Alma Mater Studiorum – Università di Bologna

Dottorato di Ricerca in:

Scienze della Nutrizione e degli alimenti – Feed and Food Science

Ciclo XX

Settore scientifico disciplinare: AGR 18

Pet food: quality and quality improvement

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Esame finale anno 2008

Abstract

Today's pet food industry is growing rapidly, with pet owners demanding high-quality diets for their pets. The primary role of diet is to provide enough nutrients to meet metabolic requirements, while giving the consumer a feeling of well-being. Diet nutrient composition and digestibility are of crucial importance for health and well being of animals. A recent strategy to improve the quality of food is the use of "nutraceuticals" or "Functional foods". At the moment, probiotics and prebiotics are among the most studied and frequently used functional food compounds in pet foods.

The present thesis reported results from three different studies.

The first study aimed to develop a simple laboratory method to predict pet foods digestibility. The developed method was based on the two-step multi-enzymatic incubation assay described by Vervaeke *et al.* (1989), with some modification in order to better represent the digestive physiology of dogs. A trial was then conducted to compare *in vivo* digestibility of pet-foods and *in vitro* digestibility using the newly developed method. Correlation coefficients showed a close correlation between digestibility data of total dry matter and crude protein obtained with *in vivo* and *in vitro* methods (0.9976 and 0.9957, respectively). Ether extract presented a lower correlation coefficient, although close to 1 (0.9098). Based on the present results, the new method could be considered as an alternative system of evaluation of dog foods digestibility, reducing the need for using experimental animals in digestibility trials.

The second parte of the study aimed to isolate from dog faeces a *Lactobacillus* strain capable of exert a probiotic effect on dog intestinal microflora. A *L. animalis* strain was isolated from the faeces of 17 adult healthy dogs..The isolated strain was first studied in vitro when it was added to a canine faecal inoculum (at a final concentration of 6 Log CFU/mL) that was incubated in anaerobic serum bottles and syringes which simulated the large intestine of dogs. Samples of fermentation fluid were collected at 0, 4, 8, and 24 hours for analysis (ammonia, SCFA, pH, lactobacilli, enterococci, coliforms, clostridia). Consequently, the *L. animalis* strain was fed to nine dogs having lactobacilli counts lower than 4.5 Log CFU per g of faeces. The study indicated that the *L animalis* strain was able to survive gastrointestinal passage and transitorily colonize the dog intestine. Both *in vitro* and *in vivo* results showed that the *L. animalis* strain positively influenced composition and metabolism of the intestinal microflora of dogs.

The third trail investigated *in vitro* the effects of several non-digestible oligosaccharides (NDO) on dog intestinal microflora composition and metabolism. Substrates were fermented using a canine faecal inoculum that was incubated in anaerobic serum bottles and syringes.

Substrates were added at the final concentration of 1g/L (inulin, FOS, pectin, lactitol, gluconic acid) or 4g/L (chicory). Samples of fermentation fluid were collected at 0, 6, and 24 hours for analysis (ammonia, SCFA, pH, lactobacilli, enterococci, coliforms). Gas production was measured throughout the 24 h of the study. Among the tested NDO lactitol showed the best prebiotic properties. In fact, it reduced coliforms and increased lactobacilli counts, enhanced microbial fermentation and promoted the production of SCFA while decreasing BCFA. All the substrates that were investigated showed one or more positive effects on dog faecal microflora metabolism or composition. Further studies (in particular *in vivo* studies with dogs) will be needed to confirm the prebiotic properties of lactitol and evaluate its optimal level of inclusion in the diet.

1. Introduction

Today's pet food industry is growing rapidly, with pet owners demanding high-quality diets for their pets. This demand is creating a search for new strategies to improve pet foods quality and/or the health status of the animal through the diet.

In Italy, more than 3 million dogs are registered in the "Anagrafe Canina Nazionale" data bank (Anagrafe Canina Nazionale, march 2008), and the estimated real number of dogs present is close to 7 million (Zoomark, 2005). In 2004, 454 million of euro were spent in Italy for commercial foods for dogs (Zoomark, 2005). Nowadays, pets are kept as part of the family and thus pet owners feel responsible for their quality of life and longevity.

The primary role of diet is to provide enough nutrients to meet metabolic requirements, while giving the consumer a feeling of well-being. Recent knowledge, however, supports the hypothesis that, beyond meeting nutritional needs, diet may modulate various functions in the body and play detrimental or beneficial roles in some diseases. Concepts in nutrition are expanding to include an emphasis on the use of foods to promote a state of well-being and better health and to help to reduce the risk of diseases.

Diet nutrient composition and digestibility are of crucial importance for health and well being of animals. Although great attention is paid to nutritional quality in the marketing of dog foods there is usually limited information on digestibility. The most highly recognized dog food brands claim to have optimum nutritional quality and high digestibility, without or with few scientific data and no controlled trial to support their statements and claims. The pet food industry traditionally uses a wide range of protein sources, including meat and bone meals, poultry meals, poultry by-product meals, and soybean meal. Significant variation in the nutritional quality of ingredients directly affects the nutritional value of the finished product. Moreover, processing of foods can influence the availability of nutrients, either positively or negatively.

A recent strategy to improve the quality of food is the use of "nutraceuticals" or "Functional foods". Nutraceutical (a term coined by the fusion of nutrition and pharmaceutical) refers to extracts of foods claimed to have a medicinal effect, while a functional food is a part of an everyday diet which is demonstrated to offer health benefits and reduce the risk of chronic disease beyond the widely accepted nutritional effects. The term 'functional foods' was introduced in Japan in mid 1980s. This type of foods is known on the Japanese market as "FOods for Specified Health Use" (FOSHU). The functional foods comprise: conventional foods containing naturally occurring bioactive substances

(e.g., dietary fiber), foods enriched with bioactive substances (e.g., probiotics, antioxidants), and synthesized food ingredients introduced to traditional foods (e.g., prebiotics).

Among the functional components, probiotics and prebiotics, soluble fiber, omega-3 – polyunsaturated fatty acids, conjugated linoleic acid, plant antioxidants, vitamins and minerals, some proteins, peptides and amino acids, as well as phospholipids are frequently mentioned. At the moment, the most studied and frequently used functional food compounds in pet foods are probiotics, prebiotics, plant antioxidants and vitamins.

2. The digestive tract of dogs

It is well known that a close relationship exists between gastro-intestinal characteristics, natural feral diet and nutrient requirements. The gastro-intestinal morphology and physiology are greatly influenced by nature of food consumed, frequency of meals, body size, and several other factors. Dogs are omnivorous, derived from carnivorous ancestors.

Compared to herbivorous and "earlier" omnivorous species, dog digestive tract is relatively short and simple. The average ratio of body length to intestine length of carnivorous is 1:6 and 1:4, for dog and cat respectively, compared to an average ratio of 1:22 for ruminants and of 1:14 for swine (Stevens, 1977). Table 2.1 shows the principal measures that characterise the dog gastro-intestinal tract.

Table 2.1: Organ volumes and length in the dog (Stevens, 1977)

Region	**Measures**	
	Relative length (%)	Average absolute length (m)
Small intestine	85	4.14
Cecum	2	0.08
Colon	13	0.60
Total	100	4.82
	Relative volume (%)	Average absolute volume (L)
Stomach	62.3	4.33
Small intestine	23.3	1.62
Cecum	1.3	0.09
Colon	13.1	0.91
Total	100	6.95

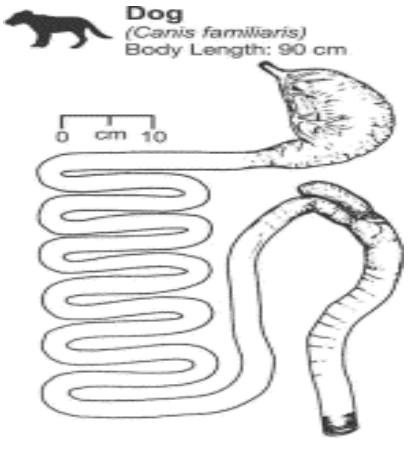


Figure 2.1: Dog gastro-intestinal tract (Stevens, 1977)

The digestive process begins in the mouth. The first step of digestion is the secretion of saliva during mastication by four pairs of salivary glands. The amount and composition of the saliva secreted depends on the type of food ingested (particularly the water content). Saliva consists of about 99% water, the remaining 1% is made of

mucus, inorganic salts (calcium, chloride, potassium, sodium and bicarbonate) and enzymes (Maskell & Johnson, 1993). In dog saliva there isn't the starch digestive enzyme α-amylase.

2.1 Stomach

The stomach controls the rate of entry of ingesta in the small intestine. This fact is very important in dogs who tend to eat large meals. The stomach participates in the initial stages of digestion by secreting hydrochloric acid and pepsinogen. Electrolyte concentrations in the stomach reported in literature vary widely, ranges of reported values are shown in Table 2.2.

Table 2.2: Electrolyte composition of gastric juice in dogs (Altman & Dittmer; 1968).

Electrolyte	**Range (mmol/L)**
Bicarbonate	5-33
Potassium	7-28
Sodium	22-155
Chloride	123-173
Calcium	0.5-4
Phosphate	0.026-12
Magnesium	0.021

The major enzymes secreted in the lumen of the stomach are gastric lipase and pepsin. Gastric lipase, in contrast to pancreatic lipase, is characterized by his high stability and high level of activity under acidic pH condition (Carrière *et al.*, 1991). The secretion of gastric lipase is stimulated by food ingestion. During the peak output, which happens during the first hour after meal, gastric lipase secretion is three times higher than the basal secretion rate, with a total output three hours after meal of about 7 mg (Carrière et al., 1993). Pepsin range of secretion vary widely among individuals. Pepsin displays optimal activity at pH 2, maintained by gastric secretion of hydrochloric acid; its proteolytic activity decreases along the small intestine and is completely inactivated at neutral pH.

Gastric acid secretion data in dogs are reported in Table 2.3 (Dressman & Yamada, 1991). In dogs, the gastric acid secretion rate at the basal state is low. Therefore, the stomach pH can be as high as the duodenal one in the fasted state. Gastric secretion is

influenced by the amount of protein in the meal (Carpentier et al., 1977), hormones and the nervous system.

Table 2.4 lists the pH values of different sections of dog gastro-intestinal tract. In the stomach, the cardiac region has a higher pH value then the pyloric region, since the parietal cells (which secrete hydrochloric acid) tend to be localized in the lower part of the stomach. In the small intestine, pH becomes progressively more alkaline in the distal portions. In the large intestine, pH values are more acidic due to microbial fermentation.

Table 2.3: Gastric acid secretion data in dogs (Dressman & Yamada, 1991).

Parameter		
Basal acid output (BAO)		
volume	mL/min	0.3-1.5
rate	mEq/h	0.1
Peak acid output (PAO)		
rate	mEq/h	39
pH		
fasted		1.5
fed		2.1

Table 2.4: pH values of different parts of the alimentary tract in dogs (Smith, 1965)

Stomach		**Small intestine**						
Anterior	**Posterior**	**a**	**b**	**c**	**d**	**Cecum**	**Colon**	**Faeces**
5.5	3.4	6.2	6.2	6.6	7.5	6.4	6.5	6.2

Gastric emptying is the process by which food is delivered to the small intestine at a rate and in a form that optimizes intestinal absorption of nutrients. Physiological data reported in literature about gastric emptying in dogs comprise a wide range of times, ranging from 66 minutes to 29 hours (Wyse et al., 2003). Rates of gastric emptying are influenced by many factors related both to animal and diet characteristics. Weber et al. (2001) reported a significant positive correlation between gastric emptying time and body weight, with a lower gastric retention time of foods in giant breeds. Foods can affect ranges depending on their volume, energy content, viscosity, density, and particle size (Mizuta *et al.*, 1990; Papasouliotis *et al*., 1993; Chalmers *et al.*, 2005; Xu *et al*., 2005).

2.2 Small intestine

In dogs, the small intestine is the major site for the digestion and absorption of nutrients. Transit of acid chyme from the stomach into the small intestine stimulates the secretion of pancreatic juice into the duodenum. Bicarbonates present in the pancreatic juice and bile neutralise the acidic pH of digesta. In the duodenum, chyme is mixed with enzymes secreted by the exocrine pancreas and the duodenal mucosa. Pancreatic enzymes include inactive proteases, lipases, and amylases. The average composition of dog pancreatic juice is shown in Table 2.5 (Altman & Dittmer; 1968). The range of values is very wide because several factors affect electrolytes and enzymes secretion, as, for example, meal composition (Fink *et al*., 1982; Fink *et al.*, 1983).

Table 2.5: Composition of dog pancreatic juice (Altman & Dittmer; 1968).

		Value
pH		7.1 - 8.2
Secretion rate	mL/min	0.2 - 1.1
Water content	%	98
Ash content	g/L	8.4 - 9.7
Bicarbonate	mmol/L	93 - 143
Total nitrogen	mmol/L	71.4 - 671.4

Besides food, hormones can stimulate the exocrine secretion of the pancreas: secretin and cholecystochinin, produced by cells of the intestinal mucosa, regulate the output of pancreatic juice. Secretin stimulates the pancreas to increase bicarbonate secretion. Secretin release is caused by the acidity of small intestinal contents. Cholecystochinin stimulates the release of enzyme-rich juices and is stimulated by the presence of partially digested food in the small intestine (Maskell & Johnson, 1993).

Table 2.6: Composition of bile secreted from the gallbladder and from the liver of dogs. Values are express in g/L. (Altman & Dittmer, 1968)

	Gallbladder	Liver
pH	5.2 - 7.0	7.1 - 8.5
Dry matter	114 - 246	23 - 45
Salts	79 - 150	5 - 24
Cholesterol	0.8 - 1.4	0.04 - 0.15

The importance of bile fluid in the digestion and absorption of dietary lipids has long been recognized. Bile is continuously produced in the liver and stored, between meals, in the gallbladder in a concentrate form. Bile from the gallbladder differs in concentration from bile secreted directly from the liver (Table 2.6). In dogs, more than 99% of bile acids are coniugated with taurine (Wildgrube *et al.*, 1986; Washizu *et al.*, 1990).

The gallbladder contracts in response to food ingestion. Emptying peaks are found at 30 min after a meal and the emptying decreases 2 hours after food ingestion; gallbladder empties only partially after a meal (5-65%). Half-emptying time has been reported to be approximately 47 min (Junderko *et al*., 1994). In Table 2.7, the rate of bile flow and bile composition are given.

Table 2.7: Rate of bile flow and bile composition (Ehrlinger, 1987; Kararli, 1995).

Parameter		**Range**
Bile flow	mL/die/kg	19-36
Total bile salts (TBS) rate	mmol/die/kg	1.6-2.9
TBS	mmol/L	40-90
Na	mEq/L	141-230
K	mEq/L	4.5-11.9
Ca	mEq/L	3.1-13.8
Mg	mEq/L	2.2-5.5
Cl	mEq/L	31-107
HCO_3	mEq/L	14-61

2.3 Large intestine

The last section of the gastrointestinal tract involved in the digestion process is the large intestine. The primary role of the large intestine is to absorb electrolytes and water and serve as an environment for microbial fermentation of nutrients that escape digestion and absorption by the small intestine. The colon represents the majority of the large intestine. The large intestine mucosa has no villi and is covered by an alkaline mucus whose function is to protect the large intestine mucosa from mechanical and chemical injuries (Maskell & Johnson, 1993). Large intestinal transit time in dogs lasts approximately 12 hours (Maskell & Johnson, 1993). Weber *et al.* (2002) studied the influence of body size on intestinal transit time in dogs. Despite the relatively lower mass of the gastrointestinal tract found in large breed dogs compared to small breed ones (3-4% vs 7-8%; Meyer *et al.*, 1993), Weber *et al.* (2002) found no direct correlation between body size and oro-cecal intestinal transit time, while Hernot *et al.* (2006) demonstrated a positive correlation between large intestinal transit time and body size. The efficiency of absorption of salts and water is dependent, to a large extent, on colonic motility. Rolfe *et al.* (2002) demonstrated that a reduction in large intestinal transit time decreases the capacity for electrolyte and water absorption and results in elimination of watery faeces. On the contrary, longer large intestinal transit time promotes colonic fermentation, which has a positive impact on faecal quality (Macfarlane & Macfarlane, 2003).

The large intestine in dogs is responsible for only about 8% of the total digestion of food (Drochner & Meyer, 1991), although this percentage is affected by the diet. Meyer & Schunemann (1989) reported that colonic digestibility accounted for 1 to 4% of total digestibility when dogs were fed highly digestible diets, whereas with diets containing certain types of fiber colonic digestibility ranged from 12 to 24% of total digestibility. Nutrient digestion in the large intestine is made by colonic bacteria, which ferment dietary nutrients and endogenous secretions that escape digestion and absorption in the small intestine.

One of the fundamental properties of mucosal epithelia is their ability to directly utilize ‘topical’ nutrients, derived from the diet or the digestion of food, without reliance on the blood flow. By the time the digesta reach the colon, however, over 90% of protein and carbohydrate has been absorbed, and all that is left is fiber and ‘resistant’ starch and protein. Although the gut does not secrete enzymes that are capable of digesting these residues, the colonic microbiota does, and in an excellent example of symbiosis, the bacteria metabolize

the residues to SCFAs and gases, such as hydrogen and methane, which are chiefly absorbed and excreted via the lungs (O'Keefe, 2008).

The primary end products of bacterial fermentation are SCFAs, lactate, carbon dioxide, and hydrogen. Other fermentative end products include hydrogen sulfite, methane, ammonia, branched-chain fatty acids, amines, phenols, and indoles. The relative proportion of these compounds is influenced by colonic microflora composition, metabolic interactions among bacteria, nutrients available for fermentation, intestinal transit time, and a variety of host factors including age and immune status (Cummings & Macfarlane, 1991).

2.4 Colonic microbiota

Indigenous intestinal microorganisms play several significant roles in host health because they aid in the digestion of food, metabolize drugs and foreign compounds, produce essential vitamins, and help prevent pathogens from colonizing the gastrointestinal tract (March, 1979; Shanahan, 2002)

Quantitative and qualitative knowledge of the structure of the bacterial community in the intestinal tract is essential to understand the impact on health status of the host. Up to the present time, few works exist which describe the intestinal microbiota of dogs (Fujisawa & Mitsuoka, 1996; Greetham *et al*., 2002; Simpson *et al*., 2002; Mentula *et al*., 2005; Sochodolski *et al*., 2005; Beasley *et al.*, 2006; Kim & Adachi, 2007). In addition, many of these studies are focalized on a restricted number of bacterial species, such as lactic acid bacteria.

The colon contents of dog support at least 400 different species, with numbers as high as 10^{10} and 10^{11} viable bacteria/g of digesta (Davis *et al*, 1977). Bacterial counts reported in Table XX are extrapolated from data by Simpson *et al*. (2002). The main cultivable bacterial groups in dogs include clostridia, *Bacteroides*, streptococci, coliforms, enterococci and lactobacilli with increasing counts towards the large intestine (Davis *et al.*, 1977; Greetham *et al.*, 2002, Buddington, 2003). In their study, Sochodolski *et al.* (2005) detected a wide variability between bacterial microflora counts of dogs housed in an identical environment and fed with the same diet and they concluded that individual variability plays a major role in the composition of the intestinal microbiota. Mentula *et al* (2005), in contrast with other studies (Greetham *et al*., 2002; Buddington, 2003) where no bifidobacteria but numerous *Lactobacillus* organisms were reported, found bifidobacteria in 64% but lactobacilli only in 32% of the dogs screened.

In their study, Simpson *et al*. (2002) stated that each individual dog harbours a characteristic faecal bacterial community which is not influenced by the diet. This statement is in opposition to what is reported in others studies in which the authors observed a direct influence of diet on bacterial population in the gastrointestinal tract (Zentek, 1995a; Zentek, 1995b; Vanhoutte *et al*., 2005; Flickinger *et al*., 2003).

Table 2.8: Counts of viable bacteria in canine faecal samples (from Simpson *et al*., 2002).

Microbial group	**Counts** **Log CFU/g faeces**
Enterococci	6.91
Streptococci	8.77
Staphylococci	3.83
Bacteroides	10.05
Fusobacteria	8.67
Clostridia	6.96
Bifidobacteria	7.80
Eubacteria	8.11
Lactobacilli	9.38
Total anaerobes	10.62
Total aerobes	9.28
Yeast and moulds	2.23

3. Diet digestibility

Digestibility values provide information on the relative amounts of nutrients in the diet that can be really used by the animal and, additionally, serve as an index of overall quality of the ingredients of the diet.

In order to calculate nutrient digestibility, it is important to quantify the exact amount of nutrient consumed by the animal and the amount that is excreted in the faeces. The difference between these two quantities, divided by the amount consumed, represents the quantity that has been digested. The digestibility coefficient that is obtained with this method is an "apparent" rather than a "true" value. In fact, faeces contain a variable quantity of nutrients of non-dietary origin such as enzymes, pancreatic juice, bile, mucus, sloughed intestinal cells, and bacteria (Phillipson, 1971). Several studies have been conducted to quantify endogenous secretions using nitrogen-free diets or diets containing only low amounts of a highly digestible protein (e.g. casein), or feeding graded levels of a nutrient with extrapolation to zero intake (Hendriks *et al.*, 2002; Kendall *et al*., 1982).

Average digestibility coefficients in dogs reported in literature are shown in Table 3.1. Values are means calculated on the basis of values reported by Vhile *et al*., 2007; Guevara *et al*., 2008; Kempe *et al*., 2007; Yamba *et al*., 2006; Dust *et al.*, 2005.

Table 3.1: Average digestibility coefficients in dogs reported in literature (Vhile *et al*., 2007; Guevara *et al*., 2008; Kempe *et al*., 2007; Yamba *et al*., 2006; Dust *et al.*, 2005)

	Digestibility coefficients
Dry matter	82.3 ± 5.17
Crude protein	82.2 ± 4.50
Ether extract	92.8 ± 2.60
Starch	98.6 ± 2.24

3.1 Methods to evaluate diet digestibility

Diet digestibility can be evaluated using various methods. The use of metabolic cages, which allows a complete collection of excreta, is the oldest technique developed. However, housing in cages may influence digestive processes of the animals and the results obtained may be different when animals are kept in a normal environment (Sales & Janssens, 2003). The use of indigestible markers is an established method for determining digestibility without total collection of faeces. An inert marker must satisfy several criteria; it must be indigestible and show only little or no interaction at all with the digestive process. Furthermore, homogenous incorporation of the marker in the feed should be possible and the marker should be harmless to experimental animals, people who work with the substance and the environment (Sales & Janssens, 2003). Indigestible markers that are commonly used in digestibility studies include chromic oxide (Cr_2O_3) (Zuo *et al*., 1996; Hendriks & Sritharan, 2002; Guevara *et al*., 2008), yttrium oxide (Y_2O_3) (Vhile *et al*., 2007) or insoluble ash (celite) (Scott & Boldaji, 1997).

Nowadays, *in vitro* digestion techniques are gaining interest because *in vivo* determinations are both time consuming (about three weeks are required for the trial and analysis of the samples) and expensive (due to the cost of the dogs, the diets, the kennels and the labour). Furthermore, in Europe, the use of dogs as experimental animals is a source of great concern for most pet owners (and pet-food producers generally avoid to be involved in *in vivo* trials with dogs). Tonglet *et al*. (2001) tried to correlate *in vivo* and *in vitro* protein digestibility data obtained using the three-enzymes procedure described by Dufour-Etienne *et al*. (1992). They analyzed seventeen dry complete industrial dog foods and obtained a correlation coefficient (r^2) of 0.71 between in vitro and in vivo protein digestibility. r^2 represent the fraction of the variance between two parameters that is “shared”, and a value close to one describe two parameters that vary together. In this study digestibility coefficients determined *in vitro* explained only a 71% of the variation of *in vivo* ones. In a recent study, Hervera *et al.* (2007) tried to develop a simple and reproducible *in vitro* method for predicting the apparent energy digestibility of dry extruded dog foods. Their method was based on the two step multy-enzymatic incubation assay described by Boisen (1991). They analyzed 54 dry extruded commercial dog foods and obtained a coefficient of correlation (r^2) of 0.92 between *in vitro* and *in vivo* organic matter disappearance. This degree of correlation indicates that the proposed method could be effectively used to predict

in vivo protein digestibility with an *in vitro* system; however, other feed components are not considered using this technique.

3.2 Factors affecting diet digestibility

Many factors can affect diet digestibility, such as ingredient sources, absolute nutrient concentrations, and food processing. In a recent study, Zentek *et al.* (2004) investigated the effect of two different dietary protein sources (beef and poultry), included either in an extruded or a canned mixed diet, fed to dogs. Poultry-based and extruded diets were associated to higher digestibility coefficients than the beef-based and canned ones. Thermal processes are known to improve starch digestibility, in particular extrusion (Perez-Navarrete et al., 2007; Murray et al., 2001). In dry extruded pet foods cereal grains are a commonly used ingredient because there is a stable supply and are a relatively inexpensive source of nutrients. Dogs diets may contain up to 50% starch, derived from cereal grains (almost 60% in dry diets). Therefore, the thermal treatment could notably affect diet digestibility. In addition, also animal factors must be considered when evaluating digestibility. These include breed, age, gender, activity level, and physiological state. With regard to the effects of breed, Weber *et al*. (2003) evaluated the effects of age and body size on the apparent digestibility of a dry expanded diet. Four breeds of different body size were used (miniature poodles, medium schnauzers, giant schnauzers, great danes) and digestibility experiments were conducted at four ages (11, 21, 35 and 60 weeks). Nutrient digestibility was significantly higher in large dogs at each age, even though these dogs had lower faecal scores and increased faecal moisture concentrations.

Age too is a factor impacting nutrient digestibility. In the study by Weber *et al*. (2003), macronutrient digestibility increased significantly with age in all four dog breeds. A similar finding was reported by Swanson *et al.* (2004), in a study conducted with senior and weanling dogs to determine the effects of age and diet on nutrient digestibility.

Ahlstrøm *et al.* (2006) investigated the effect of moderate exercise or low activity on nutrient digestibility in trained hunting dogs. Digestibility values were similar in the high and low activity periods for all the nutrients.

4. Probiotics

A probiotic was defined by Fuller (1989) as a “live microbial food supplement which beneficially affects the host by improving the intestinal microbial balance”. The most studied probiotics belong to the genera lactobacilli and bifidobacteria, although bacteria belonging to other genera (e.g. enterococci) have also been used. Recently, there has been a move towards the use of probiotics in the petfood market, where animal wellbeing is a major concern.

Based on the definition of probiotics stated above, it is clear that adequate numbers of viable organisms must reach the intestinal tract. For this to happen, probiotic organisms must be able to survive transit through the acidic environment of the stomach and resist digestion by bile. Potential probiotics must possess a variety of other properties, including the ability to adhere to intestinal epithelial cells (or mucus), colonize the intestinal tract, produce antimicrobial factors, and inhibit enteric pathogens (Gibson & Fuller, 2000). Other properties, such as immunomodulation (Sauter *et al.*, 2006 e 2005) and modulation of metabolic activities (Strompfová *et al.*, 2006) are also desirable. An organism can only be considered to be a probiotic after these properties have been identified and a positive health effect has been documented.

One important criterion for the selection of a probiotic is host species specificity, which is regarded as a prerequisite for showing the beneficial characteristics of the probiotic (Fuller, 1989). However, most of the commercial probiotic strains for dogs do not have a canine origin. In a recent study, Rinkinen *et al*. (2003b) utilized an *in vitro* mucus adhesion model to demonstrate that lactic acid bacteria mucus adhesion properties are not host specific but rather are characteristic to bacterial specie. A similar finding was reported by Lauková *et al.* (2004), who tested the adhesion properties of two *Enterococcus* strains to human, porcine, and canine mucus.

Many canine probiotic products contain *Enterococcus faecium*, whose safety has been questioned due to its antibiotic resistance genes and pathogenic characteristics (Strompfová *et al.*, 2004; Rinkinen *et al*., 2003a). Interest in probiotic strains has led to recent cultural studies directed towards the isolation of lactobacilli from dog faeces. Perelmuter *et al*. (2008, in press) isolated a *Lactobacillus murinus* strain from dog faeces and evaluated its possible use as probiotic for dogs with *in vitro* trials. The isolated strain demonstrated probiotic properties. In fact, it was able to survive to different pH and bile salts conditions, to adhere to intestinal mucus and to inhibit the *in vitro* growth of *E. coli* and *C. perfringens*.

In another study, McCoy & Gilliland (2007) compared several *Lactobacillus* specie in order to evaluate their possible use as probiotics. Their study showed that *Lactobacillus reuteri* could be used as a probiotic for dogs. Similar studies were performed by other researchers (Manninen *et al* ., 2006; Beasley *et al*., 2006; Strompfová *et al*., 2006; Strompfová *et al*., 2004) and led to the identification of various lactic acid bacteria of canine origin that could be used as probiotics in dogs.

4.1 Beneficial documented health effects of probiotics in dogs

Appealing properties of probiotics include their ability to reduce antibiotic use, the apparently high index of safety, and the public positive perception about "natural" or "alternative" therapies. Probiotics are classified, and generally regarded as safe, as opposed to antibiotics, which have a number of recognized adverse effects.

Competitive exclusion of pathogens in the gastrointestinal tract is thought to be one of the most important beneficial mechanisms of probiotic bacteria. Competitive exclusion by intestinal bacteria is based on bacteria-to-bacteria interaction mediated by competition for available nutrients and mucosal adhesion sites. In order to gain a competitive advantage, bacteria can also modify their environment to make it less suitable for their competitors. The production of antimicrobial substances, such as lactic acid or bacteriocins, is one example of this kind of environmental modification (Fooks & Gibson, 2002).

The possible effects of lactic acid bacteria on dogs' health have not been extensively examined, although some lactic acid bacteria strains have been documented to have beneficial effects on the health of dogs. Pasupathy and co-workers (2001) evaluated the effect of a *Lactobacillus acidophilus* strain supplementation on food digestibility and growth parameters of puppies. They concluded that the supplementation had a positive effect during the active growth phase, although differences between the control group and the treated one were not significant. In a later work, Benyacoub *et al*. (2003) demonstrated that dietary supplementation of the diet of puppies with *Enterococcus faecium* enhanced specific immune function.

Probiotic lactic acid bacteria were also tested to verify their ability to improve health status of dogs with gastrointestinal diseases. Sauter *et al.* (2006) tested the beneficial effect of a probiotic cocktail administered to dogs with food responsive diarrhoea. At the end of the trial, all the dogs receiving the probiotic supplementation clinically improved. In a previous work, Strompfová *et al.* (2004) detected a reduction in the level of serum

cholesterol and alanine aminotranferase after oral administration of a *Lactobacillus* strain to dogs suffering from diseases of the gastrointestinal tract.

Several researchers investigated the effects of the administration of a probiotic strain on the composition of dog intestinal microbiota. The administration of *Enterococcus faecium* significantly decreased *Sthaphylococcus* spp., Pseudomonas-like bacteria (Marcinaková *et al*., 2006) and *Clostridium* spp. (Vahjen & Männer, 2003), while it increased *Salmonella* spp. and *Campylobacter* spp. counts (Vahjen & Männer, 2003) in dogs faeces. Sauter *et al.* (2006) evaluated the effects of a probiotic cocktail containing three different *Lactobacillus* spp. strains on the intestinal microbiota of dogs with food responsive diarrhoea. They detected, during the treatment, a decrease in numbers of Enterobacteriaceae and an increase in numbers of *Lactobacillus* spp..

4.2 Probiotics in pet foods

Nowadays, the pet market offers several probiotic products for use in dogs. They are available in tablet, capsule, paste, and liquid form. Some commercial dog foods also claim to contain probiotics.

Biourge et al. (1998) evaluated the feasibility of including a probiotic strain in dry dog food during the different phases of the productive process (before and after extrusion) and its stability in the final product. A probiotic preparation (spores of *Bacillus* CIP 5832) was added to the meal of a commercial diet before expansion-extrusion or to a powder that was coated on the diet after extrusion and drying. As expected, the extrusion process resulted in the loss of more than 99% of the bacteria added, while the second technique determined losses of about 45% of the added dose. After 12 months of storage, diets prepared with the second technique had lost less than 25% of spores. These studies confirmed that the addiction of a probiotic strain to a dry dog food is feasible and that it has to be added after the extrusion process at a higher concentration than the desired one.

Rules regarding probiotic supplementation to animals diets are still missing. In a study by Weese & Arroyo (2003), who evaluated several commercial foods for dogs that claimed to contain probiotics, 26% of the products did not contain any relevant bacterial population (among the ones specified in the label), none of the tested products contained all claimed strains and 58% of the tested diets contained additional, related bacteria that were not stated in the label.

5. Prebiotics

A prebiotic is "a non digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health", as it was first defined by Gibson & Roberfroid (1995).

Since its introduction, the concept of prebiotic has attracted much attention. However, many food components have been claimed to exert prebiotic activity without any consideration to the criteria required. In fact, not all dietary carbohydrates are prebiotics. To be classified as a prebiotic a food component has to respect some criteria, such as:

1. resistance to digestion
2. fermentation by intestinal microflora
3. selective stimulation of the growth and/or activity of those intestinal bacteria that contribute to health and well-being.

Recently, the beneficial effects of prebiotics have gained interest also in companion animals. Still, little is known at present about the effect that prebiotics can have in the intestine of carnivorous animals.

Targets for prebiotic effects include the colonic microflora, gastrointestinal physiology, immune function, bioavailability of minerals, lipid metabolism and gastrointestinal tract health (Roberfroid, 1999).

The main classes of dietary carbohydrates and their physiological characteristics are reported in Table 5.1 and 5.2, respectively.

Table 5.1: Principal physiological characteristics of dietary carbohydrates (Cummings & Stephen, 2007).

	Provide energy	***Increase satiety***	***Cholesterol lowering***	***Increase calcium absorption***	***Source of SCFA***	***Alter balance of microflora (prebiotic)***	***Increase stool output***	***Immunomodulatory***
Monosaccharides	√							
Disaccharides	√			√				
Polyols	√				√		√	
Maltodextrins	√							
Oligosaccharides (non-α-glucan)	√			√	√	√		√
Starch	√				√		√	
NSP	√	√	√		√		√	

Table 5.2: Classification of dietary carbohydrates by molecular size (Cummings & Stephen, 2007)

Class (DP [a])	Subgroup	Principal components
Sugars (1–2)	Monosaccharides	Glucose, fructose, galactose
	Disaccharides	Sucrose, lactose, maltose, trehalose
	Polyols (sugar alcohols)	Sorbitol, mannitol, lactitol, xylitol, erythritol, isomalt, maltitol
Oligosaccharides (3–9) (short-chain carbohydrates)	Malto-oligosaccharides (α-glucans)	Maltodextrins
	Non-α-glucan oligosaccharides	Raffinose, stachyose, fructo and galacto oligosaccharides, polydextrose, inulin
Polysaccharides (≥10)	Starch (α-glucans)	Amylose, amylopectin, modified starches
	Non-starch polysaccharides (NSPs)	Cellulose, hemicellulose, pectin, arabinoxylans, β-glucan, glucomannans, plant gums and mucilages, hydrocolloids

[a] Degree of polymerization or number of monomeric unit.

Prebiotic carbohydrates are important because of the new concept of a healthy or balanced gut flora. A healthy, or 'balanced' microbiota is one that is predominantly saccharolytic and comprises significant numbers of bifidobacteria and lactobacilli (Cummings *et al*., 2004). This concept is based on a number of observations. The genera *Bifidobacterium* and *Lactobacillus* do not contain any known pathogens, and they are primarily carbohydrate-fermenting bacteria, unlike other groups such as *Bacteroides* and clostridia that are also proteolytic and amino-acid fermenting. The products of carbohydrate fermentation, principally SCFAs are beneficial to host health, while those of protein breakdown and amino acid fermentation, which include ammonia, phenols, indoles, thiols, amines and sulphides, are not (Cummings & Macfarlane, 1991). Furthermore, lactic acid-producing bacteria such as bifidobacteria and lactobacilli play a significant role in the maintenance of colonization resistance, through a variety of mechanisms (Gibson *et al.*, 2005).

Almost any carbohydrate that reaches the large bowel will provide a substrate for the commensal microbiota, and will affect its growth and metabolic activities. This has been shown for non-starch-polysaccharides (Stephen & Cummings, 1980), and will occur with

other substrates such as resistant starch, sugar alcohols and lactose. However, stimulation of growth by these carbohydrates is a non specific, generalized effect, that probably involves many of the major saccharolytic groups in the large bowel (Macfarlane & Cummings, 1991). The selective properties of prebiotics relate to the growth of bifidobacteria and lactobacilli at the expense of other groups of bacteria in the gut, such as *Bacteroides*, clostridia, eubacteria, enterobacteria, enterococci, and so on.

5.1 Effects of prebiotics on the microbial population of small and large intestine.

Few studies have been conducted to evaluate the effects of prebiotics on bacteria in the small intestine of companion animals. Willard et al. (2000) evaluated the effect of the dietary supplementation with fructooligosaccharides (FOS) at a concentration of 1% to healthy dogs. In their study, FOS supplementation did not have a significant effect on faecal concentration of bacteria. The researchers hypothesized that the lack of effect of FOS on faecal bacterial populations might depend on the wide variation among individual dogs. In a study by Swanson *et al*. (2002b), FOS did not affect dog faecal bacterial counts when administered at a concentration of 0.5%, but the same authors reported a significant increase in faecal lactobacilli and bifidobacteria after the administration of FOS (1%) plus mannanooligosaccharides (MOS; 0.5%) (Swanson *et al*., 2002c) and FOS alone (1.3%; Swanson *et al*., 2002a). In another study, Flickinger *et al*. (2003) evaluated the effects in dogs of the dietary supplementation with FOS at four concentration levels (0, 0.3, 0.6, and 0.9%). At these concentrations, FOS did not affect lactobacilli and bifidobacteria counts but significantly decreased *C. perfringens*. In a previous study, supplemental FOS (1%) determined a significant increase in the number of faecal bifidobacteria, streptococci andclostridia (Beynen *et al*., 1998).

Grieshop *et al*. (2004) evaluated the effects of chicory (a natural source of inulin) at 1% , alone or together with MOS, in senior dogs. In their study, chicory determined a significant increase in faecal bifidobacteria concentration compared to the control group. In another study (Zentek *et al.*, 2003), administration of chicory, supplemented at 3%, did not affect bifidobacteria faecal concentration.

5.2 Effects of prebiotics on the intestinal mucosa

Prebiotics are fermented in the colon to SCFAs. Short-chain fatty acids (butyrate, acetate, propionate, and lactate) are associated with a trophic effect on the colonic epithelium (Blottière *et al*., 2003). Propst *et al.* (2003) detected a significant increase of faecal acetate, propionate and butyrate in dogs fed inulin and oligofructose at three concentrations (0.3, 0.6, and 0.9%). A similar finding was reported by Vickers et al. (2001), who detected a higher production of all the SCFAs analyzed in *in vitro* fermentation systems containing inulin and FOS compared to fermentors containing cellulose. When fermented *in vitro* with dog faecal inoculum, several prebiotics (FOS, citrus pectin, lactulose, guar gum) rapidly produced an increase of the concentration of SCFAs (Sunvold *et al*., 1995).

5.3 Protein catabolism and production of putrefactive agents

Fermentation of undigested amino acids and endogenous protein determines the production of several putrefactive compounds. These compounds include ammonia, aliphatic amines, branched-chain fatty acids (BCFA), indoles, phenols, and volatile sulphur-containing compounds (MacFarlane & Cummings, 1991).

When administered to dogs at a concentration of 1.3%, FOS determined a significant decrease in fecal ammonia, isobutyrate, isovalerate, and total branched-chain fatty acid concentrations (Swanson *et al.*, 2002a); when fed at 0.5%, FOS decreased faecal indole and phenol concentrations (Swanson *et al*., 2002b). On the contrary, in the study conducted by Flickinger *et al*. (2003), FOS administered at 0.3, 0.6, and 0.9% did not affect BCFA, ammonia, phenols, and indoles concentrations.

5.4 Effects of prebiotics on nutrient digestibility

A few studies investigated the effect of prebiotics on nutrient digestibility. In a study by MIddelbos *et al*. (2007), the addition of FOS (1.2% and 1.5%) to a dog diet caused a significant reduction of protein digestibility. Similar findings were reported by Verlinder *et al.* (2006) after the addition of inulin at 3%, Propst *et al.* (2003) after the addition of inulin and oligofructose at 0.3, 0.6, and 0.9%, and Hesta *et al.* (2003) after supplementation with FOS and isomalto-oligosaccharides (3%). In the study by Hesta *et al.* (2003),when nitrogen

digestibility was corrected for bacterial nitrogen, the differences between the control and oligosaccharide-supplemented groups disappeared. This indicates that the lower total tract nitrogen digestibility was not a consequence of a lower small intestinal digestibility but the result of a higher faecal content of nitrogen originating from bacteria grown in the large intestine.

5.4 Effects of prebiotic on mineral methabolism

Prebiotic are known to increase the absorption of several minerals (calcium, magnesium, and phosphorus) and trace elements (mainly copper, iron, and zinc). The stimulation of absorption seems to be more pronounced in deficient animals. Few data are reported in literature about mineral absorption in dogs after prebiotic administration. Beynen et al. (2002, 2001) evaluated the effect of the administration of oligofructose (1%) and lactulose (1 or 3 grams/MJ metabolizable energy) on mineral absorption in dogs. Oligofructose determined a rise of calcium and magnesium absorption, and the same was detected with lactulose.

5.5 Systemic effects of prebiotics

Some authors investigated the effects of prebiotics on plasma metabolite concentrations of dogs. Diez *et al*. (1998) measured plasma glucose and insulin concentrations in response to supplemental (7%) inulin, guar gum, or sugar beet fiber in dog diets. The investigators determined that guar gum induced lower postprandial insulin, alpha-amino-nitrogen and urea plasma concentrations and fasting cholesterolaemia, while sugar-beet fibre and inulin showed no metabolic effects. In a previous work, the same authors (Diez *et al*., 1997) detected a significant decrease in postprandial glucose, urea and triglyceride concentrations and preprandial glucose, urea and cholesterol after the administration of a blend of inulin and sugar beet fiber (4:1) when inulin reached the concentration of 4 and 8%.

Several authors have proved the ability of prebiotics to modulate immune function in humans and laboratory animals (Seifert & Watzl, 2007; Vos *et al*., 2007). Adogony *et al.* (2007) tested the ability of short-chain FOS, administered to female dogs, to enhance the mucosal immunoglobulin level in mammary secretions. Results from their study showed

that dogs supplemented with scFOS exhibited higher colostrum and milk IgM content without concomitant effect on IgG1, IgG2 and IgA.

Aim of the thesis

Aims of the present study were:

- Development of a simple and reproducible *in vitro* method for predicting the digestibility of pet-food.
- Isolation of a *Lactobacillus* strain from dogs faeces and examination of its potentially probiotic properties .
- Investigation of the effects of several non-digestible oligosaccharides on dog intestinal microflora composition and metabolism.

6. Digestibility study: Material and methods

6.1 Development of the in vitro digestion technique

6.1.1 Feed samples

Nine samples of different commercial pet foods (dry and wet) were used. Analyses of the diets (crude protein, crude fibre, ether extract, ash, and starch) were performed according to AOAC standard methods (AOAC, 2000). Table 6.1 shows the chemical composition of the diets.

Table 6.1: Chemical composition (on dry matter basis) of the diets used during the development of the *in vitro* digestion technique.

Pet-food	Crude protein	Ether extract	Starch	Crude fibre	Ash
1 (dry, cat)	32.91	11.57	42.21	1.77	7.28
2 (dry, cat)	29.85	12.97	37.92	2.47	7.25
3 (dry, cat)	37.72	16.96	35.46	1.13	6.96
4 (wet, cat)	31.75	22.62	29.16	0.79	8.65
5 (dry, dog)	31.75	16.00	39.06	1.60	6.96
6 (dry, dog)	30.84	15.49	43.20	1.18	6.57
7 (dry, dog)	26.72	11.19	39.24	3.43	8.92
8 (wet, dog)	31.70	23.50		1.56	8.64
9 (wet, dog)	35.68	30.27	19.26	0.96	11.48

6.1.2 In vitro digestion

Samples of pet foods were first digested using the *in vitro* digestion technique proposed by Vervaeke *et al*. (1989). The method can be briefly summarized as follows:

1. Sample preparation: samples of pet food were dried at 65°C overnight and finely ground (< 1 mm particle size).
2. Step 1 (gastric digestion simulation): For each pet food sample, 400 mL of a 0.2% pepsin solution (HCl 0.075N; Pepsin from porcine gastric mucosa, 600-1,800 units/mg, P7125, Sigma-Aldrich) were added in a 1 L bottle to 20 g of pet food. Bottles were incubated in a shaking waterbath at 39°C for 4 hours.
3. Step 2 (small intestinal digestion simulation): pH level was adjusted to 7.5 with NaOH (1 N)and 400 mL of a pancreatin solution (1% in phosphate buffer;

Pancreatin from porcine pancreas, P1500, Sigma-Aldrich) were added. Bottles were incubated in a shaking waterbath at 39°C for 4 hours.

4. Centrifugation: after the enzymatic digestion, the content of each bottle was centrifuged (3,000 x *g*, 10 min, 4°C), washed twice with distilled water, re-centrifuged (3,000 x *g*, 5 min, 4°C), and the residue is dried at 65°C overnight.

Phosphate buffer was prepared mixing three solutions:

- Solution 1 (g/L):
  - 48.44 g of Na_2HPO_4
  - 49.0 g $NaHCO_3$
  - 2.35 g of NaCl
  - 2.85 g of KCl
- Solution 2 (g/L):
  - 60 g of $MgCl_2$
- Solution 3 (g/L):
  - 12.89 g $MgCl_2 \bullet 6\ H_2O$
- 500 mL of solution 1, 5 mL of solution 2 and 5 mL of solution 3 were mixed, and the volume was adjusted to 1 L adding distilled water. Final pH was adjusted to 7.5 with HCl 1 N.

6.2.3 Calculation and data analysis

In order to determine diet digestibility, the residue obtained from each bottle after the *in vitro* digestion was weighed and digestibility was calculated with the following equation:

100 – [(residue weight x 100) / sample weight]

The un-digested fraction was then analysed for crude protein, ether extract, crude fibre, starch and ash, according to AOAC standard methods (AOAC, 2000). Nutrient digestibility was calculated with the following equation:

100 – {[nutrient percentage in residue x (100 – diet digestibility)] / nutrient percentage in diet}

Digestibility data obtained with the *in vitro* technique were compared to digestibility data from the literature (obtained from *in vivo* trials). Because digestibility coefficients obtained with the *in vitro* method proposed by Vervaeke *et al*. (1989) differed from those that are reported in the literature, particularly the results regarding digestibility of lipids (see Table 3.1, Table 7.1), the method proposed by Vervaeke *et al.* (1989) was modified, in order to develop a new method that could better represent the peculiar digestive physiology of dogs and cats. In particular, the following critical points were considered during the study:

- Food sample and digestive solution ratio;
- Addition of lipase and/or emulsifiers to the digestive solutions;
- Pancreatin concentration in the second phase solution;
- Duration of each digestion phase (gastric and intestinal);
- Food characteristics (dry, wet, for dog, for cat).

After several *in vitro* digestion trials, the following new method was developed.

1. Sample preparation: each pet foodsample is dried at 65°C overnight and finely ground (< 1 mm particle size).
2. Step 1 (gastric digestion simulation): 10 g of pet food sample are added with 400 mL of a 0.2% pepsin solution (HCl 0.075N;) containing 0.1% gastric lipase (Rhizopus lipase, F-AP15, Amano Enzyme Inc.). and incubated in a 1 L bottle in a shaking waterbath at 39°C for 2 hours.
3. Step 2 (small intestinal digestion simulation): pH level is adjusted to 7.5 with NaOH (1 N). Then, 400 mL of a 1% pancreatin solution in phosphate buffer (prepared as described before) are added to each bottle. Immediately prior to addition of the pancreatin solution, bile salts (Cholic acid-Deoxycholic acid sodium salt mixture, 48305, Fluka) are added to each bottle at the final concentration of 2.5%. The bottle is placed again in the shaking waterbath at 39°C for 4 hours.
4. Centrifugation: after enzymatic digestion, the preparation is centrifuged (3,000 x *g*, 10 min, 4°C), washed twice with distilled water, re-centrifuged (3,000 x *g*, 5 min, 4°C), and the residue is dried at 65°C overnight.

6.2 Validation of the new in vitro method

In order to validate the new *in vitro* method, an *in vivo* digestibility study with dogs was performed. Three dry extruded diets for dogs were digested both *in vitro* and *in vivo*.

6.2.1 Animals

A total of 18 dogs (different breeds, same environment, with an average body weight of 24.9 ± 6.39 kg) were used for the *in vivo* digestibility trial. Before the beginning of the trial, all dogs were screened for intestinal parasites and infected ones were treated (DRONTAL, Bayer S.p.A). Dogs were randomly assigned to three different diets (six animals for each group) and individually housed in boxes. After a 5-day adaptation period (during which dogs were progressively adapted to the experimental diets), dogs received for 12 days the experimental diets. . During the last 5 days, all faeces excreted by each dog were collected, weighed and immediately frozen. Dogs were fed once daily according to their maintenance energy requirement, had free access to water and were allowed daily exercise outside of their boxes.

6.2.2 Diets

Three dry extruded diets were evaluated in this study. Celite, a source of acid-insoluble ash, was used as a digestion marker at 1.5% of the diet. Chemical analysis of the dietary treatments are presented in Table 6.2.

Table 6.2: Chemical analysis of diets used in *in vivo* trial (percentage on dry matter basis).

	T1	T2	T3
Dry matter	94.50	93.73	94.86
Crude protein	23.81	24.81	23.97
Ether extract	16.41	18.35	15.47
Crude fibre	2.36	1.65	2.57
NDF	17.59	12.66	18.21
ADF	9.11	7.85	10.59
ADL	3.54	3.21	2.83
Ash	9.67	8.80	9.47
Insoluble ash	1.78	1.59	1.28
Starch	29.97	34.57	34.74

NDF: Neutral Detergent Fibre; ADF: Acid Detergent Fibre; ADL Acid Detergent Lignin

6.2.3 Samples analyses

The frozen faecal samples from every single dog were freeze-dried, finely ground (< 1 mm particle size), mixed and analysed (crude protein, crude fibre, ether extract, starch, ash and insoluble ash) according to AOAC standard methods (AOAC, 2000). All samples were analyzed in duplicate.

6.2.4 Calculation and data analysis

Diet and nutrients digestibility was calculated as previously described.

In order to compare *in vivo* and *in vitro* digestibility data, the same three diets were digested using the new *in vitro* method (see chapter 6.1.2). Each diet was digested in triplicate. Analyses (crude protein, crude fibre, ether extract, starch, ash and insoluble ash) of the un-digested residue were performed according to AOAC standard methods (AOAC, 2000). All samples were analyzed in duplicate.

Linear regression was used to determine the precision and accuracy of the established relationship between *in vivo* and *in vitro* data.

7. Digestibility study: Results

7.1 Development of the in vitro digestion technique

7.1.1 Method proposed by Vervaeke et al. (1989)

The results obtained with the method proposed by Vervaeke *et al.* (1989) are shown in Table 7.1. The chemical composition of the tested pet-foods is shown in Table 6.1.

Table 7.1: Digestibility (Mean ± SEM) of different dry pet-foods determined with the method proposed by Vervaeke *et al.* (1989)..

Pet-food		**Digestibility**			
	N	**Dry matter**	**Crude protein**	**Ether extract**	**Starch**
1	3	72.9 ± 0.54	87.5 ± 0.63	32.5 ± 4.53	94.7 ± 0.49
2	3	72.2 ± 0.18	85.1 ± 0.37	41.6 ± 0.75	96.1 ± 0.26
3	3	73.5 ± 0.40	79.9 ± 0.26	52.3 ± 0.77	95.5 ± 0.21
5	3	72.4 ± 0.42	84.7 ± 1.00	28.9 ± 1.05	96.3 ± 0.19
6	3	73.2 ± 0.31	79.2 ± 0.23	45.3 ± 1.32	95.6 ± 0.24
7	3	65.7 ± 0.39	83.1 ± 0.58	33.8 ± 1.31	96.6 ± 0.16
Literature[a]	50	82.3 ± 5.17	82.2 ± 4.50	92.8 ± 2.60	98.6 ± 2.24

[a] Means obtained from: Vhile *et al*., 2007; Guevara *et al*., 2008; Kempe *et al*., 2007; Yamba *et al*., 2006; Dust *et al*., 2005.

7.1.2 Effect of the food / digestive solution ration food digestibility

The ratio between food and digestive solutions in the method proposed by Vervaeke *et al*. (1989) is 1:40 (see procedure described in chapter 6.1.2). Because this ratio influences the quantity of enzymes that are available to digest the substrate, different food / digestive solution ratios (1:20, 1:40, and 1:80) were tested (Table 7.2).

Table 7.2: Dry matter digestibility (Mean ± SEM) using different food/digestive solutions ratios (1:20, 1:40, and 1:80).

Ratio	N	Digestibility (%)
1:20	3	66.5 ± 5.79
1:40	3	70.9 ± 3.79
1:80	3	75.3 ± 2.31

It was decided to further use the 1:80 food to solution ratio, because this ratio lead to lipid digestibility values that were closer to those reported in literature.

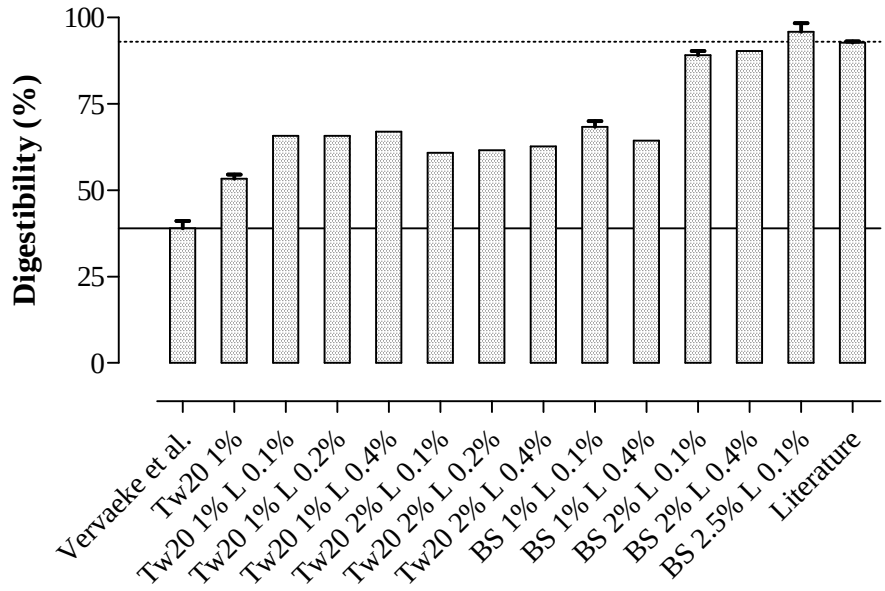
7.1.3 Addition of lipase and emulsifiers

In order to further improve lipid digestion, gastric lipase and emulsifiers were added to the digestive solutions.

The addition of gastric lipase (Rhizopus lipase, F-AP15, Amano Enzyme Inc., Japan) at different concentrations to the pepsin-HCL solution was tested in combination with the addition of a non-ionic surfactant (Tween 20, Polyoxyethylene sorbitan monolaurate) or bile salts (Cholic acid-Deoxycholic acid sodium salt mixture, 48305 Fluka) to the pancreatin solution (Figure 7.1).

The addition of gastric lipase (0.1 and 0.4%) and bile salts at the final concentration of 2% improved lipid digestibility and the data that were obtained were more consistent with data reported in literature.

Figure 7.1: Ether extract digestibility obtained using different digestive solutions compared with results reported in literature [a] (dotted line indicates literature results[a], solid line indicates results obtained with the technique proposed by Vervaeke *et al.*, 1989).



Legend:

- Tw20: Tween 20
- L: Lipase
- BS: Bile salts

[a] Means obtained from: Vhile *et al.*, 2007; Guevara *et al.*, 2008; Kempe *et al.*, 2007; Yamba *et al.*, 2006; Dust *et al.*, 2005.

7.1.4 Pancreatin concentration

We also considered the effects of different pancreatin concentrations in the second phase digestive solution..

Two different pancreatin concentrations were tested using a wet diet for dogs (pet-food number 9). This diet was chosen for its high fat content in order to verify the effectiveness of the method. Table 7.3 shows the results obtained using pancreatin at 1 and 1.25%.

Table 7.3: Dry matter, crude protein, ether extract and starch digestibility obtained using pancreatin at 1 and 1.25%. The pet food used in the trial was 9 (wet, dog). Mean ± SEM.

Bile salts (%)	Pancreatin (%)	N	Digestibility			
			Dry matter	Crude protein	Ether extract	Starch
2	**1**	2	84.8 ± 0.28	85.2 ± 0.28	86.0 ± 0.26	*
2	**1.25**	2	84.8 ± 0.40	89.3 ± 0.28	85.2 ± 0.39	*
2.5	**1**	2	85.3 ± 0.07	87.1 ± 0.06	90.9 ± 0.05	*
2.5	**1.25**	2	84.8 ± 0.20	86.7 ± 0.17	89.4 ± 0.14	*

* Starch present in traces in undigested residue.

Digestibility data obtained with pancreatin at 1.25% did not significantly differ from those obtained with 1% addition.

7.1.5 Duration of each digestive phase

Duration of each digestive phase directly affects the time of substrate exposure to digestive enzymes. The method proposed by Vervaeke *et al.* (1989) consisted of two digestive phases of 4 h each.

In order to better represent the digestive physiology of carnivores, it was decided to reduce duration of the gastric phase from four to two hours (Table 7.4).

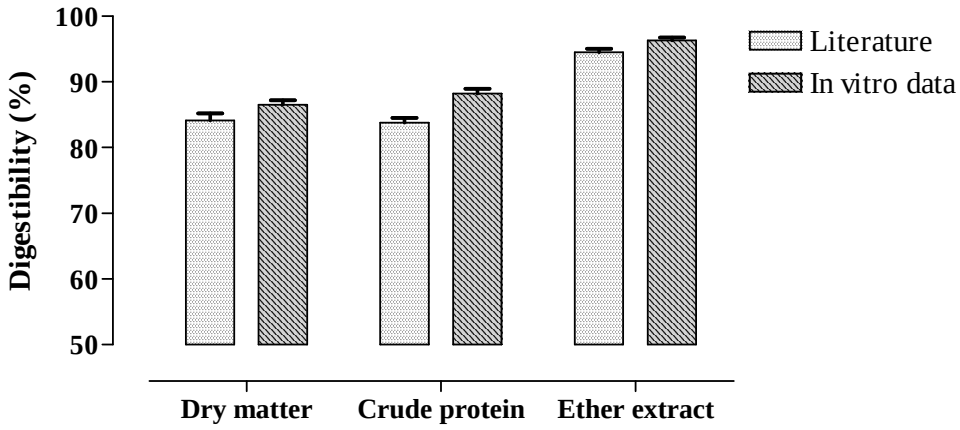
Table 7.4: Dry matter, crude protein, ether extract, and starch digestibilities obtained with different combinations of times. Pet foods used in the trial were 1 (dry cat) and 8 (wet dog), which composition is shown in Table XX. Data are expressed as Means ± SEM.

	Phase Duration (h)	N	Digestibility			
			Dry matter	Crude protein	Ether extract	Starch
Dry	2 + 4	8	86.4 ± 1.46	91.5 ± 1.10	94.9 ± 3.69	*
	4 + 4	8	84.2 ± 2.20	91.1	94.5 ± 4.26	*
Wet	2 + 4	5	87.5 ± 0.26	89.2 ± 0.20	87.7 ± 0.82	*
	4 + 4	3	89.3 ± 0.34	91.7 ± 0.26	88.4 ± 0.36	*

* Starch present in traces in undigested residue.

Comparing the digestibility results obtained with the two different durations of the gastric phase, no significant difference was observed..

Figure 7.2: Comparison of digestibility coefficients reported in literature and obtained applying the protocol proposed in the present study.



7.2 Correlation of in vitro and in vivo digestibility coefficients

A trial was conducted to compare *in vivo* digestibility of pet-foods and *in vitro* digestibility using the newly developed method.

The results obtained from the trial are presented in Table 7.5. Table 7.6 and Figures 7.3, 7.4 and 7.5 show the characteristics of the regression equations calculated.

Table 7.5: Digestibility coefficients of three pet-foods evaluated *in vivo* and *in vitro* (Means ± SD).

Digestibility	**T1**		**T2**		**T3**	
	in vivo	***in vitro***	***in vivo***	***in vitro***	***in vivo***	***in vitro***
Dry matter	81.55 ± 0.85	80.65 ± 0.30	76.19 ± 1.09	80.00 ± 0.19	79.73 ± 0.48	80.40 ± 0.12
Crude Protein	82.59 ± 0.92	83.24 ± 0.86	76.49 ± 1.52	85.52 ± 0.60	81.23 ± 0.89	83.95 ± 1.07
Ether extract	96.52 ± 0.32	94.61 ± 0.42	95.03 ± 0.64	96.35 ± 0.19	96.73 ± 0.25	93.34 ± 0.55
Crude Fiber	26.87 ± 7.32		16.82 ± 2.22		24.54 ± 0.76	
Starch	*	*	*	*	*	*
Crude ash	49.90 ± 3.45		33.60 ± 3.64		52.75 ± 2.14	

* Starch present in traces in undigested residue.

Table 7.6: Characteristics of regression equations calculated fro Dry mater, Crude protein and Ether extract.

	Dry matter	**Crude protein**	**Ether extract**
Equation	y = 8.30 x – 588.2	y = - 2.57 x + 295.5	y = - 0.57 x + 149.7
Correlation coefficient	0.9976	0.9957	0.9098

y: *in vivo* digestibility coefficient
x: *in vitro* digestibility coefficient

Figure 7.3: Dry matter digestibility coefficients of three pet-foods evaluated *in vivo* and *in vitro*.

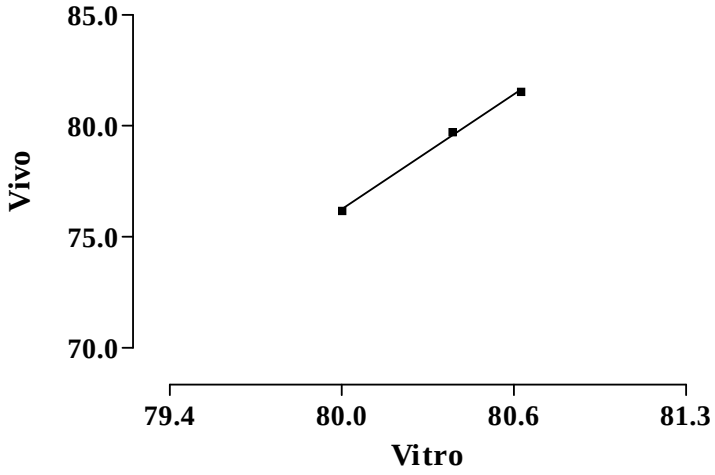


Figure 7.4: Crude protein digestibility coefficients of three pet-foods evaluated *in vivo* and *in vitro*.

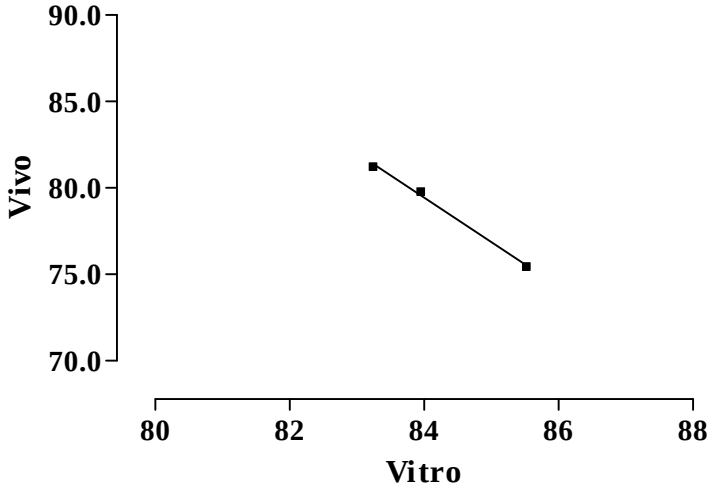
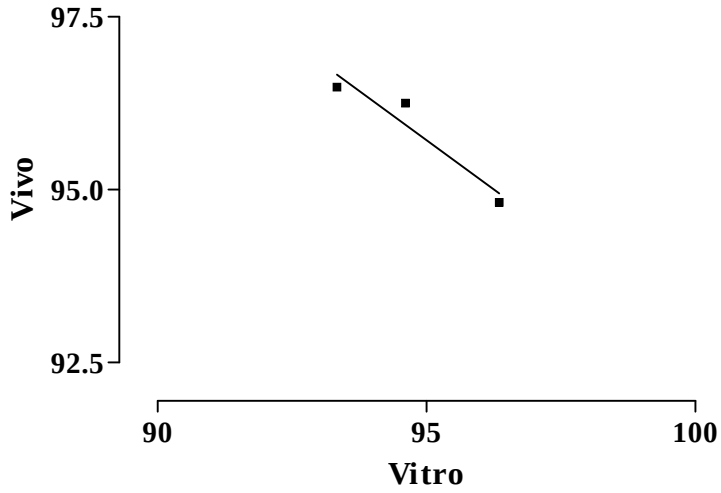


Figure 7.5: Ether extract digestibility coefficients of three pet-foods evaluated *in vivo* and *in vitro*.



8. Digestibility study: Discussion

8.1 Development of the in vitro digestion technique

Mean total digestibility of pet-foods digested with the method proposed by Vervaeke *et al.* (1989) was far below the digestibility values that are reported in the literature (65-73% *vs* 74-80%). In particular, using the method proposed by Vervaeke *et al.* (1989), lipid digestibility was very low (29-52% *vs* 76-97%) and seemed to be the factor that affected total digestibility. It has to be considered that Vervaeke *et al.* (1989) proposed their method to determine the digestibility of diets for pigs which usually contain much lower concentrations of lipids than diets for dogs and cats. In particular, the Vervaeke method does not imply the utilization of gastric lipase and bile salts, the latter an essential factor in the digestion of lipids. Conversely, protein and starch digestibility data were consistent with those reported in literature (Table XX).

The addition of Tween 20 determined a higher lipid digestibility. These results are consistent with data reported by Shome *et al*. (2007) who detected an increase in lipase activity, ranging from 26 to 72%, in presence of non ionic surfactants. However, digestibility coefficients obtained were lower than those that are reported in literature. After the addition of bile salts and gastric lipase lipid digestibility resulted more consistent with data reported in literature. It is known,that one characteristic of digestive lipase is its specificity to act on a specific emulsion interface (Armand *et al*., 1999). The emulsion interface properties, namely, droplet size and specific surface area, govern the activity of lipase on dietary fat emulsion. Therefore, changes in the emulsion droplet size and surface area might have an important role in modifying fat digestion and absorption. Emulsification of dietary fats *in vivo* is accelerated greatly as the chime enters the small intestine and is mixed with bile and pancreatic secretion. In the gut, bile salts and phospholipids cooperate in the emulsification of dietary triglycerides and other fat soluble nutrients improving the activity of lipase. Also in dogs, lipid digestion is increased by the concomitant presence of bile salts. In fact, Meyer *et al.* (1994), in a study conducted *in vivo*, detected a positive linear correlation between lipid digestion and taurocholate molar concentration in dog's intestine.

Duration of each digestive phase directly affects the time of exposure of substrate to digestive enzymes. Physiological data reported in literature about gastric emptying and intestinal transit time in dogs comprise a wide range of times (Wyse et al., 2003). Rates of gastric emptying and intestinal transit time are influenced by many factors related both to

animal and diet characteristics. In the present trial, reducing the duration of the gastric phase lead to digestibility coefficients that were coherent with data reported in literature (Table XX).

8.2 Correlation of in vitro and in vivo digestibility coefficients

The results obtained in the present study indicate that *in vivo* digestibility coefficients can be predicted quite accurately using the proposed *in vitro* method. Correlation coefficients showed, for dry matter and crude protein, a close similarity between digestibility data obtained with *in vivo* and *in vitro* methods (0.9976 and 0.9957, respectively). Ether extract presented a lower correlation coefficient, although close to 1 (0.9098).

It is well known that the microflora of the digestive tract can affect the nutritional status of the host, changing the digestibility and absorbability of nutrients (March, 1979). Karr-Lilienthal *et al.* (2004) estimated that approximately 50% (49.6-51.4%) of the dry matter of dog faeces is of bacterial origin. This can significantly affect nutrients content in faeces creating a discrepancy between *in vivo* and *in vitro* data. In fact, chemical analysis are not able to discriminate between nutrients of "faecal" or bacterial origin. Several authors (Sunvold et al., 1995; Muir et al., 1996; Flickinger et al., 2000) found evidence of an apparent inhibition of nitrogen digestion *in vivo* when diets containing fermentable fibre were administrated. They postulated that this finding was due to increased bacterial metabolism associated with the production and excretion of greater quantities of nitrogenous constituents. Moreover, we have to consider that not all the components that are solubilised *in vitro* and are considered as digested are really digestible and absorbable *in vivo*. This condition creates a tendency to overestimate digestibility coefficients using *in vitro* enzymatic methods.

Nevertheless, the aim of this study was to assess the existence of a correlation between digestibility coefficients obtained *in vivo* and *in vitro*, and to obtain equations which relate *in vivo* digestibility coefficients to *in vitro* ones. On the basis of collected data, the proposed *in vitro* method provided digestibility coefficients which correlated well with *in vivo* ones.

9. Probiotic study: Material and methods

9.1 Isolation of the probiotic strain

Seventeen healthy adult dogs (household dogs, different breeds, fed different commercial dry diets and living in different environments; between 1 and 3 years of age), that had followed a pre/probiotic-free diet for 1 month and had not been treated with antibiotics for at least 3 months, were screened for faecal LAB and bifidobacteria contents. Fresh faeces were collected immediately after excretion in sterile vessels and frozen at –18°C within 20 min. Within 10 days from collection, faeces were homogenized and serially diluted in half-strength Wilkins-Chalgren Anaerobe Broth (WCAB 0.5x, Oxoid LTD, Basingstoke, Hampshire, UK) added with L-cysteine HCl (0.5 g/L). Dilutions were plated on Raffinose Bifidobacterium Agar (RB Agar; Hartemink et al., 1996) and LAMVAB Agar (Hartemink et al., 1997), for bifidobacteria and lactobacilli counts, respectively. Plates were incubated in an anaerobic cabinet (Anaerobic System, Forma Scientific Co., Marietta, USA) under a N_2 85%, CO_2 10%, H_2 5% atmosphere at 37°C for 48 h (results shown in Table 10.1).

Attribution to the genus *Bifidobacterium* of the colonies isolated on RB agar was achieved by assaying fructose-6-phosphate phosphoketolase activity, the key enzyme of *Bifidobacterium* carbohydrate metabolism (Scardovi, 1986). In order to confirm that new isolates belonged to this genus, colonies were picked for amplification with the 16S rDNA primer set Bif164/Bif662 specific for this genus, according to Kok *et al*. 1996, to identificate the proper 523 bp amplicon.

Attribution to the specie of RB and LAMVAB colonies was obtained by subculturing on MRS. Pure MRS coltures were ribotyped for speciation using the automated ribotyping device, RiboPrinter Microbial Characterisation System (Qualicon Inc., Wilmington, DE, USA). Bacterial colonies were picked from agar plates, suspended in sample buffer, inactivated by heat kill step, and treated with lytic enzymes to release the DNA. The DNA was cut with EcoRI and the fragments were electrophoretically separated and simultaneously transferred to a nylon membrane. A DNA probe for the Escherichia coli rrnB operon was then hybridized to the genomic DNA on the membrane. Each clone was identified by comparison of the RiboPrint pattern with an identification database of EcoRI RiboPrint patterns created by E. I. DuPont de Nemours and Company (Qualicon Inc.). The taxonomic attribution was confirmed by rDNA sequence analysis. The proper primer set was used to amplify the ribosomal fragments comprising the Internal Transcribed Spacers

(ITS) and the flanking 16S and 23S rDNA regions. The amplified products were separated by gel electrophoresis, the fragments of ca 550 bp were purified using a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), and then subjected to automated sequence analysis of both DNA strands.

9.2 Preparing of the supplement

After speciation, it was decided to use one of the isolated strains belonging to the specie *Lactobacillus animalis* in a feeding trial with adult dogs for its high biomass yield.

The *L. animalis* strain was grown on the following complex medium called CM:

- phytone, 10 g/L (Difco Laboratories, Sparks, USA);
- casein hydrolisate, 10 g/L;
- sodium acetate, 2.5 g/L;
- yeast exctract, 10 g/L (Difco Laboratories, Sparks, USA);
- Tween 80, 1 g/L;
- L-cysteine HCl, 0.5 g/L;
- MnSO4 $7H_2O$, 7 mg/L;
- KH_2PO_4, 0.15 g/L;
- $MgSO_4$ $7H_2O$, 0.5 g/L;
- pH was adjusted to 6.8. The medium was, then, autoclaved for 30 min at 110°C.
- Glucose was autoclaved separately and added to the sterile basal medium to obtain the final concentration of 20 g/L.

The *Lactobacillus* strain was subcultured in Lactobacilli MRS broth (Difco Laboratories, Sparks, USA) containing 0.5 g/L L-cysteine HCl and anaerobically incubated at 37°C for 24 h.

Cells from the MRS cultures were inoculated (5% v/v) into CM medium and incubated anaerobically at 37°C for 48 h. After the incubation time the biomass was harvested by centrifugation and resuspended in preservation Suspending Fluid (skim milk 50 g/L; lactose 30 g/L; yeast exctract 50 g/L; ascorbic acid, 5 g/L) for the lyophilization process.

The freeze-dried probiotic product contained about 10^9 CFU/g.

9.3 In vitro trial

In order to prepare faecal cultures, fresh faecal samples (from two adult healthy dogs) were suspended 1% (w/v) in pre-reduced WCAB 0.5x. Faecal suspension was added (1% v/v) to 100 mL anaerobic serum bottles containing 80 ml of Faecal Extract Medium. Faecal Extract Medium was obtained following the procedure described by Benno and Mitsuoka (1992) using fresh faeces collected from 15 adult healthy dogs. Faecal cultures were inoculated (1% v/v) with the freeze-dried *L. animalis* strain resuspended in WCAB medium at a concentration of 10^8 CFU/mL (in order to achieve in the faecal cultures a final *L. animalis* concentration of 10^6 CFU/mL) or, as a negative control, with the same volume of sterile WCAB medium. Each bottle received the addition of 1 g of *in vitro* digested dry food for adult dogs (Table 9.1) suspended in 10 mL of physiological solution. The in vitro digested food simulates the undigested fraction of the diet that reaches the hindgut and is obtained by in vitro digestion (2 h incubation with HCl + gastric lipase + pepsin followed by a 4 h incubation with pancreatin + bile salts) of a commercial dry food for adult dogs (Vervaeke et al., 1989; modified method).

Table 9.1: Analyzed chemical composition of the commercial dry dog food used in the study before and after enzymatic digestion (%DM)[a].

	Before digestion	After digestion
Crude protein	23.1	12.1
Ether extract	8.6	2.0
Starch	41.2	traces

[a] Food in vitro total digestibility was 79.7%

Faecal cultures were incubated at 39°C in anaerobiosis and samples were collected for chemical and microbiological analyses at 0, 4, 8 and 24 h. All preparations were done in an anaerobic cabinet.

9.4 In vivo trial

Nine dogs, belonging to the initial pool, screened during the first phase of the trial, having lactobacilli counts lower than 4,5 Log CFU for g of faeces were selected to assess the *in vivo* effect of the *L. animalis* strain.

Selected dogs received for 10 d a single oral daily dose of 0.5 g of the freeze-dried probiotic. Faecal samples were collected the day before probiotic administration started (Day 0) and again 1 and 5 d after withdrawal of the probiotic administration (Day 11 and 15, respectively). Faecal samples were collected immediately after excretion and frozen at –18°C within 20 min. for chemical and microbiological analyses.

9.5 Chemical and microbiological analyses

Ammonia in faecal cultures and homogenized faeces samples was measured using a commercial kit (Urea/BUN – Color, BioSystems S.A., Barcelona, Spain).

Short-chain fatty acids (SCFA) in faecal cultures and homogenized faeces samples were analyzed by gas chromatography (Varian 3400, Varian Analytical Instruments, Sunyvale, CA 94089, USA) with Carbopack B-DA/4% CW 2M and 80/120 packed column (Supelco, Sigma Aldrich s.r.l., 20151 Milano, Italy). The faeces were homogenized and diluted 1:1 with distilled water and centrifuged (3,000 × g, 15 min.) and 1 mL of the supernatant was deproteinized with 50 µL perchloric acid (Merck, Darmstadt, Germany). Finally, both faecal culture and faeces supernatant samples were centrifuged (14,000 x g, 10 min.) and added with pivalic acid as an internal standard (Fussel and McCalley, 1987) prior to injection.

Immediately after sampling, faecal cultures samples were serially diluted with prereduced half-strength WCAB. From each of the dilutions, 0.1 mL was plated in triplicate onto selective media: MacConkey Agar (Merck, Darmstadt, Germany) for coliforms, OPSP Agar (Oxoid, Basingstoke, UK) for *Clostridium perfringens*, LAMVAB Agar (Hartemink et al., 1997) for lactobacilli, Azide Maltose Agar (Biolife, Milano Italy) for enterococci, and RB Agar (Hartemink et al., 1996) for bifidobacteria. All media were kept ≥ 24 h in the anaerobic chamber before use. MacConkey agar plates were incubated aerobically at 37°C for 24 h; Azide agar plates in aerobiosis for 48 h; all other media were incubated anaerobically at 37°C for 48-72 h.

Within 10 days from collection, faecal samples were homogenized and plated onto the same selective media (with the only exception of RB Agar), following the same procedures previously described.

Representative colonies grown onto LAMVAB plates were identified at genus level by standard bacteriological procedures (Gram stain reaction, colonial and cellular morphology). After genus identification, rDNA sequence of colonies apparently belonging to L. animalis species was determined for strain level identification

9.6 Statistical analysis

Data from the in vitro trial were analyzed using the Student-Newman-Keuls test. Differences were considered statistically significant at $P < 0.05$.

In the in vivo trial, data from measurements at Day 0, 11 and 15 were analyzed by one-way ANOVA using the GLM procedure of SAS (SAS Inst., Inc., Cary, N.C.) with time as the main factor; the differences among means of groups were analyzed using the Student-Newman-Keuls test. Differences were considered statistically significant at $P < 0.05$.

10. Probiotic study: Results

10.1 Isolation of the probiotic strain

Lactobacilli and Bifidobacteria counts in faecal cultures are reported in Table 10.1.

Table 10.1: Counts of viable lactobacilli and bifidobacteria (log CFU/ml) in dog faecal samples, bold data correspond to subjects selected for the in vivo trial.

	Lactobacilli	**Bifidobacteria**
1	3,30	<3
2	<3	<3
3	6,85	6,34
4	7,40	7,95
5	<3	3,85
6	<3	7,20
7	<3	9,30
8	3,90	3,00
9	<3	<3
10	5,08	5,08
11	5,08	5,08
12	<3	<3
13	5,85	6,96
14	6,85	6,98
15	6,90	6,90
16	4,30	<3
17	3,72	<3

Among the 17 dogs that were sampled, LAB faecal counts were higher than 10^5 CFU/g in three subjects and than 10^6 CFU/g in four. On the contrary, of the remaining nine dogs, four had LAB faecal counts between 10^3 and 10^5 CFU/g and six were under the detection limit of 10^3 CFU/g.

10.2 In vitro trial

Bacterial counts in faecal cultures are reported in Table 10.2 and Figures 10.1,10.2,10.3,10.4 and 10.5. Ammonia and SCFA faecal concentrations and pH values are reported in Table 10.3 and Figures 10.6 and 10.7.

Table 10.2: Counts (Log CFU/mL) of viable coliforms, enterococci, Clostridium perfringens, bifidobacteria, and lactobacilli in dog faecal cultures added (Lac +) or not (Lac -) with *Lactobacillus animalis* LA4. Values are the mean of four replicates ± SD.

	0 h		**4 h**		**8 h**		**24 h**	
	Lac -	**Lac +**	**Lac -**	**Lac +**	**Lac -**	**Lac +**	**Lac -**	**Lac +**
Coliforms	6.67	6.67	6.62 ± 0.03	6.65 ± 0.03	6.69 ± 0.07	6.68 ± 0.02	7.27 ± 0.26	7.31 ±0.34
Enterococci	6.40	6.40	7.71 ± 0.08	7.13 ± 0.17*****	7.69 ± 0.07	6.82 ± 0.06*****	7.71 ± 0.18	6.62 ± 0.20*****
Bifidobacteria	5.30	5..30	5.35 ± 0.09	5.41 ± 0.08	5.47 ± 0.08	5.76 ± 0.54	6.41 ± 0.28	6.53 ± 0.08
C. perfringens	6.48	6.48	6.42 ± 0.13	6.33 ± 0.23	6.63 ± 0.27	6.37 ± 0.09	6.82 ± 0.21	6.33 ± 0.11*****
Lactobacilli	5.70	5.70	6.26 ±0.19	8.51 ± 0.16*****	7.18 ± 0.21	9.18 ± 0.13*****	8.17 ± 0.24	9.61 ± 0.18*****
L. animalis		6.18		8.51 ± 0.16		9.18 ± 0.13		9.59 ± 0.18

* indicates a significant difference ($P < 0.05$) from the corresponding Lac- value

Figure 10.1: Counts of viable coliforms (Log CFU/ml) in dog faecal cultures added (Lac +) or not (Lac -) with a strain of *Lactobacillus animalis*. Values are the mean of four replicates ± SEM.

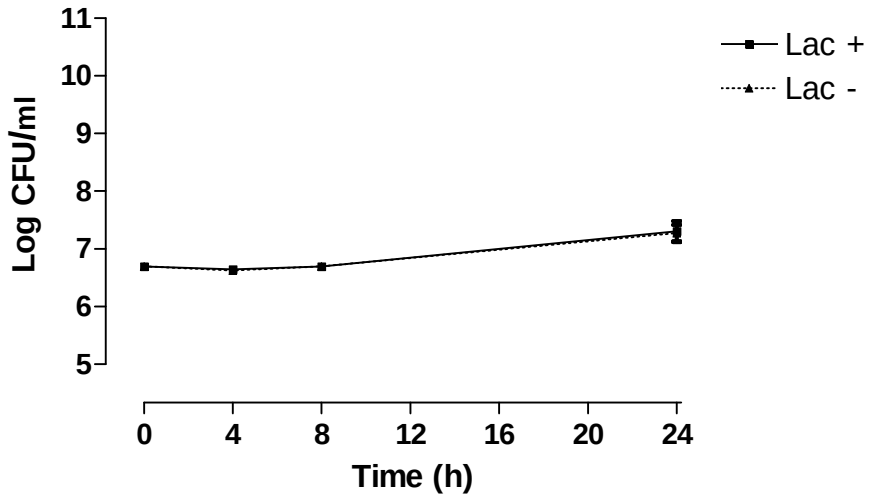
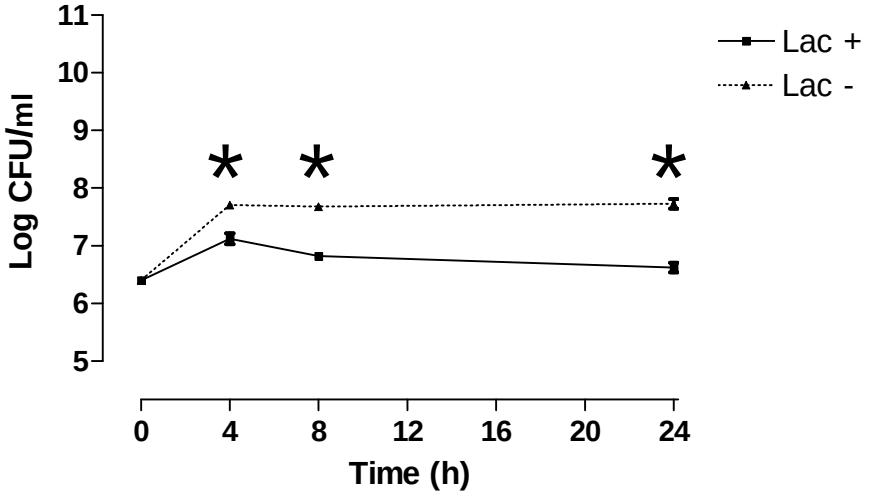


Figure 10.2: Counts of viable enterococci (Log CFU/ml) in dog faecal cultures added (Lac +) or not (Lac -) with a strain of *Lactobacillus animalis.* Values are the mean of four replicates ± SEM.



* indicates significant difference ($P < 0.05$)

Table 10.3: Counts of viable bifidobacteria (Log CFU/ml) in dog faecal cultures added (Lac +) or not (Lac -) with a strain of *Lactobacillus animalis*. Values are the mean of four replicates ± SEM.

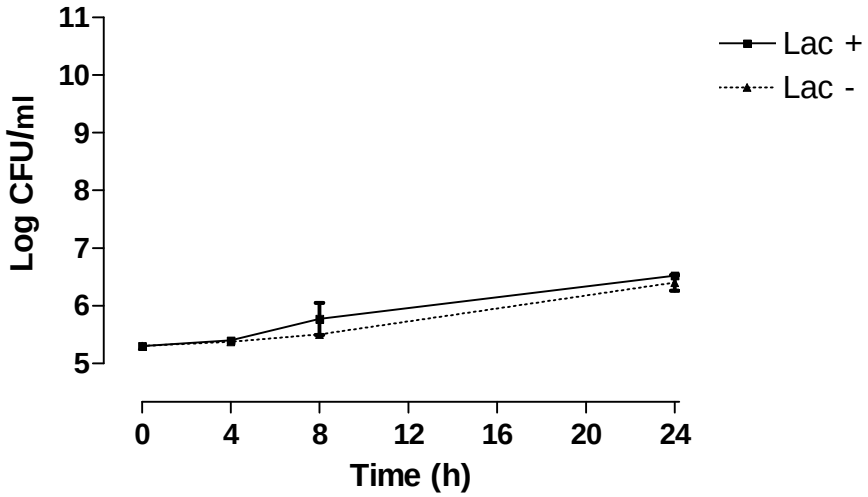
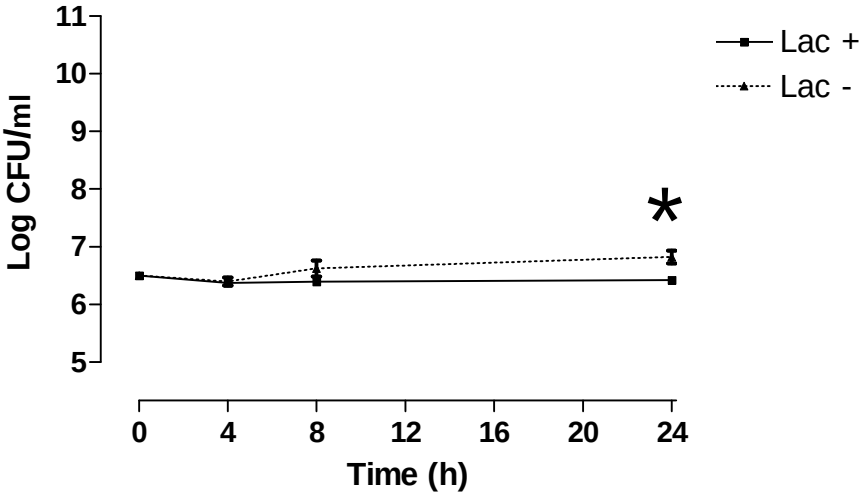
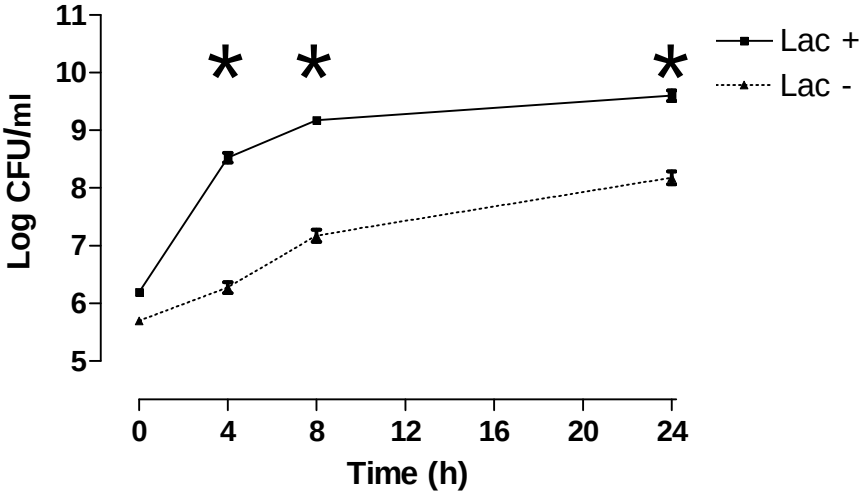


Figure 10.4: Counts of viable *Clostridium perfringens* (Log CFU/ml) in dog faecal cultures added (Lac +) or not (Lac -) with a strain of *Lactobacillus animalis*. Values are the mean of four replicates ± SEM.



* indicates significant difference ($P < 0.05$);

Figure 10.5: Counts of viable lactobacilli (log CFU/ml) in dog faecal cultures added (Lac +) or not (Lac -) with a strain of *Lactobacillus animalis*. Values are the mean of four replicates ± SEM.



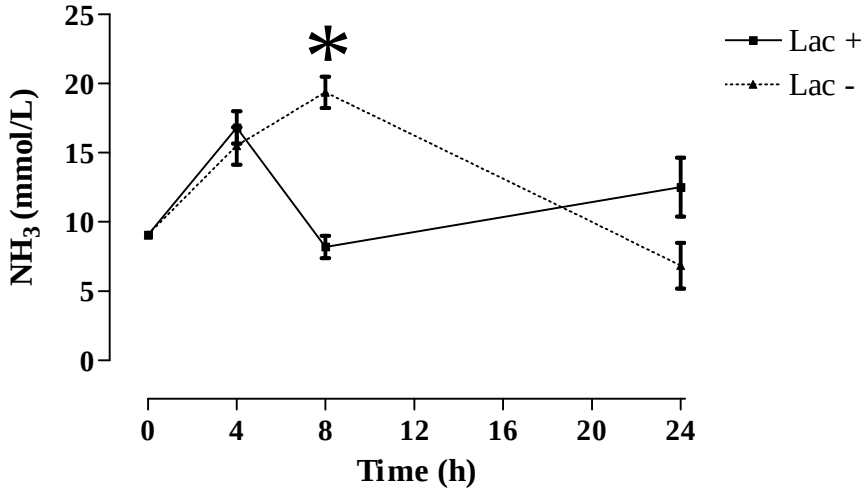
* indicates significant difference ($P < 0.05$)

Table 10.3: pH values and ammonia and short-chain fatty acids (mmol/l) concentrations in dog faecal cultures added (Lac +) or not (Lac -) with a strain of *Lactobacillus animalis*. Mean ± SEM.

	4 h		8 h		24 h	
	Lac -	**Lac +**	**Lac -**	**Lac +**	**Lac -**	**Lac +**
pH	6.14 ± 0.01	6.13 ± 0.01	6.12 ± 0.01	6.07 ± 0.02	5.73 ± 0.14	5.59 ± 0.02
Ammonia	7.74 ± 0.67	8.41 ± 0.59	9.68 ± 0.57	4.10 ±0.40*****	6.73 ± 0.84	9.91 ± 1.56
Acetic acid	16.1 ± 0.47	17.3 ± 1.46	18.6 ± 1.02	18.3 ± 0.98	21.3 ± 0.88	21.5 ± 0.73
Propionic acid	5.33 ± 0.18	5.58 ± 0.51	5.74 ± 0.31	5.82 ± 0.31	11.3 ± 0.99	11.4 ± 1.62
isoButyric acid	0.27 ± 0.01	0.28 ± 0.02	0.26 ± 0.01	0.26 ± 0.01	0.27 ± 0.01	0.31 ± 0.02
***n*-Butyric acid**	1.24 ± 0.04	1.24 ± 0.10	1.22 ± 0.06	1.22 ± 0.07	2.55 ± 0.09	2.25 ± 0.40
isoValeric acid	0.25 ± 0.01	0.25 ± 0.01	0.22 ± 0.01	0.22 ± 0.01	0.22 ± 0.01	0.25 ± 0.02
Lactic acid	1.16 ± 0.09	1.44± 0.06*****	1.57 ± 0.16	1.67 ± 0.11	0.98 ± 0.09	1.18 ± 0.16
Total SCFA	24.4 ± 0.77	26.2 ± 2.06	27.7 ± 1.51	27.6 ± 1.41	36.7 ± 1.84	37.0 ± 2.50

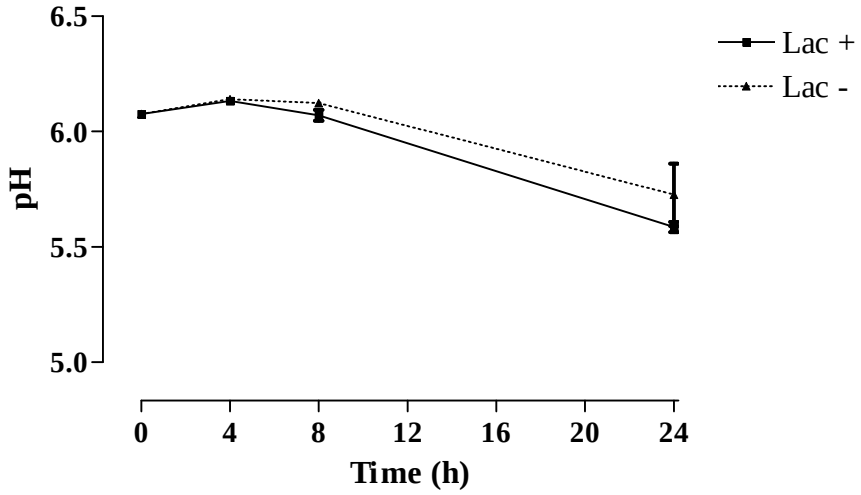
* indicates significant difference ($P < 0.05$); values are the mean of four replicates

Figure 10.6: Ammonia concentration (mmol/L) in dog faecal cultures added (Lac +) or not (Lac -) with a strain of *L. animalis*. Values are the mean of four replicates ± SEM.



* indicates significant difference ($P < 0.05$)

Figure 10.7: pH in dog faecal cultures added (Lac +) or not (Lac -) with a strain of *L. animalis*. Values are the mean of four replicates ± SEM.



Enterococci were significantly reduced and lactobacilli increased ($P < 0.05$) by *L. animalis* addition throughout the study. After 24 h of incubation, *C. perfringens* counts were significantly reduced in the bottles containing the *L. animalis* strain ($P < 0.05$). Bifidobacteria and coliforms counts were not affected by treatment.

After 8 h of incubation, ammonia was significantly reduced (4.10 vs 9.68 mmol/L; $P < 0.001$) by *L. animalis*. At 4 h, lactic acid concentration was significantly higher in faecal cultures containing the *L. animalis* strain (1.44 vs 1.16 mmol/L; $P < 0.05$).

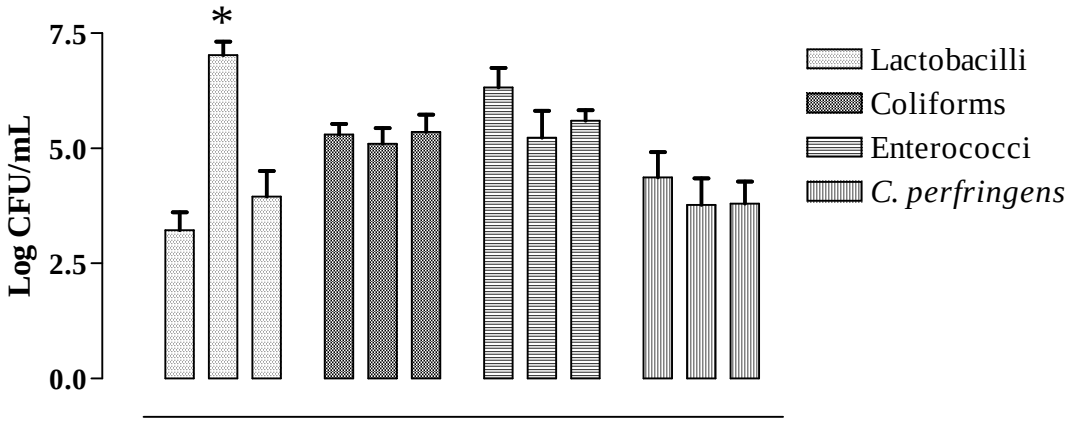
10.3 In vivo trial

All dogs remained in good health during the administration of the *L. animalis* strain. Faecal microbial counts before and after administration of the probiotic strain are shown in Table 10.4 and Figure 10.8. On Day 11, lactobacilli faecal counts were significantly higher than at trial start (6.99 vs. 3.35 Log CFU/g of faeces; $P < 0.001$). The *L. animalis* probiotic strain was recovered in all faecal samples collected on Day 11 and in faeces of four dogs out of nine at Day 15. Ammonia and SCFA faecal concentrations were not influenced by the probiotic strain (Table 10.5).

Table 10.4: Counts (Log CFU/mL) of viable coliforms, enterococci, *Clostridium perfringens*, bifidobacteria, and lactobacilli in dog faecal samples of nine dogs before (T0), 1 (T1) and 5 (T2) days after a 10 d administration of a strain of *L. animalis*.

		Lactobacilli	**Coliforms**	**Enterococci**	***C. perfringens***
	T0	2,30	5,15	4,60	3,00
1	**T1**	6,20	4,90	< 4	3,00
	T2	< 2	5,00	4,81	3,60
	T0	2,48	4,90	7,71	3,00
2	**T1**	7,08	3,79	6,74	2,41
	T2	3,78	4,67	5,41	3,38
	T0	3,11	5,00	5,00	3,11
5	**T1**	7,30	5,41	8,08	3,00
	T2	3,96	6,51	6,00	3,00
	T0	4,08	6,00	8,24	3,18
6	**T1**	7,73	4,26	5,30	2,00
	T2	4,04	6,00	7,00	3,00
	T0	< 2	5,26	5,78	3,60
7	**T1**	5,67	4,61	< 4	3,00
	T2	2,30	4,41	4,81	2,36
	T0	3,36	6,70	7,00	5,70
8	**T1**	8,20	6,62	5,90	3,30
	T2	4,43	6,04	5,60	2,48
	T0	4,48	5,18	6,30	7,30
12	**T1**	6,95	4,80	6,00	7,26
	T2	4,00	3,70	5,12	6,00
	T0	3,54	4,20	5,30	6,30
16	**T1**	7,92	6,85	5,45	6,00
	T2	7,00	7,18	5,90	6,38
	T0	4,70	5,30	7,00	4,18
17	**T1**	6,16	4,66	4,00	4,00
	T2	5,08	4,68	5,76	4,00

Figure 10.8: Counts of viable bacteria in the faeces of nine dogs before (day 0), 1 (day 11) and 5 days (day 15) after a 10 days administration of *L. animalis* LA4 (log CFU/g); values are means ± S.E.M..



* indicates significant difference ($P < 0.001$)

Table 10.5: Ammonia and short-chain fatty acids concentrations in the faeces of nine dogs before (T0), 1 (T1) and 5 (T2) days after a 10 d administration of a strain of *L. animalis* (mmol/L)

	Ammonia	**Acetic acid**	**Propionic acid**	**iso-Butyric acid**	***n*-Butyric acid**
Day 0	43.8	76.3	48.8	2.02	16.2
Day 11	43.7	71.1	40.3	2.21	17.5
Day 15	44.7	67.0	37.3	2.15	14.8
Pooled SEM	7.75	5.32	4.63	0.40	2.04
Anova *P*	0.54	0.27	0.95	0.73	0.99

11. Probiotic study: Discussion

11.1 In vitro trial

The bacterial faecal counts of the 17 dogs screened for the selection of the animals to be used in the probiotic trial confirmed that a very high variability exists in the intestinal bacterial concentrations within the canine population. In the present study, dogs were housed in different environments and fed different commercial dry diets but it is known from the literature (Suchodolski et al., 2005) that the intestinal microflora of dogs shows big differences even among dogs similarly housed and fed identical diets. Moreover, while there is some evidence that the diet may influence the intestinal microflora composition in dogs (Zentek, 2000), Simpson *et al.* (2002) concluded that individual dogs have their own characteristic faecal bacterial microflora and that this is unique and stable, and not influenced by the diet.

The strain selected for the feeding trial was identificated as *L. animalis*, a common inhabitant of canine gut (Kim & Adachi, 2007; Fujisawa & Mitsuoka, 1996).

L. animalis was not previously studied for its probiotic properties in dogs. However, Ehrmann *et al.* (2002) tested 112 strains of lactic acid bacteria for their use as a probiotic supplement in poultry. Their *in vitro* and *in vivo* trials demonstrated the ability of *L. animalis* to grow in presence of bile salts, tolerate acidic pH, and persist in the crop and caecum of ducks for a period of 18 days. The ability to survive under low pH conditions and high bile salts concentrations are desirable features for a successful passage through the gastrointestinal tract, which is a recognized prerequisite for potential probiotics (Dunne *et al*., 2001).

In another study, Chen & Yanagida (2006) found a *L. animalis* strain (C060203) capable of producing, in presence of surfactants, a bacteriocin-like inhibitory substance with a wide inhibitory spectrum against Gram-positive bacteria. In a study conducted by Gusils *et al.* (1999) with chickens intestinal fragments, *L animalis* was able to inhibit the adhesion of *S. pullorum*, *S. enteritidis*, and *S. gallinarum* to host-specific epithelial fragment. The production of substances capable of inhibit growth or adhesion of phatogenic microorganism is a desired characteristic of probiotic strains.

These data, apart from the absence of studies in dogs, suggested a potential use as a probiotic for the isolated *L. animalis* strain.

In the present study, addition of *L. animalis* determined a significant decrease of enterococci throughout the study and *C. perfringens* after 24 hours of incubation, while lactobacilli were significantly increased throughout the study. *C. perfringens* is a potential pathogen, naturally

harbored in the digestive tract of dogs. Some strains of *C. perfringens* are able to produce toxins which can cause diarrhoea. In the dog, *C. perfringens* has been associated with 28-34% of diarrheic cases, ranging in severity from mild to potentially fatal (Kather *et al.*, 2005). Enterococci are commensal bacteria of the gastro-intestinal tract of dogs. However, they are frequently isolated in intestinal content of dogs with small intestinal bacterial overgrowth (SIBO) (Rutgers *et al.*, 1995), and in case of nosocomial infections. Therefore, reducing the number of clostridia and enterococci in the intestine could decrease the risk of intestinal disease in dogs.

The competitive exclusion of pathogens in humans and animals is a well-known beneficial effect of probiotics lactic acid bacteria (Rolfe, 2000; Reid and Burton, 2002). However, effects of various lactic acid bacteria species on intestinal pathogen bacteria are different from each other. Perelmuter *et al*. (2008, in press), in a study conducted with a strain of *L. murinus* isolated from the canine intestinal tract, demonstrated its ability to inhibit the growth of two *C. perfringens* and two *E. coli* strains during an agar spot test. Similarly, several strains of *L. reuteri* (McCoy & Gilliland; 2007), isolated from canine faeces, inhibited the growth of *S. typhimurium*. However, in another study conducted by Swanson *et al.* (2002a), a *L. acidophilus* strain administered to dogs did not affect *C. perfringens* and *E. coli* faecal concentrations. The absence, in environments like faecal cultures or the microbial ecosystem of the gastrointestinal tract, of significant effects on bacterial counts could be due to the complexity and hostility of the environment, which hardly could be influenced by a single bacterial strain.

Ammonia is a toxic compound which is produced in the hindgut as a result of bacterial proteolytic activity and has a negative impact on intestinal mucosa and enterocytes (Blachier *et al.*, 2007). In vitro, the probiotic strain reduced ammonia concentration by 58% after 8 h of incubation. This finding could be the consequence of the partial inhibition of proteolytic bacteria by the probiotic strain, as suggested by the reduction of *C. perfringens* observed after 24 h of incubation. Similarly, during a 24 h in vitro study with swine cecal chyme (Piva *et al*., 2005), a *L. brevis* strain reduced ammonia concentrations after 8 h of incubation but not after 24 h. Both studies suggest that LAB strains can reduce in vitro intestinal proteolysis and ammonia concentrations but that this effect disappears when energy sources such as starch and other fermentable carbohydrates are depleted (Russel *et al*., 1983). In the study by Piva *et al.* (2005), the reduction of ammonia concentrations was more effective when the tested LAB strains were associated to lactitol, a non-digestible disaccharide. In the present study, the dry food contained after enzymatic digestion only traces of starch, thus limiting the energy available to lactic acid bacteria. This seems to be confirmed by the fact that the addition of the probiotic did not affect pH and increased lactic acid only at 4 h, but not later during the study.

11.2 In vivo trial

The faecal recovery of *L. animalis* LA4 used as a probiotic and the high lactobacilli faecal counts on day 11 showed that LA4 survived gastrointestinal passage. In fact, after 10 days of probiotic administration, faecal counts of lactobacilli increased by about 4 log units. On day 15, lactobacilli counts dropped close to their initial values. In two studies with *Lactobacillus* sp. strains in dogs (Weese and Andersen, 2002; Baillon *et al*., 2004), the probiotic strains were recovered in the faeces during administration but not a few days after.

When the *L. animalis* strain was fed to adult dogs, faecal counts of *C. perfringens*, coliforms and enterococci were not significantly influenced by the probiotic. The relative low number of animals used in this study and the high individual variability may explain the lack of a significant effect of the probiotic on faecal counts of undesired microbes. However, enterococci and *C. perfringens* showed a trend towards a numerical reduction after administration of the probiotic, and the reduction of faecal enterococci came close to a significant difference (*P* of the model = 0.08). In another study, when a *Lactobacillus acidophilus* strain was fed to dogs (Baillon *et al*., 2004), clostridia faecal counts were significantly reduced during probiotic administration. Because enterotoxigenic *C. perfringens* can be responsible of diarrhoea in dogs (Weese *et a*l., 2001), lactic acid bacteria probiotics might help reducing the incidence in dogs of *C. perfringens* enteric disease and environmental shedding. The latter might be important for dog-owners, because *C. perfringens* enterotoxin has been associated with diarrhoea and food poisoning in humans (Li *et al*., 2007).

Changes in the intestinal microflora were not reflected by differences in faecal concentrations of ammonia and SCFA. From the literature, it is known that the concentration of bacterial metabolites can vary dramatically while digesta move from the colon to the rectum, especially for those metabolites, such as ammonia and volatile fatty acids that are able to cross the intestinal mucosa and be absorbed. As such, faeces might not reflect the changes in the concentration of metabolites that the probiotic might have induced in the hindgut (Stevens & Hume, 1998).

12. Prebiotic study: Materials and methods

12.1 In vitro fermentation

Fresh faeces from eight healthy adult dogs (household dogs, different breeds, fed different commercial dry diets and living in different environments; between 1 and 5 years of age), which had not received antibiotic treatment for at least 3 months prior to experimentation, were collected immediately after excretion in sterile vessels, homogenized using stomacher for 5 minutes, and then suspended 10% (w/v) in half-strength pre-reduced Wilkins Chalgren Anaerobe Broth (WCAB 0.5x).

Faecal suspension was used to inoculate (3,3% v/v) five 30 mL anaerobic serum bottles (containing 21 mL of medium prepared according to Sunvold et al., 1995), and five 10 mL glass syringes (containing 4,85 mL of medium) per treatment.

The composition of the medium used to culture the microflora is presented in Table 12.1. All medium components, except the vitamin mixes, were added before autoclaving. The vitamin mixes were aseptically added after they were filter-sterilized.

Table 12.1: Composition of medium used (Sunvold et al., 1995, modified).

Component	**Concentration in medium**
	ml/L
Solution A [(1)]	330.0
Solution B [(2)]	330.0
Trace mineral solution [(3)]	10.0
Water-soluble vitamin mix [(4)]	20.0
Folate:biotin solution [(5)]	5.0
Riboflavin solution [(6)]	5.0
Hemin solution [(7)]	2.5
Distilled water	302.5
	g/L
Yeast extract	0.5
Trypticase	0.5
Na_2CO_3	4.0
Cisteyn HCl H_2O	0.5

[(1)] Composition g/L: NaCl, 5.4; KH_2PO_4 2.7; $CaCl_2$ H_2O 0.16; $MgCl_2$ $6H_2O$, 0.12; $MnCl_2$ $4H_2O$, 0.06; $CoCl_2$ $6H_2O$, 0.06; $(NH_4)_2SO_4$, 5.4.
[(2)] Composition g/L: K_2HPO_4, 2,7.
[(3)] Composition mg/L: EDTA (disodium salt), 500; $FeSO_4$ $7H_2O$, 200; $ZnSO_4$ 7 H_2O, 10; $MnCl_2$ 4 H_2O, 3; H_3PO_4, 30; $CoCl_2$ 6 H_2O, 20; $CuCl_2$ $2H_2O$, 1; $NiCl_2$ $6H_2O$, 2; Na_2MoO_4 $2H_2O$, 3.
[(4)] Composition mg/L: thiamin HCl, 100; d-pantothenic acid, 100; niacin, 100; Pyridoxine, 100; p-aminobenzoic acid, 5; vitamin B_{12}, 0,25.
[(5)] Composition mg/L: folic acid, 10; d-biotin, 2; NH_4HCO_3, 100.
[(6)] Composizione: riboflavin 10 mg/L in 5 mmol/L of HEPES.
[(7)] Hemin, 500 mg/L in 10 mmol/L NaOH.

Bottles and syringes also contained an in vitro digested commercial dry food for dogs at the final concentration of 10 g/L. The in vitro digested food simulates the undigested fraction of the diet that reaches the hindgut and is obtained by in vitro digestion (2 h incubation with HCl + gastric lipase + pepsin followed by a 4 h incubation with pancreatin + bile salts) of a commercial dry food for adult dogs (Vervaeke et al., 1989; modified method) (Table 12.2).

Table 12.2: Analyzed chemical composition of the commercial dry dog food used in the study before and after enzymatic digestion (%DM)[a].

	Before digestion	After digestion
Crude protein	20.5	10.3
Ether extract	11.4	2.4
Starch	42.2	traces

[a] Food in vitro total digestibility was 78.1%

In total, 6 substrates were studied: fructo-oligosaccharides (FOS), inulin, pectins, gluconic acid, lactitol, and a fiber rich ingredient (chicory). Substrates were added at the final concentration of 1g/L (inulin, FOS, pectin, lactitol, gluconic acid) or 4g/L (chicory). These concentrations should reflect the amount of fiber that reaches the hindgut when non-digestible oligosaccharides (NDO) and fiber-rich ingredients are included in the feed at a concentration of 1% and 4%, respectively. In fact, if we estimate that the average digestibility of a commercial dry food for dogs is 90% and assuming that all soluble fiber will reach the large intestine, the ratio between the undigested food fraction (in vitro digested diet) and the soluble fiber source in the hindgut will approximately be 10:1 for NDO and 10:4 for fiber-rich ingredients.

Five bottles and five syringes were prepared without any experimental substrate as a negative control, while lactitol was used as positive control in all the experiments.

Faecal cultures (bottles and syringes) were incubated for 24 h at 39°C under anaerobic conditions and samples of fermentation fluid were collected from each bottle at 0, 6, and 24 hours for analysis (ammonia, SCFA, pH, lactobacilli, enterococci, coliforms).

Gas production was measured as described by Menke et al. (1979) measuring the amount of gas produced in the glass syringes throughout the 24 h of the study.

12.2 Chemical and microbiological analyses

Ammonia in samples of fermentation fluid was measured using a commercial kit (Urea/BUN – Color, BioSystems S.A., Barcelona, Spain).

Short-chain fatty acids (SCFA) in samples of fermentation fluid were analyzed by gas chromatography (Varian 3400, Varian Analytical Instruments, Sunyvale, CA 94089, USA) with Carbopack B-DA/4% CW 2M and 80/120 packed column (Supelco, Sigma Aldrich s.r.l., 20151 Milano, Italy). The samples of fermentation fluid were centrifuged (3,000 × g, 15 min.) and 1 mL of the supernatant was deproteinized with 50 µL perchloric acid (Merck, Darmstadt, Germany). Finally, samples of fermentation fluid were centrifuged (14,000 x g, 10 min.) and added with pivalic acid as an internal standard (Fussel and McCalley, 1987) prior to injection.

The second sample of fermentation fluid was homogenized and serially diluted with prereduced half-strength WCAB 0.5x. From each of the dilutions, 0.1 mL was plated in triplicate onto selective media: MacConkey Agar (Merck, Darmstadt, Germany) for coliforms, LAMVAB Agar (Hartemink et al. 1997) for lactobacilli, and Azide Maltose Agar (Biolife, Milano Italy) for enterococci. MacConkey agar plates were incubated aerobically at 37°C for 24 h; Azide agar plates in aerobiosis for 48 h; LAMVAB Agar plates were incubated anaerobically at 37°C for 48 h.

12.3 Statistical analysis

A modified Gompertz bacterial growth model was used to fit gas production data and obtain the following gas production parameters: total gas production (mL, the amount of gas produced during the 24 h study), maximum rate of gas production (mL/h, the highest velocity reached by gas production), log time (h, the duration of gas production phase), and lag time (h, the time between fermentation start and when bacteria start producing gas)

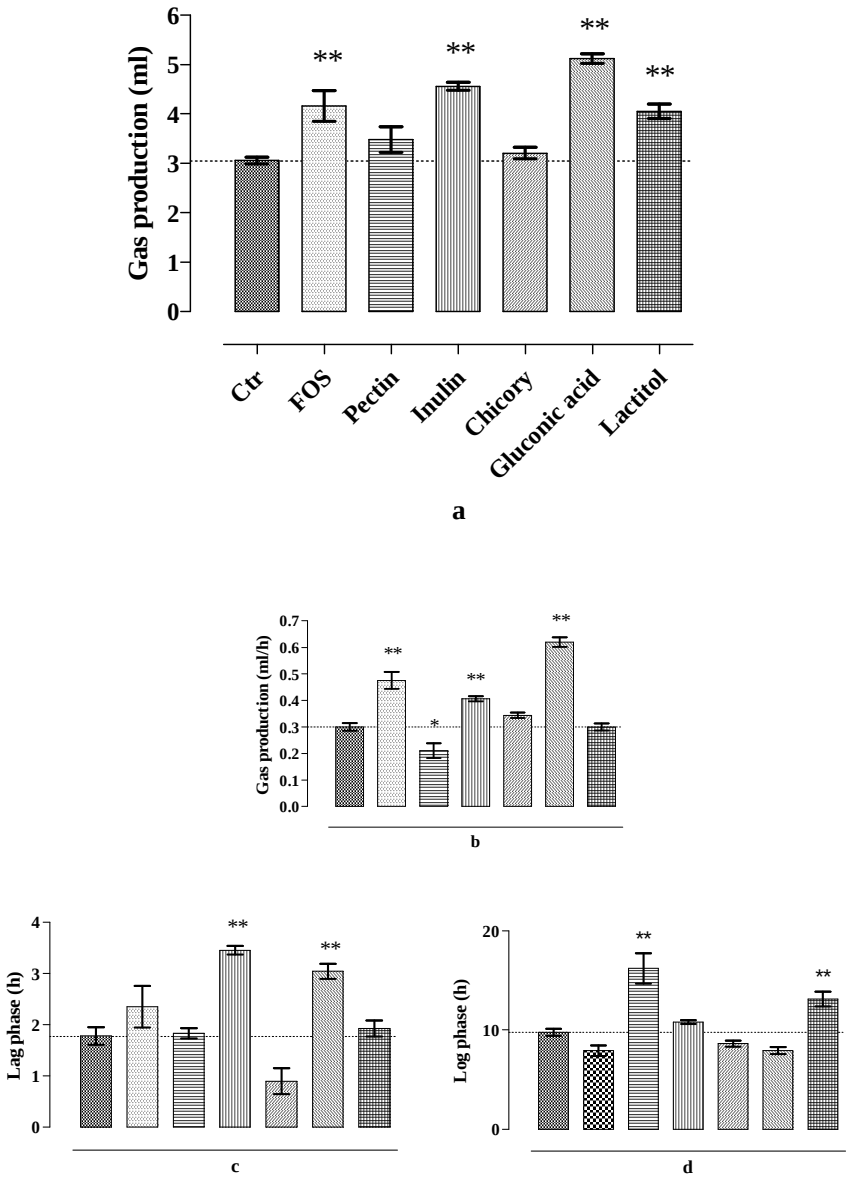
Gas production parameters, ammonia, pH, and SCFA data, as well as counts of coliforms, lactobacilli, and enterococci were analyzed by one-way ANOVA, with the Dunnett test as the post test. Each syringe and bottle formed the experimental unit. Differences were considered statistically significant at $P<0,05$.

13. Prebiotic Study: Results

Gas production parameters, pH, SCFA concentrations, and ammonia data are reported from Figure 13.1 to Figure 13.4. Major SCFAs proportion are reported in Table 13.1.

Counts of viable coliforms, lactobacilli and enterococci are reported from Figure 13.5 to Figure 13.7.

Figure 13.1: Gas production parameters. Values are the means of five replicates ± SEM.

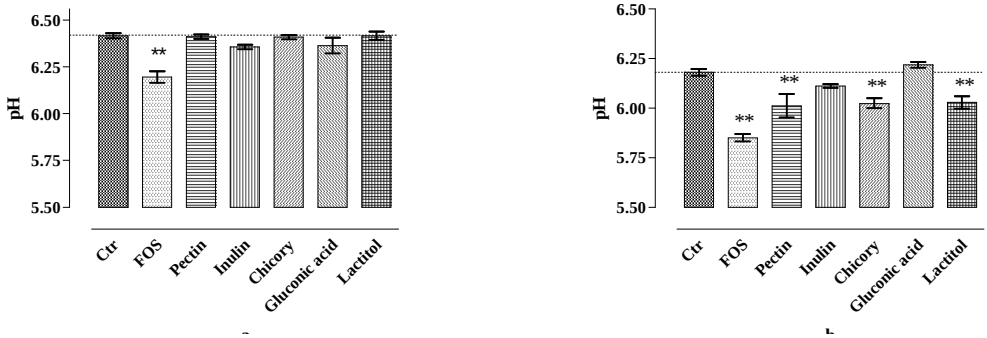


Legend:

- **a:** total gas production
- **b:** maximum rate of gas production
- **c:** lag time
- **d:** log time

* indicates significant difference ($P < 0.05$)
** indicates significant difference ($P < 0.01$)

Figure 13.2: : pH in faecal slurry. Values are means of five replicates ± SEM.



Legend:

- **a:** 6 h
- **b:** 24 h

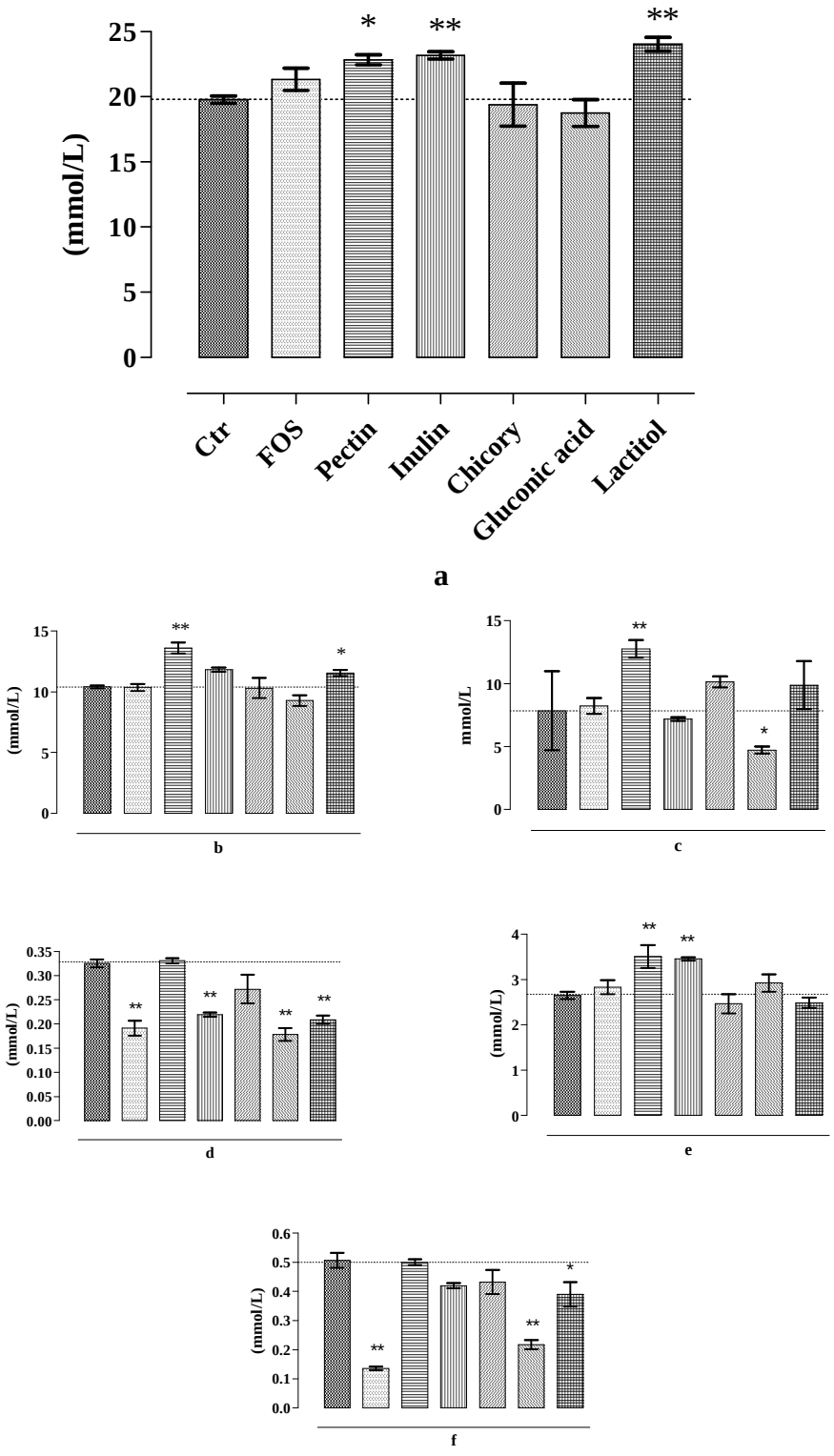
* indicates significant difference ($P < 0.05$)
** indicates significant difference ($P < 0.01$)

FOS, inulin, gluconic acid and lactitol significantly increased total gas production, compared with the control diet (+ 45%, + 50%, +68%, and +26%, respectively). While FOS, inulin, and gluconic acid increased velocity of gas production (+ 33%,+37%, and +107%, respectively), pectin resulted in lower velocity than control (- 30%). Pectin and lactitol determined a significant increase in the duration of the Log phase (+ 65%, and + 34%, respectively).

pH was significantly reduced by FOS throughout the study, while pectin, chicory, and lactitol significantly reduced pH after 24 hours of fermentation.

After 24 hours of fermentation total SCFA were significantly increased by pectin, inulin, and lactitol (+ 15%, +17%, and +19%, respectively). Pectin also increased acetic acid (+31%), lactic + propionic acid (+63%), and n-butyric acid (+36%) concentrations. Lactitol determined a significant increase in acetic acid (+10%), while propionic + lactic acid showed only a tendency to increase ($P = 0.0537$). The addition of inulin resulted in a significant increase in n-butyric acid (+34%) and in a tendency to increase of acetic acid ($P = 0.0876$). FOS and chicory did not affect SCFA concentrations. Gluconic acid determined a significant reduction of propionic + lactic acid concentration (-40%).

Figure 13.3: SCFA in faecal slurry (mmol/L). Values are means of five replicates ± SEM.



Legend:

- **a**: concentration of total SCFA in faecal slurry
- **b**: acetic acid concentration;
- **c**: lactic + propionic acid concentration;
- **d**: iso-butyric acid concentration;
- **e**: nor-butyric acid concentration;
- **f**: iso-valerianic acid concentration

* indicates significant difference ($P < 0.05$)
** indicates significant difference ($P < 0.01$)

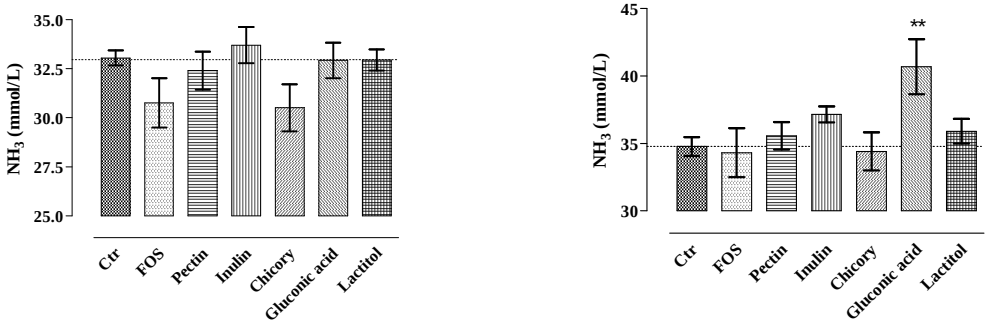
Table 13.1: Molar proportion of major SCFA (acetate : propionate+lactate : butyrate). Values are means of five replicates ± DS.

	Acetic	Propionic+lactic	Butyric
Control	55.7 ± 2.49	30.5 ± 2.69	13.9 ± 0.91
FOS	49.4 ± 2.18 *	37.1 ± 2.14 *	13.5 ± 0.90
Pectin	57.2 ± 1.79	28.0 ± 1.99	14.8 ± 2.28
Inulin	52.6 ± 0.35	32.0 ±0.28	15.4 ± 0.16
Chicory	55.8 ± 2.69	30.8 ± 2.94	13.3 ± 0.73
Gluconic acid	55.8 ± 0.97	26.8 ± 0.81	17.5 ± 0.80 *
Lactitol	52.1 ± 5.68 *	36.2 ± 6.53 *	11.6 ± 1.40 *

* indicates significant difference ($P < 0.05$)

The addition of FOS and lactitol determined a significant decrease in the molar proportion of acetic acid, while increased propionate + lactate one; lactitol reduced also butyrate molar proportion. Gluconic acid significantly increased butyrate molar proportion.

Figure 13.4: Ammonia concentration in faecal slurry (mmol/L). Values are means of five replicates ± SEM.



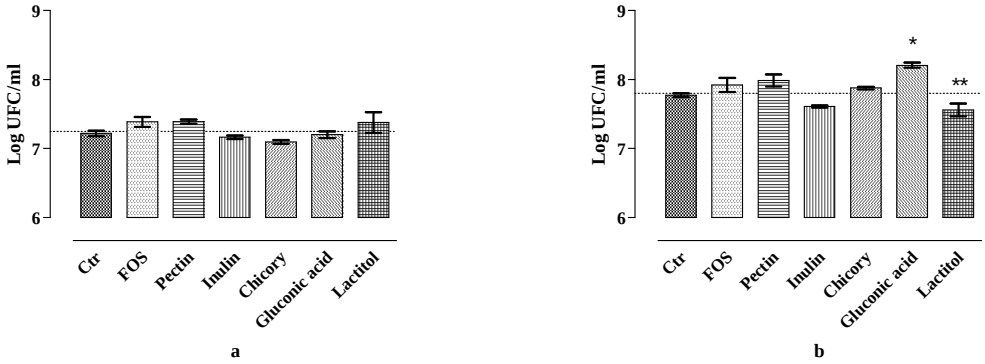
Legend:

- **a:** 6 h
- **b:** 24 h

* indicates significant difference ($P < 0.05$)
** indicates significant difference ($P < 0.01$)

Ammonia concentration in the faecal slurry wasn't affected by the addition of any prebiotic substance after 6 hours of fermentation, while it was significantly increased by gluconic acid after 24 hours.

Figure 13.5: Counts of viable coliforms (Log CFU/mL).

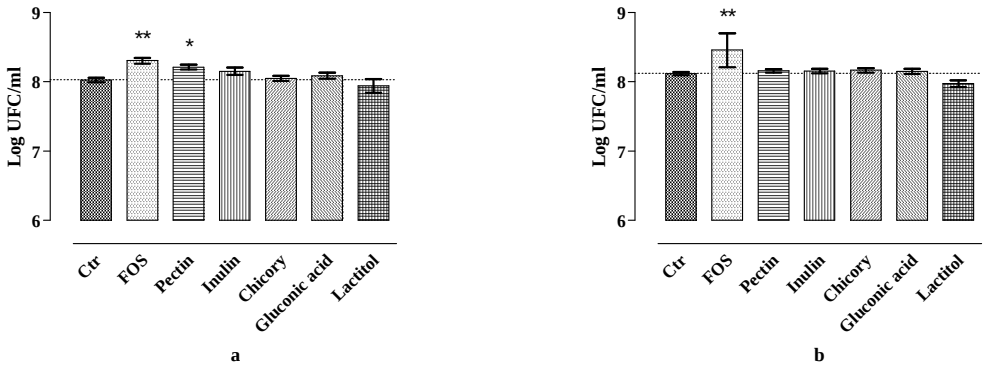


Legend:

- **a:** 6 h
- **b:** 24 h

* indicates significant difference ($P < 0.05$)
** indicates significant difference ($P < 0.01$)

Figure 13.6: Counts of viable enterococci (Log CFU/mL).

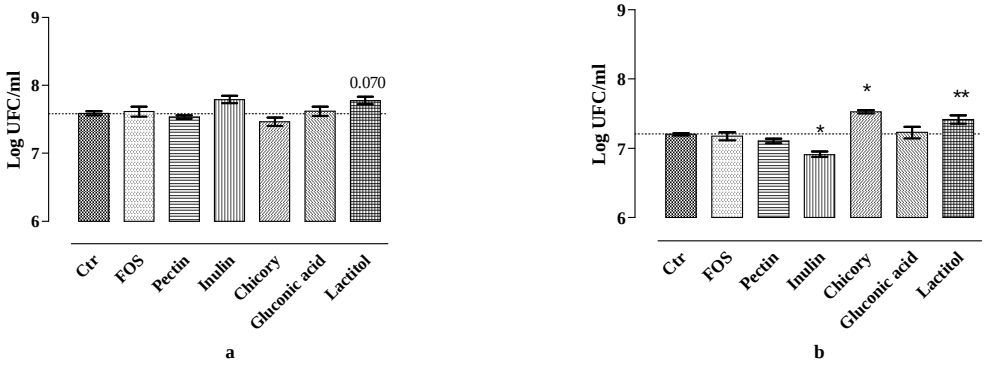


Legend:

- **a:** 6 h
- **b:** 24 h

* indicates significant difference ($P < 0.05$)
** indicates significant difference ($P < 0.01$)

Figure 13.7: Counts of viable lactobacilli (Log CFU/mL).



Legend:

- **a:** 6 h
- **b:** 24 h

* indicates significant difference ($P < 0.05$)
** indicates significant difference ($P < 0.01$)

Addition of FOS, after 6 and 24 h of fermentation, and pectin, after 6 h, significantly increased enterococci counts. Lactitol, after 24 h of fermentation, determined a significant reduction in coliforms; moreover, at the same time point, lactitol significantly increased lactobacilli. After 24 h of fermentation, the addition of inulin, while having no effect on enterococci end coliforms counts, significantly decreased lactobacilli counts. Lactobacilli counts were higher than control with supplemented chicory and lactitol ($P < 0.05$) after 24 hours of fermentation, and tended to be grater after 6 hours of fermentation with lactitol ($P = 0.0701$).

14. Prebiotic study: Discussion

In *in vitro* systems, gas production is generally recognized as a product, and an index, of the activities of the total microflora present in the fermentors. The amount and composition of gas produced could be affected by many variables such as the chemical structure of the carbohydrate (chain length, monosaccharide compositions) as well as the composition of the fermentative microflora (Spears *et al*., 2007). In literature few data exist about the gas production properties of prebiotic substances, and rarely data are compared to a negative control. In a study by Ghoddusi *et al.* (2007), who evaluated the effects of different carbohydrate sources on gas production by human fecal inocula, inulin determined the highest gas production compared to other carbohydrates, particularly if compared to FOS, which produced the lower amount of gas. Rycroft *et al.* (2001), comparing the prebiotic properties of several oligosaccharides using human fecal microflora, found the highest levels of gas production with inulin, lactulose, FOS and XOS. In a previous study by Piva *et al*. (1996) with swine cecal microflora, lactitol, compared to a negative control, determined a significant increase in the amount of gas produced when added to a low-fiber diet. In our study, inulin, FOS, gluconic acid, and lactitol increased the amount of gas produced, while pectins and chicory did not; conversely, in an *in vitro* trial with a canine fecal inoculum conducted by Swanson *et al*. (2001), pectins, when compared with fruits and vegetables fibers, determined the highest amount of gas production.

The discrepancy of some results obtained in the present trial with those reported from previous studies could be explained taking into account two main factors. First of all, we have to consider the “bifidogenic nature” of prebiotics which, in fact, selectively stimulate bifidobacteria (Gibson *et al*., 1994). Bifidobacteria are not frequently isolated from dog faeces. Kim & Adaki (2007) screened 36 samples of dog faeces for lactic acid bacteria and bifidobacteria counts and found bifidobacteria only in 6.8% of the samples. In the study by Greetham *et al.* (2002) bifidobacteria were not detectable at all in the faeces of four dogs. Therefore, the inconstant presence of bifidobacteria in the experimental faecal inoculum could explain the different results obtained in different studies.

Moreover, the different chemical structure of each prebiotic could affect the selectivity and intensity of fermentation. Roberfroid (2001) compared the fermentation of inulin and FOS by different bacterial specie; in his study, FOS were more intensely fermented by all the bacterial species tested, resulting in a lower selectivity of fermentation but in a higher intensity. A similar comparison, between FOS and inulin, was conducted by van de Wiele *et al*. (2007), who obtained the same results of Roberfroid (2001). Fructans of longer chain length, like inulin, are less (or

more slowly) fermentable than compounds of shorter chain length. However, if administered for longer periods of time they show a more pronounced beneficial effect than oligofructoses of a shorter chain length (van de Wiele *et al.*, 2007). Olano-Martin *et al.* (2002) investigated the prebiotic properties of pectins and pectic-oligosaccharides (obtained from controlled hydrolysis of pectins) with different degrees of esterification. In this case, too, molecules with shorter chain length (pectic-oligosaccharides) were more intensely fermentable. Moreover, the authors detected a clear influence of the degree of esterification on fermentation, with highly methylated carbon sources giving lower growth rates than the lower methylated ones.

Among the substrates tested in the present study, pectin, inulin, and lactitol significantly increased total SCFA concentrations after 24 hours of fermentation (Figure XX). It is well konown that substrates (dietary fibre) which escape digestion and reach the terminal tract of the intestine are broken down by the resident microflora to the SCFA acetate, propionate, butyrate and the gases hydrogen and carbon dioxide (Wang & Gibson, 1993). Lactate is an intermediate which is also converted to SCFA by the intestinal microflora (Hume *et al.*, 1995). When SCFA are produced by bacterial fermentation in the intestine they are rapidly absorbed, with only 5% to 10% being excreted in the faeces (Ruppin *et al.*, 1980). The role of SCFA comprehends various local and systemic effects. Increases in intestinal SCFA results in decreased pH, which influences the composition of colonic microflora, increases absorption of minerals (butyrate and propionate stimulate fluid absorption of calcium, magnesium, and other cations in the colon; Scholtz-Arhens & Schrezenmeir, 2002) and reduces ammonia absorption by the protonic dissociation of ammonia and others amines (Cummings, 1981). Of the three major SCFA produced (acetate, propionate, and butyrate), butyrate is the major energy source for the colonocytes and exerts an important trophic effect increasing colonic crypt depth (Velázquez *et al.*, 1997).

Published data about the influence on SCFA production of the addition of prebiotic substances in dog are contrasting and, above all, few works reported a direct comparison against a control (no supplemental prebiotic) on SCFA production. Flickinger and co-workers (2003) and Propst and co-workers (2003) studied the effects of the addition of oligofructose in the diet of dogs. Propst *et al.* (2003) detected a significant increase of all the SCFA produced, compared to the control group, while Flickinger *et al.* (2003) reported a significant increase only in propionate concentration. The different results could be explained by differences in the intestinal microbial population of the dogs that have been used. Other studies (Sunvold *et al.*, 1995; Vickers *et al.*, 2001; Swanson *et al.*, 2001) reported in literature compare the effects of several prebiotic substances without any control group. These results are hardly interpretable because none of the studies provide a “no addition effect” to which compare “prebiotic” results.

Molar proportion of SCFA can provide additional information about the quality of the substrate. In our study, molar proportions of butyric acid were low, compared with average data from the literature (60:20:20) (Cummings *et al*., 1979), for all substrates tested. The carbohydrates present in the diet could influence not only the amount but also the molar proportion of the acid produced during fermentation. Carbohydrates that are reported in literature to stimulate the formation of butyric acid are resistant starch fractions and β-glucans, while pectin and xylan are associated with low butyrate-high acetate production (Knudsen *et al*., 2003). In our study, FOS and lactitol determined a significant increase of the molar proportion of propionic + lactic acid, accompanied by a proportional reduction of acetic acid. FOS behaviour was in agreement with data from the literature. In fact, Vickers *et al*. (2003) and Liong & Shah (2005) reported a similar variation in SCFA molar proportion after the addition of FOS. Conversely, lactitol data are in disagreement with the findings of Nilsson & Nyman (2005) who reported different molar proportions. Macfarlane & Gibson (1995) demonstrated that environmental and microbial variables, particularly carbonium availability and growth rate, could affect SCFA production by pure cultures of colonic microorganism. Their results showed that, in different experimental conditions, the same bacterial specie can produce different SCFA molar ratios from the same substrate..

After 24 hours of fermentation, BCFA were lowered by the addition of FOS, inulin, gluconic acid, and lactitol. Branched SCFAs (iso-butyrate and iso-valerate) are, together with ammonia, phenols and amines, the end-products of the fermentation of dietary proteins and amino acids by proteolytic bacteria and are responsible for the odour of faeces. At 24 h, FOS, inulin, gluconic acid and lactitol significantly reduced iso-butyrate concentrations ($P < 0.001$) and FOS and gluconic acid also reduced iso-valerate concentration ($P < 0.001$).

Gluconic acid significantly increased ammonia concentration after 24 h of fermentation, while the other treatments did not affect ammonia concentration. Literature data about the ammonia concentration during prebiotic trials are contrasting. Propst *et al.* (2003) detected a significant increase in ammonia concentration in the faeces of dogs fed oligofructose and inulin enriched diets, while in a study by Flickinger *et al.* (2003) ammonia concentration tended to decrease in the faeces of dogs fed the oligofructose supplemented diet. Ammonia is a product of protein catabolism by bacteria. In close systems, like fermentors, energy sources such as starch and other fermentable carbohydrate are rapidly depleted in the initial phase of the fermentation, and protein becomes an energy source for bacterial metabolism.

When lactitol was used, lactobacilli tended to increase after 6 hours and significantly increased after 24 hours, while, at the same time point, coliforms were significantly decreased.

These results are in agreement with some data from literature. Chen *et al*. (2007) investigated the effects of lactitol on intestinal microflora of healthy humans and reported a significant increase in lactobacilli and bifidobacteria after three weeks of administration. A similar study was conducted by Ballongue *et al.* (1997), who detected an increase of probiotic bacteria and a decrease of putrefactive bacteria after the administration of lactitol. Finney *et al.* (2007), on the contrary, did not find any significant effects of lactitol on lactobacilli and enterobacteriaceae, but only a significant increase in bifidobacteria. On the other hand, other studies reported opposite results. Probert *et al*. (2004) investigated the effects of lactitol on human microflora using an in vitro model. In their study, lactitol determined a significant decrease of bifidobacteria and bacteroides and an increase in clostridia.

Chicory, apart from the absence of any sign of increased bacterial activity, determined a significant increase of lactobacilli after 24 hours of fermentation, while inulin determined a significant decrease in lactobacilli at the same time point. The latter is in disagreement with data from literature (van de Wiele *et al*., 2007) which report an increase in lactobacilli counts in presence of inulin. FOS significantly increased enterococci after 6 and 24 hours of fermentation. These findings could be explained by the lack of selectivity of FOS, as previously reported by Robertfroid (2001) and van de Wiele *et al*. (2007). Also pectin determined a significant increase in enterococci counts after 6 hours of fermentation. Langhout *et al.* (1999) tested the effects of two differently methylated citrus pectins on the intestinal microflora of broilers. The effects of pectins varied on the basis of the degree of methylation. The high-methylated one determined a significant increase in enterococci, bacterioides, clostridia and E. coli; the low-methylated one increased only the clostridia number. Gluconic acid determined a significant increase in coliforms counts after 24 hours of fermentation. Few data are present in literature about the effects of gluconic acid on intestinal microflora. In a previous study, Biagi *et al*. (2006) evaluated the effects of gluconic acid on swine intestinal microflora using an i*n vitro* system. The authors detected no significant effect of gluconic acid on microflora at any concentration tested.

As previously stated, the initial bacterial population plays a major role in determining the prebiotic results. In fact, bacterial population composition at the beginning of the study could affect the microbial balance reached at the end, particularly in short-time studies, as *in vitro* ones are, which not supply an adequate adaptation period to the microflora.

On the basis of data collected in the present study lactitol could be considered as a potential prebiotic for dogs. Other substrates need to be tested in other trial to confirm the positive results obtained in the present study and to further investigate the doubtful results.

15. Conclusions

Digestibility study

Results from the present study show that the *in vitro* method developed to predict dog foods digestibility can be considered as an affordable alternative to *in vivo* digestibility trials, thus reducing the utilization of dogs as experimental animals. Nevertheless, further studies will be needed to confirm the positive results observed in this trial.

Probiotic study

The present study indicates that the isolated *Lactobacillus animalis* strain (LA4) was able to survive gastrointestinal passage and transitorily colonized the dog intestine. *In vitro*, the *L. animalis* strain positively influenced composition and metabolism of the intestinal microflora of dogs. These results suggest that *L animalis* LA4 can be considered as a potential probiotic for dogs.

Prebiotic study

Among the tested substrates, lactitol reduced intestinal coliforms and increased lactobacilli. Moreover, lactitol promoted the production of SCFA and decreased the production of BCFA. Further studies, in particular in vivo studies with dogs, will be needed to confirm the prebiotic properties of lactitol and to evaluate the optimal level of its inclusion in a dog diet.

Furthermore, more investigation is needed to evaluate the properties of the other substrates and achieve a better understanding of their effects on the dog intestinal ecosystem.

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