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Analysis of Digestibility, Glycemic Index and Gut Microbiota in Vitro Activity in Different Formulations of Complete Dog Dry Food

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Abstract

The pet food industry for dogs is making considerable progress in the selection of raw materials; the quality of a feed is certainly dictated by the list of ingredients, but other variables for proper nutrition such as the digestibility of macronutrients, the activity of the gut microbiota of an organism fermenting dietary nutrients, and how much the carbohydrates contained affect peak glycemic index are to be considered. In addition, pet food production processes affect the final quality of the feed and its digestibility.

In this study, we compared 4 dry dog feeds from the same manufacturer (A), including one suitable for puppies and three for adult maintenance, to other six dry foods (two for puppies and four for adults) from competing companies by analysis on: starch, protein, dry matter digestibility analysis; glycemic index and load; in vitro fermentability and short-chain fatty acids (SCFAs) activity. The main purpose of this analysis was to highlight how digestibility in comparison with the activity of the gut microbiota, representing a very reliable picture regarding the metabolic utilization of the nutrients.

The results showed that feeds formulated with appropriate production technology and containing fresh ingredients are preferable for the parameters taken into consideration and for the effects on metabolism, digestibility and positive modulatory effects on gut microbiota. Feeding an animal with less digestible dietary principles will not only result in greater excretion of them through the feces but also in dangerous deficiencies with negative health consequences. It is therefore evident that only from an in-depth analysis of the digestibility of a food it is possible to understand what the intestinal fermentation trend may be from a nutritional point of view

Keywords: Digestibility; Glycemic Index; Gut Microbiota; Dog; Dry Food; Vitro Activity

Introduction

Advances in canine and feline nutrition contributed to improved pet longevity and well-being, and dietary fiber has received renewed interest in the pet food industry as it manages to improve the health and intestinal quality of feces. More recently, due to increased awareness of the beneficial effects of dietary fiber on health, as well as the popularity of functional foods and holistic and natural diets, "alternative" carbohydrates have become popular in pet and human diets.

In general, the health benefits of fermentable and soluble fibers are related to increasing digestive viscosity, decreasing gastric emptying rate, increasing satiety, decreasing the glucose absorption rate, reducing blood cholesterol concentration and promoting the development of intestinal

commensal bacteria [1-3]; conversely, nonfermentable fiber may decrease gastric transit time and caloric density of the diet, increase fecal bulk and moisture, and promote a laxative effect [4].

In pet nutrition, rice and rice bran are commonly used ingredients; however, there are still few studies on their potential health benefits. Spears, et al. [5] evaluated nutrient palatability and digestibility, fecal characteristics, blood lipid profile and selected immune mediators in dogs fed dry food containing 12% stabilized rice bran, produced by lipase inactivation, or defatted rice bran. More recently, the fermentation profile of rice bran, alone and in combination with probiotics (Lactobacillus acidophilus 1415B or Bifidobacterium longum 05), was evaluated in an in vitro study using a canine fecal inoculum in an anaerobic, pH-controlled batch culture system [6]. Large variations in postprandial glucose concentration and insulin responses to different foods have been demonstrated in dogs [7,8]. Diets high in carbohydrates and fiber improve peripheral glucose disposal and reduce insulin requirements in insulin-dependent diabetic subjects. Food processing may be especially important for dog foods: the type of food, dry, canned, or soft moist, affects maximum postprandial glucose concentration as much as the time at which the peak occurs [7].

Evaluating the nutritional quality of small animal foods, estimating the digestibility of various nutrients is of paramount importance. In fact, good quality dog food is characterized by high digestibility coefficients of macronutrients, especially protein. In relation to a particular food component (e.g., protein or dry matter), the digestibility of a food can be defined as the ratio of the amount of the component absorbed to the amount ingested. The digestibility coefficient of the food from which the macronutrient comes indicates the percentage of food actually absorbed. This value is reduced by the presence of fiber, which reduces the residence time of the food in the intestine and thus the absorption capacity. The digestibility coefficient in the canine species is virtually constant in lipids and carbohydrates (98% to 90%), while it is more variable for protein (97% in meat and eggs versus 78% in legumes). On average, the digestibility coefficient is 97% for carbohydrates, 95% for lipids and 92% for proteins). *In vivo* determination of protein digestibility is a laborious and expensive analysis, which is why much effort has been devoted to the development of in vitro procedures. The in vitro measurement of nutrient digestibility, obtained using enzyme activity, aims to provide information on the digestibility and thus the quality of nutrients, with techniques that are simple and accurate and, above all, applicable to a wide range of foods, ensuring some reproducibility of results. These aims can be achieved using much lower costs than in vivo testing.

In addition, in recent years, scientific research regarding the gut microbiota of dogs and cats has paid much attention to fecal metabolomics compared to fecal microbiota analysis: the presence or absence of certain metabolites is of greater importance to the intestinal health of dogs than the presence or absence of some specific bacterial strains [9]. By constantly gathering new information about the microbiota, it was realized that possible benefits to the body could come from molecules produced by the microbiota from the materials it processes. Bacteria in the colon ferment dietary nutrients and endogenous secretions that escape digestion and absorption in the small intestine (non-starch polysaccharides, unabsorbed sugars, oligosaccharides, and dietary proteins). The end products of fermentation and bacterial metabolism are short-chain fatty acids (SCFA): acetate, propionate, and butyrate, as well as lactate, carbon dioxide, and hydrogen. The gut microbiota can produce various metabolites from dietary nutrients, and SCFAs are the tools by which the microbiota "talks" with the host, conditions its response, influences its activities, and can contribute to the maintenance or loss of the body's gut homeostasis.

In general, with the consumption of a fiber-rich (soluble) ration, the intestinal microbiota produces more SCFA that improve the host's metabolism and immune function. In contrast, a low-fiber diet induces a growth of bacteria that degrade intestinal mucus leading to an alteration in the integrity of the intestinal mucosa, with an increase in the percentage of Gram-negative bacteria and thus the production of endotoxins with pro-inflammatory effects. Acetic acid promotes the secretion of ghrelin, the hormone that increases hunger and thus food intake. Propionic acid activates intestinal gluconeogenesis and improves systemic glucose homeostasis, while butyric acid has an anti-inflammatory function, increasing levels of regulatory T lymphocytes and gut barrier function.

The purpose of this study was to compare different types of dry dog feeds by evaluating their digestibility, glycemic index, and fermentability.

Materials & Methods

4 dry dog feeds from the same manufacturer (A), including one suitable for puppies and three for adult maintenance, were compared with 6 dry foods (2 for puppies and 4 for adults) from competing companies (B).

The scientific investigation compared them for the following parameters: starch digestibility, dry matter digestibility, protein digestibility, amino acid digestibility analysis, glycemic index and load, in vitro fermentability, and short-chain fatty acids (SCFA).

Glycemic Analysis

Glycemic index is the ability of a food - or rather of carbohydrates, more commonly called simple, complex sugars or carbohydrates - to raise the blood sugar level after a meal. The glycemic index is not an absolute value, rather a level that is measured with reference to a standard measurement: the rise in blood sugar given by the intake of pure glucose.

However, the term refers to specific values: the glycemic index (GI) is technically the speed at which blood sugar increases after taking 50 g of carbohydrates. The GI of 50 g of pure glucose is equal to 100. Foods can be classified according to their glycemic index: up to 40 the glycemic index is considered very low; from to 41 to 55 is considered low; from 56 to 69 the glycemic index is defined as moderate; over 70 is considered high.

Glycemic load (CG) is obtained by multiplying the glycemic index (GI) of carbohydrates by the amount of carbohydrates in the food and provides the most accurate measure of how foods affect the glycemic levels of dogs - Glycemic Load = (Glycemic Index x grams of carbohydrates) ÷ 100. A glycemic load above 20 is considered high; 11-19 medium; 10 or less is considered low [10].

Fermentation Profile Analysis

For the fecal inoculum preparation, we collect 3 fecal samples from 3 healthy adult dogs. They were not given antibiotics for at least 6 months before the study, had no history of gastrointestinal diseases, and were not consumers of probiotic/prebiotic supplements. 30 g of fecal sample (10 g from each donor) was collected in a plastic bag under sterile conditions. First, a reducing solution was added to the bag to obtain a 1:5 dilution in anaerobic phosphate buffered with saline (0.1 m; pH 7.4), which was then homogenized in a Stomacher for 2 min. The obtained fecal products were aliquoted into 5-mL Falcon vials and stored at -80°C.

We proceeded with the in vitro fermentation procedure through a pilot fermenter (Applikon fermentation system, Applikon biotechnology), stirred at controlled pH and temperature, to carry out the anaerobic culture of the batch. The medium used is complex and is based on that used by Zampa, et al. [11]. To simulate canine large intestine conditions, the experiment was performed under anaerobic conditions (the system was permanently gassed with N2-15 ml/min feed), at 39°C ± 1°C and pH 6, 80 ± 0.2, for a period of 24 h. The stirrer was set at 50/55 rpm. During fermentation, a 10-mL aliquot of sample was collected at three specific times: T0, T6 and T24 h, i.e., at the beginning of fermentation (T0), after 6 h of fermentation (T6) and after 24 h of fermentation (T24) for bacterial enumeration by real-time PCR and quantification of short-chain fatty acids. Samples were stored at -20°C before analysis.

Inoculation of different products into the fermentation system: fermentation containing fecal slurry (1%) was named and inoculated as follows: Complete Feed with 0.5% (i.e., 5 g) of Complete Feed as inoculum. The chosen substrate concentration simulates the amount expected for the actual use of the product.

For the numeration of the selected bacterial population by Real-Time PCR, extraction of bacterial DNA was performed by the modified benzyl chloride method preceded by a cleaning protocol [12]. Real-Time PCR was performed to enumerate the selected bacterial group as Bifidobacterium spp., Bacteroides-Prevotella-Porphyromonas spp., Lactobacillus spp., Enterobacteriaceae, Staphylococcus spp. and Clostridium coccoides - Eubacterium rectale using specific primers as described by Nasuti, et al. [13]. Real-time PCR amplification by Syber green was performed in duplicate using the iCycler IQ real-time detection system (Stratagene) associated with MXP software using the conditions reported by Nasuti. Standard curves, previously generated for each of the previously mentioned groups of target microorganisms, were used for their quantification [14].

Gas Chromatographic Analysis

Samples were taken from the culture vessel at the level of the three previously described time points (T0, T6, and T24 h) and were analyzed by gas chromatography (GC) to quantify the short-chain fatty acids (SCFAs) content. Extraction of SCFAs was performed following the procedure reported by Cresci, et al. [15] with some modifications: 0.5 g of sample was weighed and 200 µl of sulfuric acid (50 w/v%) was added. The suspension was vortexed for 1 min, 800 µl of ether was added, and then 10 µl of pentanoic acid (12000 ppm in diethyl ether), used as an internal standard. After a 5-min centrifugation, the supernatant was transferred, then 800 µl of ether was added to the bottle containing the lower aqueous phase; the extraction protocol was repeated 2 times. Eventually, each bottle would contain 2.4 mL of diethyl ether and analytes; the GC conditions used are reported by Fiorini, et al.[16].

Digestibility

For the preparation of the dry dog feed samples, we proceeded by subjecting the kibble to grinding (1 mm). They were then oven-dried twice at 60°C for 24 hours.

The determination of crude protein digestibility of the samples, during method optimization, was performed

according to the reference method used is the one described by Boisen and Fernàndez in 1995 (multi-enzymatic method-two-step system) [17].

Results

Digestibility

From the analysis of the results (Table 1), puppy feed A has a high dry matter digestibility (92.34%), compared with competitors (91.11% and 90.74%, respectively).

For adults with fresh ingredients, feed A has higher dry matter digestibility (95.13%) given by higher digestibility of starch (although lower than competitors) and crude protein.

In fresh/flour blends for Adults, Company A is more digestible than competitor B with a digestibility of 94.42% even though A has more starch.

For flour-based adult foods, Company A has higher starch digestibility (95.77%) with less starch than competitors.

CATEGORY	FEED	Type of ingredients	Starch	DRY MATTER	CRUDE PROTEIN
PUPPY A	Sea bass and sea bream	fresh	96,02%	92,34%	98,93%
ADULT A	Pork	fresh	95,13%	88,76%	97,79%
ADULT A	Beef	Mix fresh/flours	94,42%	83,14%	94,65%
ADULT A	Lamb and chicken	flours	95,77%	82,91%	93,09%
PUPPY B	Chicken, salmon and peas	fresh	95,51%	91,11%	98,12%
PUPPY B	Biologically appropriate	fresh	95,68%	90,74%	97,91%
ADULT B	Beef	fresh	92,88%	83,48%	92,15%
ADULT B	Pork	Mix fresh/flours	92,23%	82,04%	91,18%
ADULT B	Beef	flours	91,89%	81,66%	92,33%
ADULT B	Salmon	Fresh cold pressed	89,77%	81,24%	79,04%

Table 1: Feed digestibility analysis.

Glycemic Analysis

From the analysis of the results (Table 2), Puppy feed A has more starch (32.18%) but with a very good glycemic index 44.12% and glycemic load 14.20% compared with competition B.

As for Adult with fresh ingredients, A is the best in terms of glycemic index (40%).

Regarding the Adult blend with fresh ingredients and animal meal, A is the best product in terms of glycemic index (42%) even though it has high levels of starch (35.61%).

A is the best flour-based adult product; it is possible to improve the glycemic index by increasing the percentage of fiber.

CATEGORY	FEED	Type of ingredients	Starch (%)	Glycemic index	Glycemic load
PUPPY A	Sea bass and sea bream	fresh	32,18	44,12	14,20
ADULT A	Pork	fresh	33,66	40,04	13,48
ADULT A	Beef	Mix fresh/flours	35,61	42,01	14,96
ADULT A	Lamb and chicken	flours	34,91	44,91	15,68
PUPPY B	Chicken, salmon and peas	fresh	34,67	45,88	15,91
PUPPY B	Biologically appropriate	fresh	32,11	46,79	15,02
ADULT B	Beef	fresh	38,15	43,48	16,59
ADULT B	Pork	Mix fresh/flours	32,14	46,74	15,02
ADULT B	Beef	flours	18,43	48,91	9,01
ADULT B	Salmon	Fresh cold pressed	39,94	41,12	16,42

Table 2: Feed glycemic analysis.

Fermentation Profile Analysis

Results (fecal microbiota)

In in vitro fermentation, the bacterial genera Lactobacilli and Bifidobacteria were analyzed at the beginning of treatment (T0), after 6 hours and 24 hours.

In Table 3, the Puppy feed of A shows a significant increase in Lactobacilli even when starting from a lower bacterial load at T0, and in general, a better influence of feed A on bacterial growth is noted.

Regarding the analysis of bacterial growth results in the cold-pressed competitive feeds (Table 4), no increase in the population of Lactobacilli and Bifidobacteria is actually observed.

Feed A				Lactobacilli (log UFC/ml)			Bifidobacteria (log UFC/ml)		
Category	**Feed**	**Type of ingredients**	**F.G.**	**T0**	**T6**	**T24**	**T0**	**T6**	**T24**
PUPPY	Sea bass and sea bream	Fresh	2,14	6,09	7,35	9,18	3,91	4,15	4,68
ADULT	Pork	Fresh	1,98	6,24	7,69	8,67	3,42	3,97	4,67
ADULT	Beef	Mix fresh/flours	2,11	6,15	7,48	8,91	3,54	4,05	4,87
ADULT	L:amb and chicken	Flours	2,54	6,28	7,25	8,86	3,26	3,98	4,65

Table 3: Dog feed A fermentability analysis (fecal microbiota).

Feeds B				LACTOBACILLI (log UFC/ml)			BIFIDOBACTERIA (log UFC/ml)		
Category	**Feed**	**Type of ingredients**	**F.G.**	**T0**	**T6**	**T24**	**T0**	**T6**	**T24**
ADULT	Beef	Fresh	2,78	6,01	7,15	8,19	3,65	3,89	4,12
ADULT	Pork	Mix fresh/flours	1,99	6,35	7,55	8,65	3,05	3,55	4,15
ADULT	Beef	Flours	1,89	6,26	7,68	8,26	3,24	3,78	4,19
PUPPY	Chicken, salmon and peas	Fresh	2,54	6,33	7,14	8,27	3,68	3,77	4,26
PUPPY	Biologically appropriate dog food	Fresh	4,08	6,12	6,98	8,01	3,12	3,64	4,01
ADULT	Salmon	Cold pressed	1,96	6,14	6,69	7,14	3,33	3,36	3,68

Table 4: Dog feed B fermentability analysis (fecal microbiota).

Results (faecal metabolites - SCFAs)

For the evaluation of intestinal bacterial fermentation activity, the 3 main volatile fatty acids (SCFAs) from the fermentation activity of intestinal bacteria were analyzed: acetic acid, propionic acid, and butyric acid.

Regarding the analysis of results (Tables 5 and 6), for puppy feeds there were no differences, while in adult dog feeds butyric acid A was always higher.

Cold-pressed competitive feed, besides stimulating good acetic acid production, does not actually affect the amount of butyric acid and therefore could potentially pose a risk to gut and body health in the medium and long term.

Feed A				Acetic acid (mmol/kg)			Propionic acid (mmol/kg)			Butyric acid (mmol/kg)		
Category	**Feed**	**Type of ingredients**	**F.G.**	**T0**	**T6**	**T24**	**T0**	**T6**	**T24**	**T0**	**T6**	**T24**
PUPPY	Sea bass and sea bream	fresh	2,14	0,54	4,54	20,01	0,12	0,37	10,12	0,24	0,95	5,16
ADULT	Pork	fresh	1,98	0,58	5,01	21,15	0,18	0,95	10,68	0,12	0,96	4,98
ADULT	Beef	Mix fresh/flours	2,11	1,12	4,65	20,98	0,54	0,96	11,21	0,21	0,99	5,60
ADULT	Lamb and chicken	flours	2,54	0,95	4,98	21,74	0,26	0,83	11,02	0,38	0,94	5,23

Table 5: A dog feed fermentability analysis (fecal metabolomics).

COMPETITORS				Acetic acid (mmol/kg)			Propionic acid (mmol/kg)			Butyric acid (mmol/kg)		
Category	Feed	Type of ingredients	F.G.	T0	T6	**T24**	**T0**	**T6**	**T24**	**T0**	**T6**	**T24**
ADULT	Beef	Fresh	2,78	1,24	5,21	21,58	0,35	0,88	11,67	0,45	0,90	5,01
ADULT	Pork	Mix fresh/flours	1,99	0,78	4,98	21,87	1,01	0,93	11,93	0,60	0,78	4,12
ADULT	Beef	Flours	1,89	1,15	5,60	21,36	0,73	0,87	11,85	0,53	0,84	4,25
PUPPY	Chicken, salmon and peas	Fresh	2,54	1,38	5,88	21,68	0,79	1,06	11,23	0,24	0,78	5,16
PUPPY	Biologically appropriate dog food	Fresh	4,08	1,12	5,89	21,83	0,54	0,94	11,24	0,34	0,95	5,12
ADULT	Salmon	Cold pressed	1,96	1,54	7,68	25,09	0,02	0,32	6,32	0,13	0,40	2,10

Table 6: Competitor food fermentability analysis for dogs (fecal metabolomics).

Discussion

The overall digestibility data (measured in vitro) testify to a higher digestibility of commercial A products in terms of both dry matter and crude protein as well as starch. This naturally leads to an increase in the digestible energy of the products with direct consequences on the amount of feed required to meet an animal's nutritional needs since, for the same daily amount of feed ingested, the digested portion of the A feed will be higher with a decrease in the conversion index (higher feed efficiency).

As for feeds with fresh ingredients, they are the best in terms of glycemic index (40%). One suggestion would be to decrease the starch content (ideally, a starch content of less than 30 percent instead of the current 33.6 percent, with a glycemic index around 40 percent with a higher percentage of fiber).

Regarding fecal microbiota analysis, as with the results of feed A in general, a better influence on intestinal bacterial growth is noted. This finding correlates with the results on digestibility: as dry matter digestibility increases, the pabulum of bacterial fermentation varies. There is no direct correlation between the percentage of crude fiber and bacterial growth, but scientific research on the gut microbiota shows an increase in varieties of microbial populations with the addition of soluble fiber [18,19].

As for the analysis of the results of the bacterial growth of the cold-pressed feed, we note that in fact no increase in the population of Lactobacilli and Bifidobacteria is observed; most likely this finding is to be related to the low protein digestibility of the feed itself, which in fact results in undigested nitrogenous substances arriving in the large intestine, representing a potential fermentation substrate for alkaline bacterial populations to the detriment of acidophilic strains such as those we tested. This also often results in the production of nitrogen-derived metabolites, such as indole, skatole, and cresol, which increase intestinal pH with negative consequences for the maintenance of intestinal homeostasis.

In the results on SCFAs, butyric acid is consistently higher in feed A. Butyric acid is currently considered the intestinal fermentative metabolite most involved in maintaining the health of the intestinal barrier. Colonocytes oxidize butyrate in greater amounts than glucose and it is therefore the main fuel of intestinal cells, resulting in colonocyte proliferation and mucosal growth. It is also involved in the regulation of cell proliferation and differentiation and has anti-inflammatory properties; in fact, a deficiency of butyrate appears to contribute to the onset of intestinal inflammatory conditions, phenomena related to the lack of energy for colonocytes.

The latest research has shown that butyric acid increases mucosal sodium absorption [20], reduction of fluid losses following acute episodes of diarrhea, and has antibacterial properties due to the reduction of the pH of the intestinal lumen; it also contributes positively to the well-being of the microbiota and the gut, thus being a valuable aid in reducing the occurrence of pathogenic states. The acetic and propionic acid values are very similar and ensure good intestinal acidification, which is useful for maintaining intestinal homeostasis. A separate result for cold-pressed competitive

feed, which, in addition to stimulating good acetic acid production does not actually affect the amount of butyric acid and thus could potentially pose a risk to gut and body health in the medium to long term.

Conclusions

Based on the results of the analyses carried out, the importance of the quality of the raw materials used for the formulation of feed is evident in determining a better metabolic use of the ingredients it contains. The production technology certainly plays a role of primary importance in influencing the digestibility and therefore the use of food; in fact, the cold-pressed competitive feed presented the worst indices of digestibility, glycemic analysis and indicators of bacterial growth and its fermentation activity.

Another very evident aspect was the close correlation between the digestive capacity of the animal and those of its microbiota as the latter has the possibility of fermenting food ingredients not digested by the animal together with the non-absorbed nutrients.

It is therefore evident that only from an in-depth analysis of the digestibility of a food it is possible to understand what the intestinal fermentation trend may be, at least from a nutritional point of view. The digestibility values of dry matter, starch and protein, albeit calculated in vitro, could be very useful as an optional indication to be reported on the label as they would represent much more reliable indicators than just the quantitative evaluation of their respective contents.

A limitation of the study is certainly the analyzes on the fecal microbiota as for a more complete evaluation of the intestinal bacterial ecosystem, the metabolomic analysis could be extended to the parameters of protein putrefaction as indicators of an altered intestinal balance.

It is widely believed that animal by-products that might be contained in meal are more sustainable in terms of environmental impact when compared to using fresh meat or fish, originally intended for human consumption, for kibble production. While the pet food industry must aim for environmental sustainability in its goals, feeding animal better quality ingredients is also critical in the One Health perspective.

Another interesting aspect to be evaluated in the future for the feed industry, especially for intestinal fermentation aspects, is the possibility of reporting on the packaging the quantities of soluble and insoluble fiber in the food which would represent much more reliable indicators than the official raw fiber parameter. As regards the overall evaluation of the results of the feed, it would be very profitable to work in terms of formulation on the ratio between digestible carbohydrates (starch) and indigestible carbohydrates (fiber) both to better analyse the ratio between insoluble soluble fiber in the various raw materials and / or additives and for the evaluation of the choice of starch sources according to their glycemic index.

Further will be needed in vivo studies to confirm these in vitro results, to improve knowledge of the nutritional characteristics of different dry pet food formulations in relation to the digestibility of the feed.

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