusion reduces its starch gelatinization degree. This effect could be due to the insulating effect of lipids, that prevents the starch granule to absorb water (Lin et al., 1997). Another reaction that can involve lipids and starch during the extrusion is the formation of V-amylose complexes, less susceptible to the enzymatic attack (Singh et al., 2007). A specific research have been conducted on the digestibility of V-complexes in dogs, in which a lower dry matter digestibility was noted when those complexes substituted all the diet carbohydrates and 10% of the fed lipids in enteral formulas (Murray et al., 1998). If the amylose-lipid complexes are not present in a large amount, however, they do not seem to impair the fat utilization (Tran et al., 2008).

1.2.8 Effect of extrusion on Lipids

During the extrusion and the storage of pet foods, lipids contained in the formulation can be affected by chemical modifications such as oxidation, hydrogenation, isomerization and polymerization (Singh et al., 2007). The most influent of them is the oxidative reaction that causes the development of rancid odors during storage. Many factors can influence the rate of lipid oxidation, as for example fat type, moisture content and the degree of kibble expansion. Lin and colleagues (1998) studied the effects of the type of fat (beef tallow or poultry fat), its concentration in the diet (0, 25, 50 and 75 g/kg), the feed moisture content (160, 180 and 200 g/kg) and the speed of the extruder screw (200, 300, 400 rpm) on the lipid oxidation of extruded dry diets. They found that all the parameters influenced the oxidation rate, but the most significant effect resulted the expansion ratio of the kibble: a higher surface exposed to air corresponded to a higher lipid oxidation rate.

In extruded products the formation of complexes between lipids and amylose (described in the previous paragraph) and lipids and proteins has been observed.

1.2.9 Effect of extrusion on Fiber

Results of some researches on wheat flour, beet fiber, corn meal and barley flour (Björck et al., 1983; Lue et al., 1991 Vasanthan et al., 2002) have shown that extrusion process causes an increase of the total dietary fibre (TDF) content of the diet, due to the formation of soluble dietary fibre (SDF) from non fibrous components, and it has been hypothesized that this change was due to the reaction of transglycosidation between reactive anydro-compounds and fragmented starch, forming non digestible branched glucans (Björck et al., 1983). Another explanation for the increase in TDF content was the formation during extrusion of resistant starch (Vasanthan et al., 2002). Studies also showed an increase on the SDF content of the extruded material, caused by the shift from insoluble fibre fraction (IDF) towards the SDF (Björck et al., 1983; Vasanthan et al., 2002, Dust et al., 2004). The same structural changes described above could occur in extruded dry dog feeds, but no studies, to the author's knowledge, have been already published on this specific topic.

1.2.10 Effects of extrusion on Vitamins

As vitamins differ greatly in chemical structure and composition, they have different stability properties. Extrusion process is usually associated to vitamins degradation depending on specific parameters during food processing and storage, such as temperature, oxygen, light, moisture, pH and time (Killeit, 1994). Minimizing temperature and shear force within the extruder protects most vitamins (Singh et al., 2007).

Some studies have assessed the effects of extrusion cooking on the retention of B group vitamins (Cheftel, 1986; Killeit, 1994). During the extrusion of crispbread products, at a retention time of 0,5 to 1,0 min at 178ºC, the levels of B group vitamins decreased (Cheftel,

1986), and thiamine and pyridoxine were the most thermo-labile with their levels decreasing linearly with temperature. Camire et al. (1990) obtained similar results, with thiamin losses increasing at increasing barrel temperature.

Other authors studied the effects of extrusion on other vitamins, (Guzman-Tello & Cheftel, 1990; Andersson & Hedlund, 1990; Chaovanalikit, 1999). When higher barrel temperatures were used (200ºC instead of 125ºC), there was a reduction of all trans-β-carotene in wheat flour by over 50% (Guzman-Tello & Cheftel, 1990), appearing that thermal degradation is the major factor that contributes to β-carotene losses during extrusion (Singh et al., 2007). Ascorbic acid is also sensitive to heat; a specific study on wheat flour (Anderson & Hedlund, 1990) reported that the concentration of this vitamin decreased when the material was extruded at a higher barrel temperature at 10% of moisture, while no effects on vitamin B2 and niacin were detected.

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General objective

In the present thesis several aspects regarding the production and the nutritional quality of dry dog foods were explored.

The first experiment aimed at evaluating the effects of the addiction of α-amylase during the productive process on the extrusion parameters and *in vivo* feed digestibility.

The second experiment aimed at identifying the influence of particle size of the raw materials and of extrusion temperature on food digestibility, gut microbiology and fermentation products formation.

The third experiment was developed in 5 consecutive assays, aiming to improve and make an *in vitro* method easily reproducible for the evaluation of dog feed digestibility.

Chapter 2: Amylase utilization for the extrusion of dog diets

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Abbreviations: KNU, kilo-novo-α-amilase-unit; ANOVA, analysis of variance; AAFCO, American association of feed control officials; CP, crude protein; OM, organic matter; NRC, National research council; CTTAD, coefficient of total tract apparent digestibility; DM, dry matter; ME, metabolizable energy; GLM, general linear model; SAS, Statistical Analysis Systems.
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Abstract

To assess the potential use of amylase to reduce the cost of extrusion, the present study evaluates the effects of amylase addition on extruder parameters, kibble quality and digestibility of dog food in two separate experiments. In experiment 1, 120 KNU/kg of heat stable α-amylase produced by *Bacillus licheniformis* was added in liquid form in the preconditioning. In experiment 2 3,684 KNU/kg of heat stable α-amylase produced by *Aspergillus oryzae* was added to the dry ingredients before the mixing phase. Both diets were processed in a single screw extruder and submitted to a digestibility test whereas only diets in experiment 1 were tested for their palatability. Digestibility was performed using 12 dogs for each experiment, six animals per diet. Data were submitted to ANOVA F test (P<0.05). Amylase addition significantly affected extrusion parameters in both experiments (P<0.05) as higher food productivity (+28% and +43% kg of DM/hour in experiment 1 and 2, respectively) and lower electric energy consumption (-22% and -29% US$/100 kg DM in experiment 1 and 2, respectively) were observed in the supplemented diets. Kibble appearance and quality (density (g/L), cutting force (g), and starch gelatinization degree (%) were not affected by the enzyme treatment (P>0.05). Likewise, enzyme addition did not change nutrient digestibility, fecal dry matter or food palatability (P<0.05). The achieved results showed that amylase inclusion reduces the dough viscosity and resistance inside the extruder allowing for higher product flow and food productivity and lower electricity energy consumption, without altering food quality.

Keywords: digestibility, enzymes, extruder productivity, kibble quality, starch

2.1. Introduction

The first application of extrusion in food production is believed to have been around 1940, when this technology was employed to produce corn flakes and pasta. The first utilization of extrusion in pet food production dates back to 1950, when the first complete dry diet for dogs was produced (Crane et al., 2000). The extrusion process is composed of a feeding system, a preconditioner, an extruder and a dryer (Riaz 2000). The principle of extruder operation is the compression of dough by either a single or twin screw that generates mechanical energy. The friction and compression of the material increase the dough temperature, and the addition of steam determines the gelatinization of the starch and the cooking of the material, increasing food digestibility and palatability (Chuang and Yeh, 2004; Ding et al., 2004). Other functions of extrusion include mixing of ingredients, food sanitization, degradation of anti-nutritional factors, and improvements in the shape and texture of the final product (Björck and Asp, 1983; Cheftel, 1986).

The combination of retention time, temperature, moisture and shear forces determines the productivity and the final quality of pet food (Riaz, 2000). During processing, the gelatinization of the starch inside the extruder causes an increase in the dough viscosity, raising the resistance of the material against flowing through the extruder. This process is important, allowing the transference of mechanical force from the screw to the dough, a process that ultimately will promote cooking. However, a parallel increase in electric consumption by the engine is required to overcome the increased resistance of the material and maintain constant productivity.

The cost of extrusion is an important factor for the pet food industry. Equipment composition, configuration and efficiency need to be carefully monitored and optimized. One potential method to reduce electricity costs could be the use of amylase during the extrusion process. This enzyme is a saccharidase which breaks α1-4-glycosidic bonds in starch (Dijkhuizen et al., 2002). The use of amylase could promote the dextrinization of the starch,

thereby decreasing the dough viscosity and consequently the friction and resistance of the material against flowing through the extruder, ultimately reducing the mechanical (and electrical) energy needed for extrusion. The purpose of the present study was to evaluate the effects of two heat-stable α-amylases (from *Bacillus licheniformis* and *Aspergillus oryzae*) during dog food production on extruder productivity, electrical consumption, kibble characteristics, food starch gelatinization degree, and *in vivo* food digestibility.

2.2. Material and methods

2.2.1. Production of the experimental diets

This study included two independent experiments whose ingredients and the chemical analysis is reported in Table 1. For each experiment a different diet was formulated following the AAFCO (American Association of Feed Control Officials, 2008) nutritional recommendations for dog maintenance. Each formulation was extruded with or without the addition of a heat-stable α-amylase, therefore two experimental diets were tested on each experiment. In the first experiment, a heat-stable α-amylase produced from *Bacillus licheniformis* (Termamyl 120 L, Novozymes, Bagsvaerd, Denmark) at a dosage of 120 kilo-novo-α-amylase-unit (KNU)/kg of dry matter was used. The enzyme was diluted in water and pumped (model V-10,0 Bar, Injetronic, Sumaré, SP, Brazil) directly into the preconditioner to enrich the experimental supplemented diet. To ensure equal water addition in both treatments, the same volume of water was pumped into the control diet. In the second experiment a heat-stable α-amylase produced from *Aspergillus oryzae* (Fungamyl 4000 BG, Novozymes, Bagsvaerd, Denmark) was included in the experimental diet at a concentration of 3,684

KNU/kg of dry matter. The enzyme was in powder form and mixed with the ingredients before extrusion.

Table 1: Ingredients and chemical composition of the experimental diets

Item	Diet T[a]	Diet F[b]
Ingredients (g/kg, as fed)		
Maize	164.7	206.7
Broken rice	180.0	202.0
Poultry by-product meal	260.0	239.0
Defatted corn	120.0	-
Wheat bran	140.0	252.0
Palatability enhancer	20.0	20.0
Soybean meal	50.0	-
Poultry fat	50.0	59.0
Mineral and Vitamin premix	3.0	5.0
Potassium chloride	-	4.0
Sodium chloride	10.0	4.0
Choline chloride	-	1.0
Mold inhibitor	2.0	1.0
Antioxidant	0.3	0.4
Lysine chloride	-	0.9
Calcium carbonate	-	5.0
Chemical composition[c] (g/kg, DM)		
Dry matter	914.0	931.4
Crude protein	229.0	261.2
Acid-hydrolyzed fat	101.2	111.7
Starch	448.8	407.0
Crude fiber	25.3	38.1
Ash	95.5	83.4

[a] Supplemented with α-amylase produced by *Bacillus licheniformis* (experiment 1); [b]Supplemented with α-amylase produced by *Aspergillus oryzae* (experiment 2).
[c] Analyzed in duplicate in the laboratory of Nutrition and Nutritional Diseases of Dogs and Cats at São Paulo State University (Jaboticabal, Brazil).

All diets were processed in the extruder facility of the College of Agrarian and Veterinarian Sciences, São Paulo State University, Brazil. Dry ingredients were mixed and ground in a hammer mill (Model 4, D'Andrea, Limeira, Brazil) fitted with a 1-mm screen sieve, and further extruded in a single-screw extruder (Mab 400S, Extrucenter, Monte Alto, Brazil) with an average extrusion capacity of 150 kg/h. During the

extrusion process the amperage of the extruder engine and the extruder productivity were constantly monitored and recorded every 20 minutes. The decrease in amperage need due to the amylase addition was recorded and once the amperage became stable, the feeding flux was increased until the amperage value returned to the previous value. Productivity (kg/h) and kibble density (g/L) were measured only when the system reached stability. The extruder pre-conditioning temperature was kept above 90°C. Water, steam, screw speed and the raw materials flux were adjusted according to diet formulation, and the extrusion temperature kept above 120°C. After extrusion kibbles were dried in a dryer at 105°C and further coated with fats and palatability enhancers.

2.2.2. Production parameters and calculations

The estimation of the hourly productivity was calculated by weighing the mass of kibbles produced in two minutes and multiplying that value by 30. Samples were also collected from each diet for dry matter (DM) determination, and productivity was corrected for DM basis to avoid the interference of hydration level on the extruder capacity calculation. The productivity of the control diets was used as reference value (production equals to 100%). The degree of gelatinization of the starch was determined using a biochemical analyzer (YSI 2700, Yellow Springs, Ohio, USA) at Labtec (Campinas, Brazil). The energy expense of the engine was calculated using the following formula:

Energy expense (kW/kg DM) = Active Power x 100/productivity (kgDM/h),

where the Active Power (kW/h) is equal to: √Motor-phase x Voltage x Amperage x cosφ.

2.2.3. Animals, digestibility and palatability test

Experimental diets were submitted to a digestibility test. A total of 12 adult Beagles between 3 to 6 years old and 11.2 ± 0.7 kg body weight were used. Prior to be submitted to the

study, dogs were dewormed and subjected to a veterinary examination to ensure good health. Each experiment was organized according to a randomized design accounting for 12 experimental units (dogs) and two treatments (diets), with six dogs per diet. The dogs belong to the Laboratory of Research in Nutrition and Nutritional Diseases of Dogs and Cats, São Paulo State University (UNESP, Jaboticabal, Brazil). The Ethics Committee for Animal Well-Being of the College of Agrarian and Veterinarian Sciences, São Paulo State University approved all experimental procedures.

In vivo digestibility was determined by total fecal collection, following the AAFCO (2008) recommendations and calculation procedures. Dogs were individually housed in 1.5 x 3.0 m kennels for five days, during which they adapted to the experimental diet. In the following five days total fecal collection was carried out keeping the animals in stainless steel metabolic cages (90 x 90 x 100 cm) equipped with an apparatus to separate feces from urine. The amount of food supplied to each dog was estimated by considering the metabolizable energy content of the food and each dog's energy requirement, according to the Nutrient Requirements of Dogs and Cats (NRC, 2006). Dogs were fed their individually-calculated amounts once a day, at 09:00 h. Bowls were removed after 30 minutes and any remaining food was weighed and recorded. Fresh water was available *ad libitum*. During the collection period, fecal output from the individual dogs were collected twice a day, weighed and stored frozen at -15 ℃ until analysis. For the analysis, feces were thawed, homogenized and dried at 55 °C for 72 h in a forced air oven (320-SE, FANEM, São Paulo, Brazil). Feces and diets were then ground in a cutting mill (MOD 340, ART LAB, São Paulo, Brazil) fitted with a 1 mm screen sieve before analysis. Food and fecal samples were tested for dry matter (DM) by oven-drying the samples (method 934.01), ash composition by muffle furnace incineration (method 942.05), crude protein (CP) composition by the Kjeldahl method (method 954.01), and acid-hydrolysed fat content using a Soxhlet apparatus (method 954.02) according to the guidelines of the Association of Official Analytical Chemists (1995). Organic matter (OM) was calculated as 1000-ash. Total starch content was analyzed according to Hendrix (1993).

In experiment 1, diets were submitted to a palatability test. Dog food palatability was measured using the two-pan method (Griffin 2003) in which 38 individually housed dogs of different breeds and body weights were tested on two consecutive days. In the morning, after twelve hours of fasting, dogs received two pans, each one containing one of the experimental diets (with and without enzyme), and were allowed to eat for 30 min. The position of the food pans was alternated at the evening meal. The amount of food offered in each pan exceeded the consumption capacity of the animal to ensure leftovers to be measured. After 30 min, pans were removed, leftovers weighed and individual consumption recorded. Due to the large differences in body weights, interpretation of the results was based on the relative consumption of each diet:

$$\text{Food relative consumption} = \frac{\text{g of food without enzyme}}{\text{g of food with enzyme} + \text{g of food without enzyme}}$$

2.2.4. Cutting test

Kibbles were submitted to a cutting test performed with a texturometer (TA-XT2 SMS, Stable Micro Systems, Godalming, UK) set to operation mode strength/compression, return to start option enabled, pre-test speed of 2 mm/s, speed during the test of 0.5 mm/s and speed before test of 10 mm/s. The test was conducted on 20 units for each sample, using the probe HDP/BSK blade set with knife with a cutting distance of 10 mm. Data were analyzed with the software Texture Expert (Stable Micro Systems, Godalming, UK).

2.5. Statistical analysis

A completely randomized experimental design was applied to the palatability trial results, and a Student's t-test was used for the comparison of the relative food consumption. Data were submitted to ANOVA F test utilizing the GLM procedure of the SAS software (Version 9.1, SAS Institute Inc., Cary, NC, USA). Values of $P<0.05$ were considered significant. All data were found to comply with the assumptions of ANOVA models. Results are presented as mean ± standard error.

2.3. Results

2.3.1. Production parameters

In both experiments the addition of the enzyme affected the production parameters (Table 2). The extruder feeding rate increased significantly in both experiments in the diets added of α-amylases (by 39% in experiment 1 and by 100% in experiment 2, respectively), Enzyme addition increased the extruder productivity (on DM basis) by approximately 29% in experiment 1 and 43% in experiment 2, both values being significantly higher than the respective controls ($P<0.05$). The electrical energy cost of extrusion (100 kg/DM) decreased 27% in experiment 1 and 37% in experiment 2 with enzyme addition ($P<0.05$). Other parameters of the extruder operation (preconditioner temperature, and extruder temperature) did not change with enzyme addition ($P>0.05$). Starch gelatinization degree, kibble density and cutting force also were not different between diets ($P>0.05$).

Table 2: Extrusion parameters and kibble characteristics of the experimental diets with or without amylase addition (mean ± standard error).

Item	Experiment 1		Experiment 2	
	Diet T		Diet F	
	Without enzyme (n=4)	With enzyme[a] (n=4)	Without enzyme (n=4)	With enzyme[b] (n=4)
Preconditioner temperature (°C)	92.2±2.06	92.0±1.43	95.0±2.00	95.1±3.0
Extruder temperature (°C)	>120.0	>120.0	>120.0	>120.0
Extruder feeding (Hz)	15.7±0.1	21.9±0.9*	24.6±0.3	51.8±0.8*
Engine amperage (A)	39.6±0.2	39.7±0.2	16.6±2.2	20.1±3.2
Screw speed (Hz)	42.2±1.4	40.1±0.9	51.8±0.02	57.1±0.1
Productivity (kg of DM/h)	84.1±1.3	108.3±8.2*	97.5±6.6	139.9±5.4*
Energy expense (kW/h)	15.2±0.5	12.7±0.3*	5.5±1.1	5.7±0.2
Cost of electricity (US$/100 kg DM) [3]	1.80±0.1	1.4±0.1*	0.7±0.1	0.5±0.0*
Kibble density (g/L)	404±5.3	410±6.0	340±14.1	342±13.1
Cutting force (g)	6,256±108	6,609±57	4,468±96.0	4,107±109.0
Starch gelatinization degree (%)	91.0	88.5	92.3	98.5

[a] Heat stable α-amylase produced from *Bacillus licheniformis* (Termamyl 120 L, Novozymes, Bagsvaerd, Denmark), added directly in the preconditioner at a dosage of 120 KNU/kg of dry matter. [b] Heat stable α-amylase produced from *Aspergillus oryzae* (Fungamyl 4000 BG, Novozymes, Bagsvaerd, Denmark), at a concentration of 3,684 KNU/kg of dry matter. [c] calculated considering US$ 0.119 for KW/h. * within an experiment, statistical difference for enzyme addition ($P<0.05$)

2.3.2. Food digestibility and palatability

Table 3 reports the coefficients of total tract apparent digestibility of the foods. No changes to nutrient digestibility were observed after enzyme addition. Fecal dry matter was also not affected by treatment in either experiments. Food palatability was equivalent for the two diets tested in experiment 1.

2.4. Discussion

The current study confirms the experimental hypothesis that the addition of α-amylase to dog food reduces energy use during extrusion. We are aware of only one other study using amylase as a processing enzyme for extruded dog diets, and this study also demonstrated electricity cost reductions ranging from 17.9% to 25.8%, depending on the type of enzyme used (Froetschner et al., 2006). However, researchers observed low productivity increases, from 0.7% to 1.3%, notably lower than those found in the present study.

The observed productivity increases can be explained by the hydrolytic action of the amylase enzyme on the starch molecules in the preconditioning phase which interferes with starch gelatinization. Starch gelatinization results in swelling and water absorption, which together increase the viscosity of the dough and its resistance against flowing through the extruder. The breakdown of amylose chains by amylases reduces water absorption, swelling, and friction of the dough, thus making the product more fluid and less flow-resistant (Hsieh et al., 1991; Lin et al., 1997; Samarasinghe et al., 2000). This resulted in an immediate reduction of the work required to the engine, and this was observed by a reduction in engine amperage (data not shown). Since the experiments were conducted maintaining the amperage request constant, the higher extruder feeding rate resulted in an increased productivity.

It is worth to note that structural changes in the starch can interfere with kibble quality and appearance. The final density of the kibble is a product of the ingredient mixture density

Table 3: Coefficient of total tract apparent digestibility (CTTAD) of nutrients, fecal dry matter and food relative consumption of the experimental diets with or without amylase addition.

Item	Experiment 1 Diet T		Experiment 2 Diet F	
	Without enzyme (n=6)	With enzyme[a] (n=6)	Without enzyme (n=6)	With enzyme[b] (n=6)
Food intake (g/dog/day)	206.0 ± 1.1	196.2 ± 16.1	170.9±22.2	171.5±22.4
CTTAD				
Dry matter	0.803 ± 0.008	0.800 ± 0.005	0.762±0.004	0.761±0.002
Organic matter	0.856 ± 0.006	0.848 ± 0.004	0.814±0.003	0.818±0.002
Crude Protein	0.845 ± 0.007	0.829 ± 0.007	0.834±0.003	0.832±0.002
Acid-hydrolysed fat	0.914 ± 0.004	0.902 ± 0.005	0.869±0.001	0.861±0.002
Starch	0.991 ± 0.001	0.992 ± 0.001	0.992±0.001	0.994±0.001
Fecal dry matter (g/kg)	383±12	372 ± 23	410±42	390±57
Food relative consumption[c]	0.49	0.51	-	-

[a] Heat stable α-amylase produced from *Bacillus licheniformis* (Termamyl 120 L, Novozymes, Bagsvaerd, Denmark), added directly in the preconditioner at a dosage of 120 KNU/kg of dry matter.
[b] Heat stable α-amylase produced from *Aspergillus oryzae* (Fungamyl 4000 BG, Novozymes, Bagsvaerd, Denmark), at a concentration of 3,684 KNU/kg of dry matter. [c] Food relative consumption = (consumption of food without enzyme) / (consumption of food without enzyme + consumption of food with enzyme).

and the kibble expansion rate. The fact that the kibble density remained constant in our experiments reveals that the amylases did not reduce the dough expansion capacity. Cutting force is a measure of the force required to break the kibble, such that harder kibbles require more force and therefore more chewing effort from the dog: the increased cutting force can negatively interfere with palatability and consumption. Our data showed that amylase does not interfere with these parameters. Although dry kibbles showed no apparent changes in appearance or quality, whenever hydrated in water before being offered to the animal the amylase-treated kibbles lose their structural strength and can turn mushy (data not shown), a problem that needs to be considered by the manufacturer.

Starch cooking, measured by the degree of starch gelatinization, is affected by pressure, temperature, retention time and shear force (Riaz 2000). Given that amylase reduces dough resistance, a decrease of pressure and consequently temperature inside the extruder can occur, and starch gelatinization can be compromised. However, in our experiments, the cooking of the starch was not affected by the enzymes addition, while the increased extruder feeding rate observed in the supplemented diets probably contributed to keep the extruder pressure at optimal level.

Starch digestibility is usually high in extruded dog diets when the food is well processed (Carciofi et al., 2008), and in the current study the amylase addition did not affect starch digestibility. The experimental design did not allow for a direct comparison between experiments 1 and 2. In fact, food composition differed: in experiment 1 the diet contained more starch and less fiber and protein. The source of starch also differed, as a mixture of rice, maize and wheat were used for diets in experiment 2 whereas maize and rice were used for diets in experiment 1. Extruder screw configuration also changed; in experiment 2 a more restrictive screw was used which resulted in greater engine work (amperage) and electric energy expense (kW/h).

Food palatability depends on several characteristics of the kibble including crispness, hardness, shape, size, and taste. Protein plasticization and denaturation, starch gelatinization and

dextrinization, reducing sugar formation and browning reactions resulting from amino-carbohydrate complexation can all influence it. Results of the palatability tests in experiment 1 showed that these aspects were not influenced by enzyme addition.

Other considerations that are important for pet food manufacturers to keep in mind include the enzyme cost and the relative impact of the electric cost on the total cost of food production. Practically, adding α-amylases before extrusion allows pet food producers either to work at constant electric energy, resulting in an increased productivity, or to reduce the cost of electricity, maintaining the same productivity achieved without enzymes addition. However, it must be taken into consideration that it is important that the increased extrusion capacity and/or the reduced electric cost are able to offset the cost of the enzyme addition. Further researches should therefore evaluate the most effective enzyme dosage in relation to the specific extrusion process conditions (temperature, moisture, resident time) and food formulation, in order to obtain an appreciable reduction of the production costs.

2.5. Conclusion

The present study showed that the addition of α-amylase in the pet food process reduces the resistance of the dough against flowing through the extruder leading to an increase in extruder productivity or a reduction of electric energy costs. The enzyme addition does not interfere with food texture, starch gelatinization, nutrient digestibility or food palatability. The economic benefit of amylase utilization needs to be evaluated in different food formulations and under different extrusion systems.

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Chapter 3: Maize particle size and extruder configuration effects on energy and nutrient digestibility, fermentaion products and microbial composition of the feces of dogs fed kibble diets

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Abstract

The effects of raw material particle size and extruder conformation were evaluated on dry dog food extrusion parameters and on in vivo diet digestibility, glycemic response, short chain fatty acids (SCFA) production, intestinal flora, and fecal characteristics using 48 adult dogs. The same diet was milled for passing through sieves of 0.5, 0.8, 1,4 and 2 mm, and extruded with two different conformations, reaching high (>135°C) or low (<110°C) extrusion temperatures, for a total of 8 experimental diets. Extrusion productivity increased by 30% (linear effect, P<0.001) by increasing particle size from 0.5 to 2mm. The extrusion temperature did not show any effect on the factors tested, while the increasing particle size decreased linearly (P<0.05) the digestibility of DM, N and fat. The starch digestibility was not affected by the particle size variations, being above 99% for all the diets. To increasing particle size was associated a higher fecal output (linear effect, P<0.05), but not a different fecal score. Significant (linear effect) higher fecal pH and fecal ammonia, and lower bacterial counts (total aerobes, total anaerobes, bifidobacteria) were registered for higher particle size diets. The higher bacterial counts were also confirmed by higher SCFA production (quadratic effect, P<0.05). No different glycemic response was associated to the 8 diets. The use of higher raw material particle size can reduce the energy required for milling and can increase extruder productivity. The benefits of using higher particle size in the feeds are not only economic, while an improvement of the canine intestinal health is assured through higher microbial proliferation and fermentation.

3.1 Introduction

Many factors influence the nutritional quality and metabolic responses induced by the dry kibbles destined to dogs. Raw materials quality and food formulation are the first points to be considered, but also the processing system plays a central role (Tran et al., 2008) although it has

been much less studied. Particle size reduction is a relevant production cost (Amerah et al., 2007), usually studied with regard to extruder efficiency, starch gelatinization degree, water absorption, expansion ratio (Desrumeaux et al., 1998; Mathew et al., 1999) and kibble appearance; however, little information is known on its effect on food utilization or animal health. Cereals particle size can induce variations on nutrient digestibility in poultry and swine (Amerah et al., 2007; Wondra et al., 1995), and alter postprandial responses in humans (Holt and Miller, 1994; Pereira et al., 2002), but no information is available for dogs.

Another critical point on pet food processing is extrusion: water, steam, screw configuration and ration flux is usually adjusted to maximize productivity, reduce costs and improve kibble density and appearance. Extruder parameters such as temperature, shear force, retention time, and pressure, however, act on starch gelatinization degree, amino acid availability and vitamin and other nutrient losses (Camire et al., 1990), directly impacting on animal health. It is possible that defining proper processing parameters, adopting a specific cereal particle size and extruder configuration, benefits for animal health could be achieved by the formation of resistant starch. This particular starch fraction is widely studied as prebiotic for humans (Topping and Clifton, 2001), but it is poorly studied in animals. Considering this, the present research investigated the influence of cereal particle size and extruder configuration on energy and nutrient digestibility, fecal microbiota, fermentative end-products formation and glucose postprandial response of dogs

3.2 Material and methods

The Ethics Committee for Animal Well-Being at the College of Agrarian and Veterinarian Sciences, São Paulo State University, approved all experimental procedures.

3.2.1 Animals, Diets and Experimental Design

Table 1. Ingredient composition of the experimental dog formulation

Ingredients	%, as-fed basis
Maize grain	55.8
Poultry by-products meal	28.6
Poultry fat	8.5
Sugar cane fibre	3.0
Flavor enhancer	2.0
Vitamins and minerals premix[1]	0.5
Common salt	0.5
Potassium chloride	0.5
Choline chloride	0.2
Fish oil (salmon)	0.15
Antioxidant [2]	0.1
Calcium carbonate	0.07
Mold inhibitor[3]	0.04
Lys•HCl	0.04

[1]Addiction for kg of product: Iron, 100 mg as iron sulfate; Copper 9.25 mg as copper sulfate; Manganese 6.25 mg as manganese sulfate; Zinc 150 mg as zinc sulfate; Iodine 1.87 mg as potassium iodide; Selenium 0,13 mg as sodium selenite; vitamin A 18,750 UI; vitamin D 1,500 UI; vitamin K 0.15 mg; Thiamine 5 mg; Riboflavin 16 mg; Pantotenic acid 35.75 mg; Niacin 62.5 mg; Piroxidin 7.5 mg; Cobalamin 45 mcg; Folic acid 0.75 mg.
[2]Mold Zap (Ammonium dipropionate, acetic acid, sorbic acid, and benzoic acid; Alltech do Brasil Agroindustrial Ltd., Curitiba, PR, Brazil).
[3]Banox (BHA, BHT, propyl gallate, and calcium carbonate; Alltech do Brasil Agroindustrial Ltd.).

The experiment was conducted using 48 adult Beagle dogs, both females and males, with body condition score ranging between 4/9 and 6/9 (Laflamme, 1997), and mean body weight of 12.4 ±1.5 kg. Dogs were kept in the Laboratory of Research in Nutrition and Nutritional Diseases of Dogs and Cats at São Paulo State University (Jaboticabal, Brazil) for the entire study period. Prior to start the experiment, dogs' health condition was assessed by physical examination, complete blood count, biochemical profile, and fecal and urinary exams.

A "base diet" was formulated according to the nutritional recommendations of the American Association of Feed Control Officials (AAFCO, 2009) for adult dogs at maintenance, using maize as unique cereal source. The ingredients used for its formulation are listed in Table 1. From this formulation 4 experimental diets were produced by grinding the ingredients at 4 different particle sizes, using a hammer mill (Model 4, D'Andrea, Limeira, Brazil) fitted with 4 different screen sieves: 0.5, 0.8, 1.4 and 2-mm. Food particles were separated according to the procedure described by Zanotto and Bellaver (1996), modified for the mesh size, which measured 1, 0.5, 0.25, 0.105, and 0 mm. The mean geometric diameter (MGD) of the ingredient mixes before extrusion were then calculated with the program Gransuave (Embrapa, Brasilia, Brazil).

Diets were processed in the extruder facility of the College of Agrarian and Veterinarian Sciences, Sao Paulo State University, in a single-screw extruder (Mab 400S, Extrucenter, Monte Alto, Brazil) under 2 different die conformations: in the first one (A) the extruder die had an open output area of 63.6 mm^2 and in the second one (B) the open output area was reduced at 23.7 mm^2. This was a technical solution adopted to facilitate the achievement of different temperatures inside the extruder. Reducing the open output area (kg/h·mm$^{2(-1)}$) the amount of share force, dough resistance and the temperature inside the extruder increase consequently. Therefore, two temperatures were adopted for the extrusion: T<115°C (low temperature) for conformation A and T >135°C (high temperature) for conformation B. During the extrusion process many parameters were evaluated and recorded every 15 minutes: conditioning temperature and water addition, retention time, engine amperage, feed rate, screw and knife speed, temperature inside the extruder, kibble density at extruder output, and extruder productivity.

The experiment was organized in a 4 x 2 factorial arrangement (4 food particle size and 2 extruder configurations), in order to obtain 8 experimental diets. Chemical composition and quality parameters of the diets are presented in the Table 2. The metabolizable energy of the base diet was estimated from its chemical composition, and the amount of diet provided was calculated using the

standard equation for dog's maintenance energy requirements (ME, kcal = 130 x kg $BW^{0.75}$) (NRC, 2006). Dogs were weighed weekly and food supply was individually adjusted in order to maintain a constant BW during the entire experiment. Fresh water was always available *ad libitum*.

Since only 16 metabolic cages were available, dogs were divided into 3 groups of 16 animals each. Two dogs/group were fed one of the eight experimental diets for 21 days. During this period, a digestibility trial was performed for the first 11 days, fresh feces were individually collected on days 12 and 13 for short chain fatty acid (SCFA) analysis, individual fresh feces collection was performed on days 14 to 17 for microbiological analyses.

3.2.2 Digestibility Protocol

The digestibility trial was performed following the AAFCO (2009) guidelines and calculation procedures, allowing 6 days for diet adaptation and 5 days for total fecal collection. Dogs were individually housed in 1 x 1 x 1 m stainless steel metabolic cages. Each day, food was weighed and left out at 0800 h. Bowls were removed after 15 min, and any remaining food was weighed and recorded. On the first day of fecal collection, all feces were removed from the cages and discarded before 0800 h. Fecal output was collected for the next 5 d, at least 3 times a day. Samples were frozen (-20∘C) as soon as they were collected and pooled by dog. Feces were also scored for their texture according to the following system: 0 = watery liquid, which can be poured; 1 = soft, unformed; 2 = soft, malformed stool, which assumes shape of container; 3 = soft, formed, and moist, which retains shape; 4 = well-formed and consistent stool, which does not adhere to the floor; and 5 = hard, dry pellets.

At the end of the collection period, feces were thawed, homogenized, and pooled by dog. Before performing chemical analyses, fecal samples were dried in a forced-air oven at 55°C for 72 h (Fanem, São Paulo, Brazil) and ground in a cutting mill (MOD 340, ART LAB, São Paulo) with a 1-mm screen. Diet and feces were analyzed for DM, OM, ash, CP (Kjeldahl method), and acid-

hydrolyzed fat using AOAC (1995) methods. Total dietary fiber (TDF) was determined according to the procedure described by Prosky et al. (1992). The GE content of diets and feces were determined by bomb calorimeter (Model 1261, Parr Instrument Company, Moline, IL), and the total amount of starch was analyzed according to Hendrix (1993). All samples were analyzed in duplicate and repeated when the variation was greater than 5%.

3.2.3 Measurement of Fermentative Products in Feces

Fresh feces from each dog were collected after the digestibility trials to assess pH, SCFA, and ammonia concentrations. Feces were collected in 2 successive days, within 15 min from defecation. For SCFA, immediately after collection, fecal samples (approximately 10 g) were mixed in 30 mL 16% (v/v) formic acid solution, precipitated at 4EC for 72 h, and the supernatant centrifuged (5804R, Eppendorf, Hamburgo, Brazil) 3 times at 4,500 x g at 15°C for 15 min, and transferred to a new tube to clean the sample avoiding the obstruction of the chromatography column. Fecal SCFA were analyzed by gas chromatography (Model 9001, Finnigan, San Jose, CA) according to the method reported by Erwin et al. (1961) using a glass column 2 m in length and 3.17 mm in width covered with 80/120 Carbopack B-DA/4% Carbowax 20M (Supelco, Bellefonte, PA). Nitrogen was the carrier gas with a flow rate of 25 mL/min. Working temperatures were 220ºC at injection, 210ºC in the column, and 250ºC in the flame ionization detector. Lactic acid was measured (Pryce, 1969) using a colorimetric method (Spectrophotometer Quick – Lab, Drake, São José do Rio Preto, Brazil). Fecal pH was determined with a pH meter (model Q-400-Bd, Quimis, Brazil) on 1g of fresh feces diluted with 5 ml of Milliq water. Fecal ammonia concentration was quantified according to Vieira et al., (1980) on the same extracts used for the determination of the SCFA.

3.2.4 Microbial Enumeration

The collection of fresh feces for microbial enumeration (total anaerobes, total aerobes, *Bifidobacterium* spp, *Lactobacillus* spp, *Clostridium* spp and *Escherichia*. coli) was performed from

d 14 to d 17 keeping dogs housed individually in the 1 x 1 x 1 m stainless steel metabolic cages used for the digestibility trial. 10 g of fresh feces were weighted in a sterile container for bacterial enumeration; the analysis started within a maximum of 30 minutes after defecation. Microbial populations were determined by serial dilution (10^{-1} to 10^{-7}) of fecal samples in peptone water before inoculation onto *petri* dishes containing sterile agar. For total anaerobes and total aerobes enumeration, diluted samples were grown in Plate Count Agar; E. coli were grown on MacConkey Agar and Lactobacilli were grown on Man-Rogosa Sharpe Agar (all from Acumedia Manufacturers Inc., Lansing, MI, USA). The selective media for bifidobacteria was Bifidobacteria Agar (Himedia Laboratories, Mumbai, India). Agar used to grow *Clostridium* was Reinforced Clostridium Agar (Oxoid Ltd, Basingstoke, Hampshire, United Kingdon). Samples for total anaerobes, Bifidobacterium, Lactobacillus and Clostridium quantification were incubated anaerobically (73% N : 20% CO_2:7% H_2) at 37°C. Total aerobes and E. coli were incubated aerobically at 37°C. Plates were counted between 24 and 48 h after inoculation. Colony forming units (cfu) were defined as distinct colonies measuring at least 1 mm in diameter.

Table 2. Particle size distribution, mean geometric diameter (MGD), chemical composition and quality parameters of the experimental diets

Item	screen sieve size, mm							
	0.5		0.8		1.4		2.0	
Sieve opening, µm	----% of particles retained----							
1000	0.0		0.0		0.1		1.2	
500	0.2		15.2		17.3		26.0	
250	20.4		33.6		31.8		26.1	
105	62.9		40.2		36.0		38.3	
Pan	16.6		11.1		14.9		8.4	
MGD, µm	169		248		252		290	
MGD standard device	1.8		2.1		2.2		2.2	
	Extruder conformation							
	A	B	A	B	A	B	A	B
Chemical composition, % on DM basis								
DM	94.9	94.0	94.6	95.0	94.3	94.3	94.3	95.0
OM	88.0	87.1	87.9	88.3	86.8	86.8	87.2	87.8
CP	24.5	24.6	25.2	25.2	24.7	24.9	24.3	23.9
Fat	13.4	14.2	14.6	15.3	15.2	14.9	16.2	14.6
Starch	42.3	40.8	42.1	40.8	42.0	40.3	41.3	41.5
Quality parameters								
Kibble density, g/L	360	340	390	360	430	440	425	390
Cutting force, kgf	2.96	3.21	3.28	3.91	3.58	3.07	3.56	3.08

3.2.5 Statistical Analyses

Data were analyzed in a randomized complete block design using the GLM procedure of SAS (Version 9; SAS Inst. Inc., Cary, NC). The experimental unit was the dog. The model sums of squares were separated into diet, period (blocks), and animal effects. The interactions among the variables tested (particle size and extruder conformation) were analyzed with the SLICE statement. The averages of the food particle size were evaluated using polynomial contrasts (linear, quadratic and cubic effects). The averages for the type of extruder configuration (A and B) were compared using the F test. Repeated measures analysis of variance with 2 inter-animal factors (diet and period) and one intra-animal factor (time of sampling) was the statistical method chosen to evaluate the effects of diet and time on postprandial plasma changes. Pair-wise means comparisons were made using Tukey's test when the F-test was significant. All data were found to comply with the assumptions of ANOVA models. Values were considered significant at $P < 0.05$. Results are presented as mean $\pm$ standard error.

3.3 Results

3.3.1 Food Production Parameters

After grinding, a different particle size distribution was observed among diets (Table 2). Particles > 500µm represented only 0.2% of the food ground with the 0.5-mm screen sieve, but more than 27% of the food ground with the 2.0-mm sieve. Despite the differences on screen sieve, foods ground with the 0.8-mm and 1.4-mm sieves resulted in very similar MGD.

Temperature in the extruder preconditioner was kept above 92°C in all diets. Water addition was similar among diets, with approximately 21% of humidity in the food exiting from the extruder. Kibble density (g/L) and cutting force (kgf) also did not vary among diets ($P > 0.05$; Table 2).

The use of different output area helped in obtaining different temperatures inside the extruder. Conformation A resulted in a mean temperature inside the extruder of 113°C, lower than the mean value registered for the conformation B of 137°C ($P < 0.001$). The influence of the particle size on the extruder temperature was

different for each conformation: while for conformation A no effect of particle size was observed on extruder temperature (means ranging between $112^{\circ}C$ and $116^{\circ}C$), in conformation B the extruder temperature decreased linearly as the MGD of the food increased ($P = 0.013$), ranging from $144.7\pm1.5^{\circ}C$ for the diet ground with the 0.5-mm screen to $124.5\pm0.8^{\circ}C$ for the diet ground with the 2.0-mm screen.

The energy expense for food extrusion did not vary with the extruder configuration (69.5 ± 5.3 kW•ton^{-1}•h^{-1} for conformation A and 67.4 ± 1.9 kW•ton^{-1}•h^{-1} for conformation B), but it decreased linearly by increasing the food particle size ($r^2=0.49$; $P < 0.001$) (Figure 1).
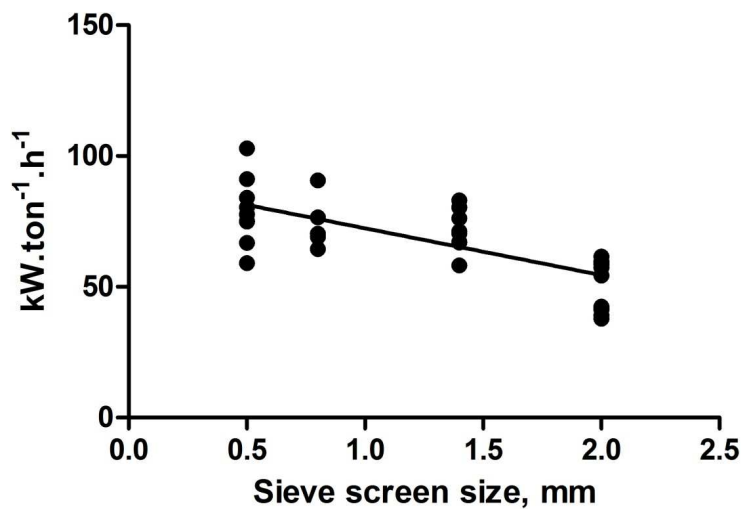
Figure 1. Energy expense for food extrusion (kW•ton^{-1}•h^{-1}) in relation to the sieve screen size (mm) used to grind the experimental diets ($r^2=0.49$; $P < 0.001$).

3.3.2 Digestibility

All dogs consumed the entire amount of offered food and their BW remained stable during the entire digestibility trial. Nutrients intake was similar among treatments (Table 3). Nutrients digestibility were not affected by the interaction between extruder conformation and particle size, so only the effect of particle size was considered (Table 3). All nutrients, except starch and fat, showed a linear reduction on digestibility as mean

particle size increased ($P < 0.01$).. Fat presented a tendency towards reduction ($P = 0.0526$) and starch digestibility remained above 99.9% for all diets.

3.3.3 Fecal production and characteristics

No interaction between extruder conformation and particle size was verified for any fecal parameter. The extruder conformation did not influence significantly the fecal characteristics (Table 4), while grinding at different particle sizes had significant effects: the daily fecal output (g/day) increased linearly by increasing the particle size of the food ($P = 0.002$), but this effect was only due to a higher fecal water content, as the daily fecal output referred on dry matter basis did not result different among treatments ($P = 0.159$). Fecal score also decreased as the food particle size increased ($P = 0.001$), but feces texture remained adequate, being the average fecal score always > 3. By increasing the diet particle size a linear decrease ($P < 0.001$) of fecal pH was observed .

3.3.4 Concentration of Fermentation Products and Bacterial Enumeration in the Feces

Food particle size significantly modified the fecal concentrations of the fermentation products ($P < 0.05$), while the extruder conformation did not (Table 5). No interactions between extruder conformation and particle size were observed. By increasing the food particle size a quadratic increase was observed in the concentration of all the SCFA (Acetic, Propionic and Butyric acid) ($P < 0.05$). Fecal ammonia, on the other hand, decreased linearly as the food particle size increased ($P < 0.001$).

For bacterial enumeration no effects of the extruder conformation or interactions were seen (Table 6). Food particle size, however significantly modified the fecal microbial counts: to an increase in particle size a linear increase in total aerobes ($P = 0.002$), total anerobes ($P = 0.014$) and Bifidobacteria ($P = 0.001$) populations was observed. No influence on the remaining considered microbic populations was observed.

Table 3. Nutrient intake and apparent total tract digestibility of nutrients by dogs fed experimental diets processed with different screen sieve sizes and extruder conformations (mean ± standard error).

Item	Extruder conformation	Screen sieve size, mm				Mean	P-value	
		0.5	0.8	1.4	2.0		Linear	Quadratic
Nutrient intake, g•dog^{-1}•d^{-1}								
	A	227.6±16.4	231.5±10.79	246.0±4.3	240.9±15.4	236.5±6.1		
Dry matter	B	248.2±10.3	226.3±15.1	228.2±11.0	240.7±12.3	235.8±6.0		
	Mean	237.9±9.7	228.9±8.9	237.1±6.25	240.8±9.4		0.665	0.469
Apparent digestibility values, %								
	A	81.5±0.40	82.3±0.92	78.3±0.64	79.6±0.71	80.4±0.46		
Dry matter	B	81.9±0.63	80.9±0.86	78.9±0.68	81.2±0.89	80.7±0.44		
	Mean	81.7±0.43	81.6±0.64	78.6±0.46	80.3±0.59		0.007	0.084
	A	85.2±0.35	85.7±0.77	82.6±0.50	83.4±0.67	84.2±0.38		
Organic matter	B	85.6±0.63	84.7±0.72	83.4±0.55	84.9±0.7	84.6±0.35		
	Mean	85.4±0.35	85.2±0.53	83.0±0.37	84.1±0.51		0.004	0.126
	A	84.6±0.43	84.17±0.92	80.9±0.93	81.4±1.13	82.8±0.54		
Crude protein	B	85.4±0.80	84.4±0.94	82.2±0.68	83.0±0.67	83.7±0.44		
	Mean	85.0±0.35	84.3±0.63	81.5±0.58	82.2±0.67		0.001	0.231
Fat	A	91.2±0.71	92.6±0.77	89.9±2.15	91.2±0.81	91.5±0.47	0.053	0.548

Item	Extruder conformation	Screen sieve size, mm				Mean	P-value	
		0.5	0.8	1.4	2.0		Linear	Quadratic
	B	92.2±0.60	91.1±0.94	91.0±0.75	90.3±0.69	91.1±0.38		
	Mean	91.7±0.47	92.2±0.64	90.6±0.77	90.7±0.52			
	A	99.9±0.03	99.9±0.02	99.9±0.02	99.9±0.03	99.9±0.01		
Starch	B	99.9±0.02	99.9±0.03	99.9±0.02	99.9±0.02	99.9±0.01		
	Mean	99.9±0.02	99.9±0.02	99.9±0.02	99.9±0.02		0.810	0.453
	A	85.7±0.3	85.9±0.7	82.9±0.5	83.8 ±0.7	84.6±0.4		
Gross energy	B	85.8±0.7	85.1±0.7	83.7± 0.5	85.2 ±0.7	85.0± 0.4		
	Mean	85.8 ± 0.4	85.5±0.5	83.3±0.4	84.5±0 .5		0.005	0.110

Table 4. Fecal production and characteristics of dogs fed experimental diets processed with different screen sieve sizes and extruder conformations (mean ± standard error).

Item	Extruder conformation	Screen sieve size, mm				Mean	*P*-value	
		0.5	0.8	1.4	2.0		Linear	Quadratic
Daily fecal output, fresh	A	104.9±8.7	112.25±9.5	143.8±5.6	144.8±17.4	126.4±6-4		
	B	114.1±8.9	116.6±11.7	130.0±7.8	133.0±12.2	123.4±5.1		
	Mean	109.4±6.1	114.4±7.2	136.9±5.0	138.9±10.3		0.002	0.844
Daily fecal output, DM-basis	A	38.8±3.0	37.2±2.7	49.0±2.4	45.0±5.5	42.5±2.0		
	B	41.9±3.3	40.1±4.5	45.9±3.9	41.1±4.4	42.2±2.0		
	Mean	40.3±2.2	38.7±2-5	47.4±2.2	43.0±3.4		0.159	0.611
Fecal DM, %	A	37.2±1.4	33.5±1.30	34.1±0.87	31.2±0.95	34.0±0.69		
	B	36.7±1.03	34.3±0.93	35.0±1.26	30.7±0.57	34.2±0.65		
	Mean	36.9±0.82	33.9±0.77	34.6±0.75	30.9±0.5		<0.001	0.714
Fecal pH	A	6.85±0.07	6.67±0.05	6.37±0.07	6.33±0.07	6.55±0.05		
	B	6.77±0.07	6.77±0.08	6.58±0.08	6.42±0.67	6.64±0.05		
	Mean	6.81±0.05	6.72±0.05	6.48±0.06	6.37±0.05		<0.001	0.930
Fecal Score[3]	A	3.71±0.06	3.45±0.09	3.32±0.04	3.14±0.08	3.4±0.05		
	B	3.55±0.06	3.53±0.07	3.54±0.07	3.23±0.03	3.5±0.05		
	Mean	3.63±0.07	3.49±0.06	3.44±0.05	3.18±0.04		0.001	0.049

Table 5. Concentration of some fermentative products on the feces of dogs fed experimental diets processed with different screen sieve sizes and extruder conformations (mean ± standard error).

Item	Extruder conformation	Screen sieve size, mm				Mean	P-value	
		0.5	0.8	1.4	2.0		Linear	Quadratic
		μmol/g Feces, DM-basis						
Fecal Ammonia	A	132.3±5.0	120.5±8.0	120.68±8.7	103.1±6.7	119.1±4.0		
	B	172.1±1.0	143.7±14.5	122.9±12.1	114.7±7.0	138.4±7.0		
	Mean	152.2±8.0	132.1±8.6	121.8±7.1	108.9±4.9		<0.001	0.623
Acetic acid	A	88.8±6.7	109.2±7.0	116.1±11.2	103.6±5.0	104.4±4.2		
	B	99.9±6.7	119.1±10.7	131.3±16.7	112.2±12.6	115.7±6.2		
	Mean	94.4±4.8	114.2±6.3	123.7±9.9	107.9±6.6		0.136	0.020
Propionic acid	A	35.4±2.6	52.6±3.7	56.4±5.7	59.4±2.8	51.0±2.7		
	B	41.4±2.2	53.1±8.4	63.9±8.6	55.1±9.0	53.4±3.9		
	Mean	38.4±1.8	52.9±4.4	60.2±5.0	57.0±4.5		0.018	0.049
Butyric acid	A	15.0±2.3	18.1±1.0	18.7±2.1	15.5±1.4	16.8±0.9		
	B	15.5±0.6	16.1±2.2	26.2±5.3	17.4±2.1	19.0±1.7		
	Mean	15.3 ±3.9	17.5±1.2	22.4±2.9	16.5±1.2		0.291	0.028
Total SCFA[3]	A	139.2±10.2	179.9±11.2	191.2±17.9	178.6±8.7	172.2±7.1		
	B	156.8±7.6	189.1±19.1	221.4±29.7	184.7±22.7	188.0±11.1		
	Mean	148.0±4.8	184.5±10.7	206.3±17.2	181.6±11.7		0.036	0.020

[3]The sum of acetic, propionic and butyric acids

Table 6. Microbial enumeration on the feces of dogs fed experimental diets processed with different screen sieve sizes and extruder conformations (mean ± standard error).

Item	Extruder conformation	Screen sieve size, mm				Mean	P-value	
		0.5	0.8	1.4	2.0		Linear	Quadratic
		log of colony-forming units/g of faecal DM						
Total aerobes	A	8.88±0.34	9.24±0.49	9.36±0.29	9.51±0.24	9.25±0.17		
	B	8.59±0.32	8.94±0.36	9.49±0.34	10.10±0.30	9.27±0.19		
	Mean	8.73±0.22	9.09±0.29	9.43±0.21	9.79±0.19		0.002	0.993
Total anaerobes	A	8.97±0.33	9.22±0.39	9.48±0.35	9.52±0.24	9.29±0.16		
	B	9.08±0.47	8.83±0.60	9.90±0.42	10.36±0.28	9.49±0.26		
	Mean	9.03±0.30	9.03±0.35	9.67±0.26	9.99±0.21		0.014	0.688
Enterobacteriaceae	A	6.42±0.44	6.37±0.23	5.28±0.47	5.55±0.29	5.90±0.20		
	B	6.37±0.33	5.95±0.39	6.99±0.69	6.00±0.36	6.30±0.20		
	Mean	6.40±0.26	6.16±0.22	6.13±0.47	5.77±0.23		0.185	0.847
Clostridium	A	4.51±0.39	4.36±0.46	4.75±0.10	4.84±0.24	4.62±0.16		
	B	4.94±0.37	4.88±0.44	4.99±0.20	4.70±0.35	4.88±0.16		
	Mean	4.71±0.27	4.62±0.31	4.87±0.10	4.77±0.20		0.679	0.977
Lactobacilli	A	8.60±0.25	9.29±0.19	9.27±0.59	9.14±0.24	9.08±0.19		
	B	8.50±0.28	8.67±0.21	8.10±0.98	9.40±0.30	8.66±0.25		
	Mean	8.54±0.18	8.95±0.17	8.74±0.55	9.27±0.18		0.182	0.853
Bifidobacteria	A	8.33±0.34	8.79±0.38	9.10±0.29	9.26±0.15	8.87±0.16		
	B	8.60±0.38	8.15±0.41	9.19±0.11	9.41±0.19	8.84±0.17		
	Mean	8.47±0.25	8.47±0.28	9.15±0.15	9.33±0.12		0.001	0.663

3.4 Discussion

In the past, food quality was associated only to high food digestibility and small amounts of fecal production. Nowadays this concept has changed, been orienting towards diets that promote other health benefits also (Carciofi et al., 2010). Changes in lifestyle, neutering, prolonged life expectancy, and new food habits have led to an increase in the incidence of metabolic diseases, obesity, and several degenerative conditions in dogs that can be potentially ameliorated by diet. Food digestibility, metabolic responses to the meal, and gut health has been investigated in the recent past using different types of fiber (Diez et al., 1998, Kimmel et al., 2000,) and prebiotics (Swanson et al., 2002). No studies, to the authors' knowledge, have however explored the characteristics of the extrusion process (i.e. open output area and temperature) and the particle size of the food on the dietary digestibility and animals' gut health.

Grinding the food by the use of a hammer mill fitted with 4 different sieves resulted in the production of 4 experimental diets characterized by different average MGD. It is worth to note that diets milled with the 0.8 and 1.4 sieves resulted in very similar average MGD.

The differences observed in the dough temperature inside the extruder barrel (113°C for conformation A vs 137° C for conformation B) may be explained by the restriction of output area in conformation B which may have increased the dough resistance, shear force, pressure and temperature consequently. On the contrary, increasing food particle size resulted in more intact starch granules, where hydration is more difficult, so the swelling and viscosity of the dough during the extrusion is lower, explaining the reduction of the temperature. When comparing foods ground with 0.5-mm and 2.0-mm screen size, energy expense for food production ($kW \cdot ton^{-1} \cdot h^{-1}$) decreased of more than 30%. This accounts for an important energy saving during food production, as it was previously reported by Mathew et al. (1999) and Al-Rabadi (2011). Larger starch granules swell more slowly and at a lesser extent, reducing the viscosity and the resistance of the dough that flows more easily inside the extruder barrel, requiring less mechanical energy.

Although the different temperatures registered inside the extruder, neither dogs' nutrient digestibility nor fecal characteristics were affected. This is probably due to the fact the lower temperature inside the extruder (average 113°C) may have been enough to achieve an optimal starch gelatinization and digestibility. The extent of starch gelatinization required to optimize food digestibility is an information that need to be established in dogs. This would be useful in order to avoid unnecessary input of energy, and to save processing cost.

The linear decrease in nutrient digestibility caused by an increase of the particle size of raw material was expected, as demonstrated by previous studies on other monogastric animals, such as poultry (Carre, 2004), piglets (Healy et al., 1994) or finishing pigs (Wondra et al. 1995). In the only one study carried out on extruded dog foods, Hilcko et al (2009) also found a linear reduction of nutrient digestibility following the increasing of particle size, but some unusual findings like the lack of effect on DM digestibility and the very low fat digestibility of the diets did not allow a deeper interpretation of the data. The reduction on nutrient digestibility is explained by a decreased contact between digestive enzymes and nutrients in the diet (Amerah et al., 2007). However, in the current study the reduction in nutrient total tract apparent digestibility was small and lower than 1.5 points of percentage; only for crude protein the digestibility was reduced more than 2.5 points of percentage. Starch apparent digestibility resulted almost complete in all diets, as previously reported for extruded dog diets (Walker et al., 1994, Murray et al., 1999, Carciofi et al., 2008), without differences between foods. One limitation to consider when interpreting these data, however, is the difference between ileal and total tract apparent digestibility. Starch fermentation on dog's colon and increased microbial protein excretion for the foods with greater MGD, as a result of greater microbial activity in the colon, may contributed for the lack of difference in starch total tract apparent digestibility among diets and the reduction of protein digestibility in diets with greater MGD. Moreover, the reduction of some nutrients digestibility should be considered beneficial for the gut health, as the supply of fermentable organic matter to the colon (as the resistant starch) favorably influenced some parameters related to gut health.

The linear increase in fecal production per dog/day, and linear reduction in fecal DM%, fecal score, and pH are explained by the reduction in digestibility and the increased fermentation activity in dog's colon after

consumption of foods with larger MGD. These alterations on dog's feces, although significant, resulted in satisfactory fecal characteristic (score >than 3). Microbial fermentation of starch and other organic matter escaped from digestion in the small intestine explain the increased fecal concentration of SCFA (Cumming and Englyst, 1995; Kienzle, 2001) and the linear reduction on fecal ammonia concentration (Birkett et al., 1996; Zentek et al., 2002) verified in the present study. The fecal ammonia is a catabolite of protein fermentation and its presence has been correlated to higher risks of tumorogenesis (Lin and Viesek 1991). The SCFA have been extensively studied for their potential health benefits, especially butyrate due to its potential to stimulate coloncytes metabolism, gut immunity and intestinal healthy (NRC, 2006). Previous studies, attempting to change canine colon fermentation activity, evaluated the effects of different fiber sources (Sunvold et al., 1995; Biagi et al., 2010; Kawauchi et al. 2011) or prebiotics such as fructooligossacharides (Vickers et al., 2001), spray-dried yeast cell wall (Swanson et al., 2002b; Middelbos et al. 2007), or others oligosaccharides (Strickling et al., 2000; Propst et al., 2003). No studies on food process as a potential tool to improve dog's gut health are available. The inclusion of prebiotics did not always result in significant alterations on SCFA formation (Swanson et al., 2002a), turning promise to explore in further studies the effect of food particle size. Moreover, it must be considered that increasing the food particle size may generate resistant starch (Topping and Clifton, 2001), which may act as a prebiotic.

Another factor that can promote gut health status of animals is the proliferation of beneficial bateria in the gut (O'Mahony et al., 2009; Carciofi and Gomes, 2010). The intestinal microbiota plays an important role in host's digestion and metabolism, and provides a natural defense mechanism against invading pathogens (Hooper et al., 2001; NRC, 2006). In the present research a linear increase in total aerobes and total anaerobes was observed as the MGD of the food increased. Among the selected bacterial populations considered in the present study, a linear increase of bifdobacteria, a population generally associated with gut health (autor) and studied as probiotic for humans and animals (Abe et al., 1995; Sauter et al., 2006), was detected. The efficacy of probiotics and prebiotics in beneficially modifying gut microbial population in dogs resulted controversial (Strickling et al., 2000; Swanson et al., 2002, Middelbos et al., 2007; Zentek et al., 2003; probiotic).

3.5 Conclusions

Although further studies are deemed to confirm our results, increasing the temperature inside the extruder did not affect either dogs' nutrient digestibility or fecal characteristics. However, increasing food particle size from 0.5 to 2.0 mm decreased DM, OM and CP digestibility; moreover benign microbial populations (aerobes, anaerobes and Bifidobacteria) and SCFA production increased, whereas fecal pH and ammonia concentration decreased, all conditions that promote intestinal health. The increase in the particle size seems a good strategy to improve canine health.

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Chapter 4: Improvements on the in vitro organic matter digestibility method applied to dog feeds

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Abstract

A recent *in vitro* method for the estimation of apparent digestibility (*in vitro* OMD) of dry dog foods using a thermostatic camera (TA) (Hervera et al., 2007) showed a good correlation with *in vivo* values, but overestimated the digestible energy content of foods. This research aimed to evaluate if the precipitation of soluble non-digested polysaccharides with ethanol can improve the *in vitro* OMD prediction of dry dog foods rich in fibre. The use of a readily available laboratory equipment (water-bath with manual -WM- or automatic - WA- agitation) instead of a TA for samples incubation was evaluated because of the easier availability, that can make the method reproducible in any simply equipped laboratory.

Dog foods previously *in vivo* tested for their OMD were used, and 4 trials were conducted. In trial 1, a precipitation step with ethanol before filtration was performed on one food sample incubated alone or with the addition of 10% of either pectin or cellulose. In trial 2, the precipitation with ethanol was tested on 3 foods rich in fibre. In experiment 3, three methods of incubation (i.e. in TA, in WM and in WA) were investigated. The two best equipments resulting from the previous experiment were tested in trial 4 for repeatability and reproducibility.

The *in vitro* OMD overestimated the *in vivo* OMD in a lesser extent when the precipitation step with ethanol was carried out, but higher number of samples should be tested in order to confirm the result.

The *in vitro* OMD obtained with TA and WM showed higher correlations with the *in vivo* OMD (r=0.99, 0.96 and 0.74 for TA, WM and WA, respectively). Similar repeatability (1.70 and 1.68) and reproducibility (2.07 and 2.93) was observed for TA and WM, respectively. The TA used in the original method can therefore be replaced by the WM, a more readily available equipment, without compromising the *in vitro* OMD estimation.

4.1 Introduction

The *in vitro* methods allow to study food digestibility without utilizing experimental animals, so the reduction of labor force and of the maintenance costs for hosting animals in adequate structures are relevant. In the case of pet animals, furthermore, the employment of animals for the experimentation can arouse ethic problems. A specific *in vitro* method for the estimation of apparent digestibility of dry dog foods, based on a previous study on pigs (Boisen 1991), has been recently presented (Hervera et al., 2007). This *in vitro* method gave higher accuracy on the prediction of energy digestibility, compared to the equations proposed by NRC (2006). However, it slightly overestimates the food energy digestibility. Possible explanations for this overestimation can be the incomplete recovery of the undigested solid material (e.g soluble fibre) as well as the use of solvents (ethanol and acetone) during the washing phase of the residues, which dissolve all fats contained in the sample, assuming that fats digestibility is 100%. Moreover, it has to be considered that data coming from *in vitro* methods are compared with the *in vivo* apparent digestibility, that is an underestimation of the true digestibility (Crane et al., 2000). A strategy to improve the *in vitro* procedure accuracy is the precipitation of non digested soluble carbohydrates with ethanol, a method already adopted in other procedures for the precipitation and the recovery of the soluble fibre fraction (Prosky et al., 1988). First aim of this study was to evaluate if the introduction of a precipitation step with ethanol in the procedure presented by Hervera et al. (2007) improves the *in vitro* prediction of OM digestibility of dry dog foods rich in fibre. The second objective was to investigate the possible reduction of costs of this above-mentioned method by using an easily available laboratory equipment.

4.2 Material and methods

Below is reported the original method published by Hervera and colleagues (2007), which represents the starting point of the experiments objective of the present study:

Dry dog foods were ground finely (<1mm) and samples were represented by 1±0.1 g of material. In a first incubation step samples were put in Erlenmayer and added of 25 mL of phosphate buffer (0.1 M, pH 6) and 10 mL of HCl 0,2 M. After adjusting pH at 2 with 1M HCl or NaOH, 1 mL of pepsin solution (10 mg of enzyme Fluka 77152 diluted in 1 mL phosphate buffer) was added. To avoid bacterial growth 0.5 mL of chloramfenicol (0.5 g in 100 mL ethanol) was also included. Samples were incubated at 39°C in a thermostated camera for 2 hours, with continuous magnetic stirring (200-250 rpm). After the incubation, Erlenmeyers were cooled and 10 mL of phosphate buffer (0.2 M pH 6,8) and 5 mL of NaOH 0,6 M were then added for the second incubation step. pH was adjusted at 6.8 with HCl or NaOH 1M and 1 mL of pancreatin (Sigma P-1750) solution containing 100mg of enzyme/g of sample was added. Samples were again incubated at 39°C in continuous magnetic stirring for 4 hours. After the incubation, Erlenmayers were cooled and 5 mL of sulfosalicylic acid solution were added in order to precipitate proteins. Samples were then poured into filtrating unit (Fibertec Tecator) with distilled water, using glass filters (pore 2). Solid residues were washed 2 times with 10 mL ethanol 96% and acetone, and each wash lasted 3 minutes. Glass filters were dried at 70°C for 18 hours, then weighted for dry weight determination. Residues were then ashed at 500°C for 4 hours and weighted.

Calculations

In vitro organic OMD was calculated with the following formula:

$$\textit{in vitro} \ \text{OMD} = \frac{[\text{Sample (g) x sample OM (gOM/g feed)}] - [(\text{residue+crucible after drying (g)}) - (\text{ashes+crucible (g)})]}{\text{Sample (g) x sample OM (gOM/gfeed)}}$$

Feeds

All food samples were previously tested for their *in vivo* digestibility following the procedure described by Castrillo et al. (2001), and analyzed for their ash, CP, EE and CF content according to AOAC (1985) methods. Gross energy was determined in an adiabatic calorimetric bomb.

In table 1 the proximate analysis and the *in vivo* digestibility of the tested dry canine diets are shown.

Experiment 1

One food sample (see Table 1 for the chemical composition) was incubated either alone or with the addition of 10% of pectin (P9135 Sigma) or cellulose (Sigmacell) in order to evaluate if a precipitation step with ethanol, performed before the filtration of the samples, could significantly help in the recovery of the soluble portion of the indigestible carbohydrates. Ethanol was added in 4:1 ratio with respect to the liquid phase. Samples were incubated in quadruplicate.

Experiment 2

Aiming at verifying if the precipitation step with ethanol could improve the estimation of digestibility of foods rich in fibres, three high fibre-diets (Table 1 shows their chemical composition) were included in this experiment. For each food six samples were analyzed: three samples were incubated following the method of Hervera et al. (2007) whereas three samples were let precipitate 1 hour with ethanol 96% in 4:1 ratio with respect to the incubation liquids before filtering. Samples were then dried overnight at 70°C, weighed, ashed at 500°C for 4 h and weighed again for the *in vitro* OMD determination. Means from the original and modified procedures were compared by a T test .

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Table 1: Chemical composition (g/kg DM) and *in vivo* OMD (%) of the experimental diets

Item	Exp. 1 Food 1	Food1	Exp. 2 Food 2	Food 3	Mean (n=8)	Exp. 3 Range	SD	Food 1	Exp. 4 Food 2	Food 3	Mean (n=4)	Exp. 5 Range	SD
DM (g/kg)	92.4	91.9	940	95.4	91.6	88.7 - 93.9	1.29	92.6	91.7	92.4	92.7	92.1-93.3	0.61
Nutrients (g/kg DM)													
OM	92.3	94.0	92.6	91.6	92.9	91.7 - 93.9	0.69	92.9	92.7	91.5	92.9	91.7-93.6	1.00
CP	26.0	34.0	31.5	32.2	28.0	18.6 - 34.0	3.14	28.3	35.6	28.3	28.9	24.8-39.5	6.78
EE	13.0	9.60	7.29	7.37	14.4	7.77 - 24.1	4.67	16.5	9.26	12.3	16.4	6.21-23.4	6.03
CF	2.08	11.5	8.85	8.66	2.44	0.61 - 9.19	2.30	1.44	2.95	8.20	1.89	1.11-6.75	0.72
NFE	51.2	38.9	44.9	43.4	48.0	37.4 - 63.9	5.51	46.7	52.2	35.4	45.0	29.4-53.8	13.5
OMD (%)	83.8	68.7	70.3	71.4	84.4	72.4 -90.8	5.39	87.9	83.8	72.9	86.7	75.3-88.9	3.20

DM= Dry matter; OM=Organic matter; CP=Crude protein EE=Ether extract; CF=Crude fibre; NFE= Nitrogen free extracts.

Experiment 3

The aim of the present experiment was to verify if the sample incubation in a water-bath instead of a thermostated camera affects the results of the *in vitro* OMD. Eight dry dog foods, previously tested for their *in vitro* OMD by Hervera et al. (2007), were considered. Samples were incubated following theoriginal method (Hervera et al., 2007), except for the equipment used as incubator: the thermostatic camera (Stuart, SI60) equipped with a multipoint magnetic stirrer (ANM 10009 -SBS) was replaced by a thermostatic water-bath (UNITRONIC 320 OR - Selecta, Barcelona, Spain) where the agitation was performed manually, (eight rapid circular movements every 15 minutes), or automatically by a horizontal shaking (70 strokes/minute). All the samples were analyzed in double. Data were analyzed with SAS package (SAS 9.2, SAS institute Inc., Cary, NC, USA) with the GLM procedure, and compared with the *in vitro* results obtained by Hervera et al. (2007). A regression analysis was also performed, in order to establish the relationship between the OMD results obtained with the original incubation method and with WM or WA.

Experiment 4

In this experiment the repeatability (RT) and reproducibility (RD) of the method was tested both when using TA and WM for the samples incubation. Three foods were chosen for their wide variability in chemical composition and *in vivo* OMD (Table 1). Foods were incubated in WM or TA in triplicate, in three different days (batches) of incubation by the same operator. Data were analyzed with SAS package (SAS 9.2, SAS institute Inc., Cary, NC, USA) with the GLM procedure; moreover, variance components of the two incubation conditions were analyzed separately, in order to calculate RD and RT of the methods.

RT, defined as the value below which the absolute difference between two single measures obtained on the same sample under the same conditions is expected to lie with a probability of 95% (International

Organization for Standardization, 1994) was computed according to the following functions of estimated variance components:

$$RT = 2x \sqrt[2]{2} \; \sigma^2_{error}$$

RD, defined as the value below which the absolute difference between two single measures obtained on the same sample under different conditions (different incubation batches) is expected to lie with a probability of 95% (International Organization for Standardization, 1994a, b), was computed according to the following functions of estimated variance components:

$$RD = 2x \sqrt[2]{2} \; \sigma^2_{day} + \sigma^2_{day \; x \; sample} + \sigma^2_{error})$$

4.3 Results and discussions

Experiment 1

In table 2 results of the *in vitro* OMD obtained by applying or not the precipitation step with ethanol are reported. Precipitating samples with ethanol resulted in a higher recovery of the fibre added to the food, leading to a lower *in vitro* OMD estimation, mainly in samples added of pectin.

Assuming that the fibre added to the samples is completely indigestible, the OM digestibility of the samples added of pectin or cellulose should be around 73%. The precipitation with ethanol seems to improve notably the *in vitro* OMD estimation.

Table 2: Mean *in vitro* OMD (%) and SD of the means obtained incubating either samples following the original method or those including a precipitation step with ethanol

| Sample	Precipitation with ethanol	Original method
Food	82.7±0.47	84.3±0.67
Food+10% pectin	71.4±0.99	83.6±0.66
Food +10% cellulose	74.2±0.79	75.5±0.48

Experiment 2

In figure 1 the *in vitro* OMD obtained applying or not the precipitation step with ethanol in 3 foods rich in fibre are shown. The use of ethanol as precipitating agent decreased significantly the *in vitro* OMD values in 2 foods out of the 3 tested, reducing the overestimation of the *in vitro* OMD in comparison to the original method; the variability obtained was however higher when the precipitation step was carried out.

A higher number of samples should be tested in order to demonstrate the effect of the ethanol precipitation.
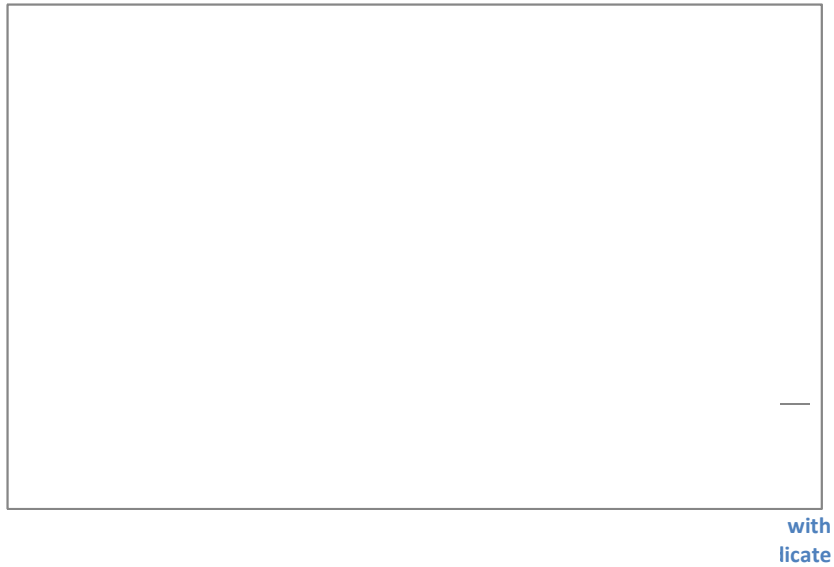
Experiment 3

Figure 2 shows the relationship between *in vitro* OMD obtained incubating samples with TA, WM (Figure 2a) or WA (Figure 2b). The correlation between WM and TA was very high, and the regression coefficient was not different from the unit, although the *in vitro* OMD in WM resulted on average 6% units lower than that obtained using the TA. By contrast, no linear relationship (P>0.05) was observed with the i*n vitro* OMD obtained with TA and using the WA (r^2=0.25). The lack of preciseness of this method is probably due to an insufficient stirring of the sample, which did not allow a proper interaction between enzymes and substrate.

a   b
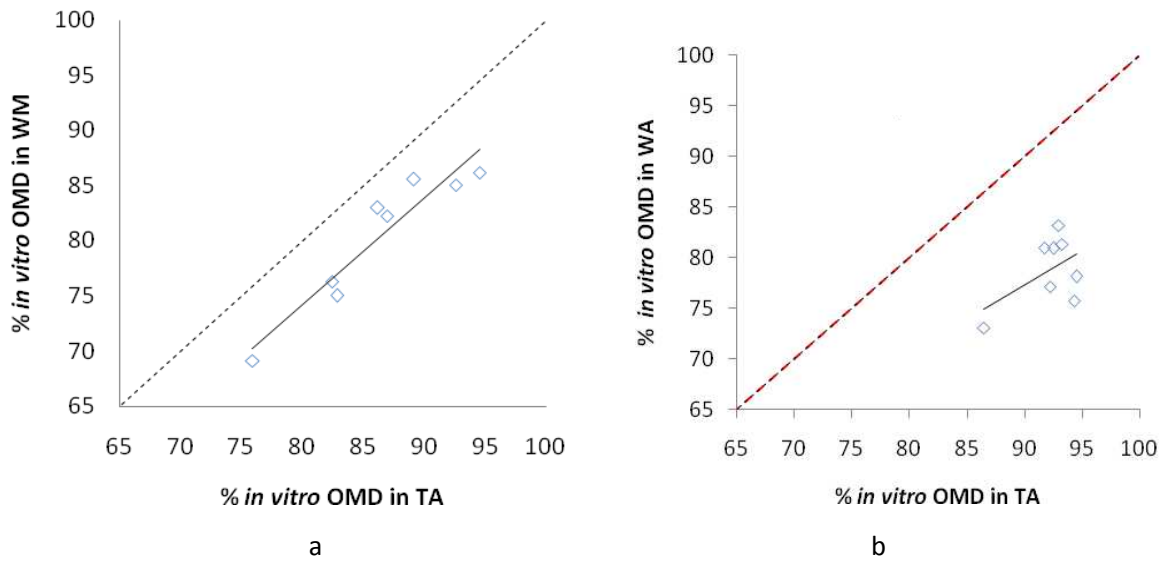
Figure 2: Relationship between the *in vitro* OMD determined in TA and in a) WM (*in vitro* OMD in WM=-3.94(±11.7)+0.98(±0.14)x *in vitro* OMD in TA; r^2=0.90; RSD=2.13; CV=2.66%); b) WA (*in vitro* OMD in WA=16.3(±43.4)+0.68(±0.47)x *in vitro* OMD TA; ; r^2=0.26; RSD=3.14; CV=3.98%)

In figure 3 the relationship between *in vivo* OMD and *in vitro* OMD using the TA (Figure 3a) and the WM (Figure 3b) is shown.

a   b
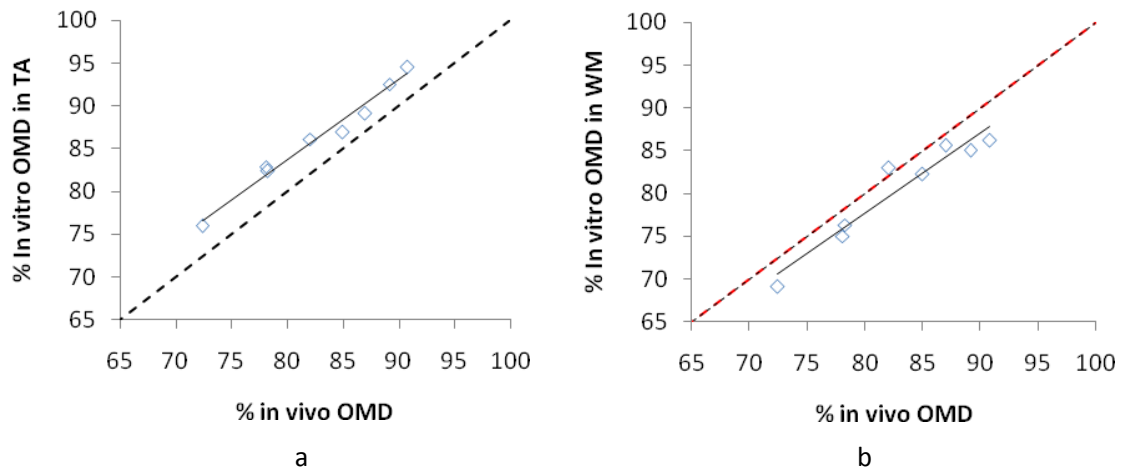
Figure 3 a and b: Relationship between OMD determined *in vivo* and a) *in vitro* in TA (*in vitro* OMD in TA=0.94(±0.06)x *in vivo* OMD + 8.62(±.4.8) , r^2=0.98 RSD=0.96 CV=1.11%); b) *in vitro* in WM (*in vitro* OMD in WM=0.94(±0.1)x *in vivo* OMD + 2.46(±9.1) r^2=0.92 RSD=1.83 CV=2.28%.

In both cases slopes were not significantly different from the unit and the ordinates in the origin were not different form zero. The *in vitro* OMD determined in TA overestimated on average 3.5- p.u. the *in vivo* OMD, whereas the *in vitro* WM underestimated on average 2.5 p.u. the *in vivo* values.. The *in vitro* OMD data obtained using the TA were more straightly correlated with the *in vivo* data (r=0.99 vs 0.96) and the regression equation showed a slightly lower coefficient of variation (1.11% vs 2.28%). Authors excluded the use of the automatic water-bath as alternative equipment for the estimation of *in vitro* OMD, and decided to test the reproducibility and repeatability only of the water-bath with a manual agitation in comparison to the thermostatically-controlled heating chamber equipped with the magnetic agitation.

Experiment 4

In table 3 the descriptive statistics of the *in vitro* OMD, sorted by food sample and incubation method are reported.

Table 3: Descriptive statistics of the *in vitro* OMD obtained with the two different incubation methods, separated for tested food

	Food 1		Food 2		Food 3	
	WM	TA	WM	TA	WM	TA
Mean (n=9)	87.14	91.66	81.68	84.68	73.21	75.58
SD	0.9	0.56	0.64	0.83	1.21	0.69
CV,%	1.03	0.61	0.78	0.98	1.65	0.91
Min	85.75	90.75	80.74	82.83	70.61	74.80
Max	88.35	92.51	82.43	85.39	74.66	76.93

As in experiment 3, the *in vitro* OMD resulted higher when the TA was used as incubation system. Differences between TA and WM are probably due to the continuous stirring, assured by the magnetic agitator, which does not allow the deposit of the solid particles, and allows the dispersed enzymes to easily get in contact with the substrate.

Values obtained in WM (Table 3) were closer to those obtained *in vivo* for foods 1 and 2 (Table 1). It is worth to note that the coefficient of variation (CV) was very low in both methods, indicating a substantial efficiency of the equipments employed. The samples, the two methods used and the day of incubation contributed for 91.0, 6.5 and 0.7% of the total variability, respectively.

The RD calculated for TA was 2.07, while a slightly higher value, 2.93, was obtained for WM. The RT values obtained were also comparable (1.70 for TA and 1.68 for WM). These values led the author to assert that the methods are similarly repeatable and reproducible.

4.4 Conclusions

The precipitation step with ethanol showed an improvement in the *in vitro* estimation of the dog feed digestibility, that however should be confirmed by a higher number of samples.

Among the 3 methods of sample incubation tested, the water-bath with automatic agitation seemed not applicable for the *in vitro* OMD at the conditions adopted in the present experiment. The use of the water-bath with manual agitation, on the contrary, gave comparable results with those obtained with the thermostated camera and the magnetic stirring. Briefly, the estimation of OMD can be performed with both the thermostated camera and the magnetic stirring obtaining the same accuracy, whereas a general underestimation of the *in vitro* OMD values has been observed using the water-bath with manual agitation. The equipment adopted in the original procedure can be replaced by the water-bath with manual agitation, and this makes the method for the *in vitro* OMD estimation reproducible in any simply-equipped laboratory.

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