Scientific Research Publishing

Food and Nutrition Sciences, 2024, 15, 1043-1054
https://www.scirp.org/journal/fns
ISSN Online: 2157-9458
ISSN Print: 2157-944X

Addition of Protease Enzyme to Dog and Cat Feed and Its Influence on the Digestibility Coefficient, Immune Response, and Metabolic Biomarkers

Bruno Giorgio de Oliveira Cécere[1], Emanuela Cristina Zatti Anzigliero[2], Angelo Schonorr[2], Thiago Pereira Ribeiro[3], Walter Hugo Cuelho Suarez[3], Daniel Pigatto Monteiro[3], Aleksandro S. Da Silva[1,2]

[1]Multicenter Biochemistry and Molecular Biology Program, Santa Catarina State University (UDESC), Lages, Brazil
[2]Department of Animal Science, Santa Catarina State University (UDESC), Chapecó, Brazil
[3]Department of Animal Nutrition, Tectron—Tecnologia e Inovação, Toledo, Paraná, Brazil
Email: *aleksandro_ss@yahoo.com.br

How to cite this paper: Cécere, B.G.O., Anzigliero, E.C.Z., Schonorr, A., Ribeiro, T.P., Suarez, W.H.C., Monteiro, D.P. and Da Silva, A.S. (2024) Addition of Protease Enzyme to Dog and Cat Feed and Its Influence on the Digestibility Coefficient, Immune Response, and Metabolic Biomarkers. *Food and Nutrition Sciences*, **15**, 1043-1054.
https://doi.org/10.4236/fns.2024.1511067

Received: September 16, 2024
Accepted: November 3, 2024
Published: November 6, 2024

Abstract

Promoting better protein digestibility through exogenous enzymes in the diet is essential in the nutrition of companion animals, mainly for better performance and maintenance of the animals' physiological and metabolic systems, promoting health and adequate growth. In addition, it reduces the cost of the diet by possibly reducing protein in the diet, which is the main and most expensive ingredient for dogs and cats. The objective of this study was to verify whether the addition of protease to dog and cat food can improve the protein digestibility of the food, thus facilitating greater absorption of amino acids and influencing metabolic biomarkers and immune response. To this end, two experiments were carried out to evaluate the protease from the fermentation of *Aspergillus niger* and *Bacillus subtilis*. Experiment 1 was carried out with ten male, non-castrated beagles divided into two groups of five animals: the control group (without enzyme) and the test group (with 250 g of protease/ton). The animals underwent two 45-day experimental periods, and in the second period, after a 15-day interval, the dogs in the control group became part of the treatment group (crossover model). Adding this enzyme to the dogs' diet had no adverse effects on the animals' health besides improving the digestibility of dry matter and crude protein consumed by the dogs. Experiment 2 was carried out with sixteen female cats of no defined breed, non-castrated, divided into four groups with four animals per group, namely: Treatment A (without enzyme), Treatment B (with protease at a dose of 100 g/ton), Treatment C

(with protease at a dose of 200 g/ton) and Treatment D (with protease at a dose of 400 g/ton). The cats underwent two 30-day experimental periods, and in the second period, after a 15-day interval, the animals switched between treatments (crossover model) to increase the power of the statistical test. The enzyme consumption did not affect the felines' metabolism and health but improved the digestibility of crude protein at doses of 200 and 400 g/ton. The results allow us to conclude that the protease used in this study can improve the digestibility of crude protein for dogs and cats.

Keywords

Canines, Diet, Enzymes exogenous, Felines, Metabolism

1. Introduction

There is an exponential growth in the number of companion animals in homes. Pets are essential family members, a situation influenced by the COVID-19 pandemic, in which, in many cases, pets were the refuge of many people, becoming a relationship of mutual benefit in terms of health and well-being [1] [2]. Due to evolution, dogs and cats are carnivorous species; therefore, the main ingredient that is strictly necessary is a protein of animal origin, which will provide the most significant load of amino acids for the animals [3] [4]. Despite adaptations acquired over time regarding eating habits, resulting from changes in how pets are raised and the search for ease and low maintenance costs, protein is still an essential ingredient that covers the most outstanding value within a diet. As a result, the industry focused on the pet food market is looking for alternatives to reduce the cost of producing a kilo of feed through changing ingredients and using additives and supplements that, in addition to modifying the price of the diet, provide the animal with an improvement in parameters for digestion and absorption of nutrients [5].

Dietary protein digestion is an essential and beneficial factor in the nutrition of dogs and cats, considering factors such as adequate development and maintenance of tissues, and strengthening of immune functions, in addition to contributing to the availability of amino acids necessary for maintaining healthy skin and fur and even energy supply as a secondary factor, thus providing adequate performance of the animals' physiological and metabolic systems [6] [7]. Protease is an exogenously supplemented enzyme designed to increase dietary protein digestibility through mainly enzymatic hydrolysis, increasing its bioavailability to the body [8] [9]. The production of protease from the fermentation process has been known for a long time, coming from some known strains such as *Rhizopus* sp., *Aspergillus* sp., and *Bacillus* sp., and with the advancement of technology, the fermentation and protease production processes are increasingly optimized and maximized [10].

Much research has been done on the addition of protease to the diet of

production animals, such as pigs and poultry [11], which aim to achieve more remarkable performance in a short period; therefore, increasing protein digestibility can be a crucial factor in the accelerated growth of these species [9] [12]. One of the main reasons for using exogenous enzymes, in addition to reducing the cost of ingredients in the diet, is that it balances the production of endogenous enzymes, compensating for this use to improve the digestion of recommended foods in the diet [13]. Another factor to take into consideration is that based on the hypothesis of greater availability of amino acids, resulting from greater protein digestibility in the diet with the use of protease, it would stimulate the synthesis of L-carnitine through lysine and methionine, which could become a source of increased energy for growth due to the ability to transport fatty acids to the mitochondria, converting them into energy for the cells [14]. In addition, the possible enzymatic loss due to the extrusion process can be mentioned [15]. Research on enzyme supplementation in dogs and cats still needs to be completed. Therefore, the objective of the present study is to evaluate whether the addition of protease to the diet of dogs and cats can improve protein digestibility and influence blood and metabolic parameters. The present work hypothesizes that with an increase in protein digestibility, there will be an increase in the bioavailability and absorption of amino acids in animal organisms, positively influencing metabolic biomarkers.

2. Materials and Methods

2.1. Enzyme

The protease tested is a commercial enzyme from TECTRON (TECMAX PRO®). This enzyme was produced from the fermentation of microorganisms *Aspergillus niger* and *Bacillus subtilis*, using carob flour as a vehicle. Protease guarantees levels of 20,000 enzymatic activity units (U/g).

The additive (protease) was added to dog and cat feed with the aid of soybean oil (40 g/kg of dog feed and 20 g/kg of cat feed), with the additive mixed with soybean oil and subsequently sprayed on the feed that was constantly homogenized. This methodology was defined in a previous test in which different diluents for the enzyme were tested. Soybean oil is the best vehicle, as it allows the enzyme to adhere to the feed pellets, resulting in a more homogeneous distribution in the amount of feed prepared. The experimental period of each of the experiments described below was based on the current regulations (MAPA 2023) of the Brazilian government for the species dogs (42 days) and cats (28 days) when tests were carried out with additives and foods.

2.2. Experiment 1: Addition of Protease to the Diet of Dogs

The extruded food used in the experiment is classified as Premium for adult dogs. Ten adult Beagle dogs, male, non-castrate, five years old, weighing an average of ten kg, in stable health conditions, vaccinated, and with antiparasitic drugs administered up to date, were used as an experimental model. The animals were

housed in an experimental kennel at the experimental farm in Guatambu-SC. The kennel has ten cages for individual feeding (1 × 1 m) and two collective kennels, with a controlled temperature of 24˚C and an outdoor area with lawn and shade, where the animals have access during the day.

The dogs were distributed into two treatments in a crossover design that aims for all animals to undergo both treatments. A sample of five animals per treatment was divided into two groups: Treatment A (without adding the enzyme) and Treatment B (with the protease at a dose of 250 g/ton). It is essential to make it clear that this study was carried out in two stages: to increase the number of animals per treatment and to increase the power of the statistical test; in the second stage, the animals that were in the control group became part of the treatment group (crossover model). There were 15 days between the first and second phases, during which all the dogs consumed the same food already available to the animals before the start of the experiment.

Each phase of the experiment lasted 45 days; for the first 40 days, the animals remained in the collective pens and were placed in the cages only for feeding, and between days 41 and 45, they were only in the cages to collect total feces for analysis of the apparent digestibility coefficient. The feed was calculated according to the animals' maintenance energy needs, NRC Dogs and Cats 2006, and the diet's metabolizable energy (ME) per gram. Food was provided in two meals during the day. It is worth noting that the animals were fed in an individual kennel, just as water was made available ad libitum.

2.3. Experiment 2: Addition of Protease to the Diet of Cats

The food used in the experiment is classified as being in the super-premium class for adult cats. Sixteen female cats of no defined breed, non-castrated aged ten months at the beginning of the experiment, were used as experimental models. The animals were housed in the experimental cattery located in Guatambu-SC. The cattery has 16 individual cages for feeding, an area where the cats were loose collectively, with a controlled temperature of 24˚C, and an external area for sunbathing and playing, which the animals had access to during dry days. The cats were raised in this facility since they were kittens, keeping their vaccination protocol up to date; all tested negative for FIV and FELV and using a current antiparasitic protocol.

The cats were distributed across four treatments in a crossover design that aims to repeat the experiment by changing the animals between treatments. A sample number of four animals per treatment, divided into four groups: Treatment A (without enzyme), Treatment B (with protease at a dose of 100 g/ton), Treatment C (with protease at a dose of 200 g/ton) and Treatment D (with protease at a dose of 400 g/ton). It is essential to highlight that this study was carried out in two stages to increase the number of animals and improve the power of the statistical test. In the second stage, there was a rotation between the groups, in which the animals from a control group were necessarily in another group. There were 15 days between the first and second phases, during which all cats consumed the

same food available before the start of the experiment.

Each phase of the experiment lasted 30 days. In the first 25 days, the animals remained in the collective areas. They were placed in cages only for feeding, and between days 26 and 30, they remained only in the cages to collect total feces for subsequent analysis of the digestibility coefficient of diet/nutritional fractions.

The feed was calculated according to the animals' maintenance energy needs, NRC Dogs and Cats, 2006, and the diet was ME per gram. Food was divided into two meals during the day, highlighting that the animals were fed in individual cages and water was available ad libitum.

2.4. Sampling

2.4.1. Blood

Blood samples from dogs were collected on days 1 and 40 of the experiment of both periods. Dogs were manually restrained, and blood was collected through the jugular vein with a 5 ml syringe equipped with a 25/7 G needle. Blood samples were collected on day 25 of both experimental periods for cats. For this, the cats were sedated with a mixture of two commercial sedatives (xylazine 0.06 to 0.1 mL/kg and ketamine 0.1 mL/kg) at the dose indicated by the manufacturer; then, the neck was shaved to facilitate collection via the jugular vein, with a 5-ml syringe equipped with a 25/7 G needle. Subsequently, the blood of both was placed in tubes containing anticoagulant (EDTA), for hematologic analysis, and tubes without anticoagulant. Samples in tubes without anticoagulant were centrifuged (10 min at 5500 rpm) to obtain serum, then frozen (–20˚C) for subsequent serum analysis.

2.4.2. Serum biochemistry

According to the manufacturer's recommendations and a semi-automatic analyzer, total protein, albumin, glucose, and cholesterol levels were measured using specific commercial kits (BioPlus, 2000). Globulin levels were obtained mathematically from the difference between total protein and albumin (total protein – albumin).

2.4.3. Hemogram

The erythrocyte and leukocyte count, leukocyte differential, hemoglobin concentration, platelets, and hematocrit were obtained using an automatic counter (EquipVET 3000).

2.4.4. Apparent Digestibility Coefficient

Samples of the feed provided to the animals throughout the experimental period of both experiments were collected, stored, and frozen (–20˚C) for subsequent bromatological analysis.

During days 41 to 45 of experiment I with dogs, the feces of animals housed in cages were collected. In the same way, on days 26 to 30 of experiment II, like cats, the total feces were collected.

Subsequently, the feed and feces samples were thawed, weighed, and placed in

an oven at 55˚C for 72 hours to determine the percentage of dry matter in the samples. Subsequently, these feces were dried, weighed, and crushed, and the chemical composition was analyzed. Based on this information, as well as the chemical composition of the food, and calculating the apparent digestibility coefficient of nutrients, analysis of dry matter, ash, ether extract, and crude protein was carried out according to literature [16].

Using the chemical composition of feces and feed and the proportion of feed consumed and feces excreted, the apparent digestibility coefficient (ADC) was calculated according to the equation described by [17]: ADC (%) = ((Nutr I (g) − Nutr E (g)) × 100)/Nutr I (g), where Nutr I = nutrient ingested and Nutr E = nutrient excreted in feces.

2.5. Statistical Analysis

All data were analyzed using the SAS 'MIXED procedure' (SAS Inst. Inc., Cary, NC, USA; version 9.4), with Satterthwaite's approximation to determine the denominator degrees of freedom for the fixed effects test. The variables were analyzed as repeated measures and were tested for fixed effects of the treatment, day, and treatment × day interaction, using animal as random effects. Day 1 results were included as an independent covariate. The first-order autoregressive covariance structure was selected according to the lowest Akaike information criterion. Means were separated using the PDIFF method, and all results were reported as LSMEANS followed by SEM. Significance was defined when $P \leq 0.05$ and trend when $P > 0.05$ and ≤ 0.10.

3. Results

3.1. Experiment 1

Table 1 describes the chemical composition and ingredients of the dog feed. It contains 26% protein and 10% ether extract.

The results of the complete blood count are presented in Supplementary Material 1. There was no difference in the erythrocyte count, leukocyte count, leukocyte differential, hematocrit, hemoglobin, and platelets between treatments ($P > 0.05$).

The results regarding serum biochemistry are shown in Table 2. We observed a significant response to the levels of total protein and urea in the dogs' serum, with the group that received protease in the diet demonstrating higher levels compared to the control group at the end of the experiment ($P < 0.05$) in addition to a trend toward higher albumin levels for the group that received protease ($P < 0.10$). We found no difference in globulin, glucose, and cholesterol between treatments ($P > 0.05$).

Table 3 shows the results of the ADC. We observed a difference in the ADC of dry matter and crude protein to where dogs that consumed protease demonstrated greater digestibility than the control group ($P < 0.05$). The digestibility of ash and ether extract did not differ between treatments ($P > 0.05$).

Table 1. Chemical composition (%) of the feed used in the experiment with dogs.

	Dogs[1]	Cats[2]
Dry matter	90.9	94.5
Crude protein	26.1	34.9
Ethereal extract	10.6	12.5
Ash	15.6	7.14

Note 1: The commercial feed used in this study contained the following ingredients: Poultry by-product meal, beef, and bone meal from cattle, ground corn*, ground whole sorghum, soybean bran*, wheat bran, rice bran, oat hulls, oil poultry, fish oil, poultry and pork liver hydrolyzate, acidifier, antioxidants (BHA and BHT), sodium chloride, vitamin A, vitamin D3, vitamin E, vitamin K3, vitamin B1, vitamin B2, vitamin B6, vitamin B12, vitamin C, niacin, choline chloride, pantothenic acid, folic acid, biotin, calcium iodate, copper amino acid chelate, iron amino acid chelate, manganese amino acid chelate, zinc amino acid chelate, selenium-enriched yeast, calcium propionate, sorbate potassium, yeast wall extract. **Note 2**: The commercial feed used in this study contained the following ingredients: Poultry by-product meal, rice grits, wheat gluten, ground whole corn*, pea hulls, corn gluten*, chicken fat, pork fat, protein flour isolated from pork, refined soybean oil*, beet pulp, dry brewer's yeast, dehydrated egg, zeolite, borage oil, fructo-oligosaccharides, marigold extract, grape and green tea polyphenols 10%, dicalcium phosphate, calcium sulfate, potassium chloride, monosodium phosphate, sodium chloride (common salt), calcium carbonate, monocalcium phosphate, vitamins (A, C, E, D3, B1, B2, B6, B12, PP), pantothenic acid, biotin, folic acid, choline chloride, iron sulfate, copper sulfate, manganese oxide, zinc oxide, calcium iodate, sodium selenite, chicken liver, natural annatto coloring, taurine, L-lysine, DL-methionine, antioxidant (BHA). Note: *ground whole corn and corn gluten genetically modified by *Bacillus thurigiensis* and *Streptomyces viridochromogenes*; refined soybean oil produced from soybeans genetically modified by *Agrobacterium* sp.

Table 2. Serum biochemistry of dogs fed exogenous protease.

Variables	CONTROL	PROTEASE	SEM	P-value: Treat × Day
Albumin (g/dL)				**0.06**
d 1	3.45	3.46	0.03	
d 40	3.21[b]	3.50[a]	0.02	
Globulin (g/dL)				0.16
d 1	3.26	3.55	0.06	
d 40	3.47	3.70	0.05	
Total protein (g/dL)				**0.05**
d 1	6.71	7.01	0.07	
d 40	6.70[b]	7.20[a]	0.05	
Glucose (mg/dL)				0.25
d 1	104	86.6	4.85	
d 40	101	90.7	4.63	
Urea (mg/dL)				**0.03**
d 1	43.5	44.9	1.09	
d 40	35.3[b]	43.1[a]	0.82	
Cholesterol (mg/dL)				0.52
d 1	130	138	4.14	
d 40	165	160	3.44	

[1]Treatments were: CONTROL treatment containing extruded commercial meat food for adult dogs + 40 g/kg soybean oil. PROTEASE treatment containing extruded commercial meat food for adult dogs + 40 g/kg of soybean oil + protease enzyme at 250 g/ton. [a-b]Within a row, differ ($P \leq 0.05$) or tend to vary ($P \leq 0.10$).

Table 3. Apparent digestibility coefficient (ADC) of dogs and cats fed protease when compared to the control.

	ADC DM%	ADC ASH%	ADC CP%	ADC EE%
Experiment I: Dogs				
Control group	79.6[b]	67.4	90.7[b]	97.2
Protease group	82.3[a]	66.2	93.3[a]	97.4
SEM	0.60	0.41	0.22	0.15
P-value	**0.03**	0.48	**0.01**	0.94
Experiment II: Cats				
Group A	82.6	49.8	83.5[b]	93.9
Group B	83.5	47.7	85.4[ab]	93.9
Group C	84.2	50.6	86.5[a]	94.1
Group D	84.8	51.5	87.3[a]	94.3
SEM	0.45	0.37	0.21	0.13
P-value	0.12	0.89	**0.01**	0.93

OBS: When P < 0.05, there is a difference between treatments, illustrated by letters (a, b) in the same column.

3.3. Experiment 2

Table 1 shows the chemical composition of the cat food and its ingredients. It is a feed with more than 34% crude protein and 12% ether extract.

The results of the complete blood count are presented in Supplementary Material 2. No difference was observed between treatments (P > 0.05) for hematologic variables.

Table 4 displays the results corresponding to serum biochemistry. There was no difference between treatments for the concentrations of glucose, cholesterol, total protein, albumin, urea, and globulin (P > 0.05).

Table 4. Serum biochemistry of cats fed exogenous protease.

Group	Glucose	Cholesterol	Total protein	Albumin	Urea	Globulin
A	125	78.0	7.10	2.47	46.3	4.63
B	106	93.4	6.97	2.60	50.4	4.37
C	102	87.9	7.60	2.60	48.9	5.00
D	111	81.7	7.17	2.31	50.4	4.86
SEM	4.36	4.02	1.05	0.25	1.95	0.22
P-value	0.21	0.13	0.84	0.91	0.89	0.49

OBS: There was no significant difference between treatments for biochemical variables (P > 0.05).

Table 3 presents the ADC of diet/nutritional fractions of cats. The digestibility of crude protein (CP) was higher for cats that consumed the diet with 200 and 400

g/ton of protease compared to the other treatments ($P < 0.05$). There was no difference in the digestibility of dry matter, ash, and ether extract ($P > 0.05$).

4. Discussion

The animals that received diets containing protease treatments had a higher CP digestibility coefficient, which was expected because it is an enzyme produced for this purpose but has yet to be tested in the diets of dogs and cats. According to Vermelho *et al.* [18], the benefits of adding exogenous enzymes, in addition to contributing to the enhancement of nutrient utilization, may have the ability to reduce possible anti-nutritional effects. Proteases act on proteins through a process called proteolytic cleavage, containing the ability to break the peptide bonds that join amino acids in proteins, resulting in the breakdown of proteins into smaller fragments, increasing the release of amino acids and peptides for use and absorption [19]. The following Villaverde *et al.* [14], tested using different exogenous enzymes for dogs, including protease from porcine pancreas. However, they did not corroborate our study, as they did not obtain a significant difference in the digestibility of the dog's protein.

Exogenous enzymes are widely used in the food industry, mainly for farm animals, where several studies have already reported significant benefits. The following authors [10], offered a low-protein diet with added protease to broiler chickens, compared to a diet with average protein values and observed that performance and digestibility were similar, thus favoring the use of the enzyme and reducing the cost of the protein ingredient in the feed. Second, Park *et al.* [9] confirmed that adding protease to piglets in the nursery phase and on a diet with low protein levels improved growth performance, nutrient digestibility, and even the intestinal morphology of the animals. Tortola *et al.* [20] also reported in their study that when exogenous protease was tested in diets for juvenile peacock bass, there was an improvement in feed conversion rates, weight gain, and specific growth. However, the focus for dogs and cats is not on growth performance; however, improving the digestion and absorption of protein and amino acids implies a reduction in the cost of food production, in addition to the benefit for these animals with carnivorous characteristics and highly dependent on protein to survive. It is essential to highlight that this type of research shows an alternative to feed with quality proteins, including those of vegetable origin, used in economically defined feeds (low selling cost).

In dogs, better protein digestibility was also reflected in blood tests because there was an increase in total protein and urea in the blood of animals that consumed the protease. The benefits of increasing protein digestion go beyond the animal itself, spreading to the environment, since the protein content that is not digested and absorbed by the animal will be excreted via feces and urine, a fact that in addition to being a protein waste in the diet, it acts as an environmental impact factor in which the protein content in excreta will be converted into ammonia and nitrate [5]. Second, Tortola *et al.* [20] raise the hypothesis that there is

a different bioavailability and absorption of amino acids arising from other protein sources in the diet, in addition to possible fermentation of the nutrient in the large intestine, affecting blood urea. However, it should be noted that further studies aimed at characterizing the types of amino acids present and the possible rate of bioavailability and absorption would be necessary to elucidate the direct action at the blood protein level without harming the health of the animal and the sustainability of the environment in which it lives. In cats, despite the greater digestibility of CP, there was no statistically significant difference for metabolic variables. We do not have an explanation for why behavior similar to that of dogs did not occur. Still, it is essential to highlight that the metabolism of these two animal species is different.

5. Conclusion

The use of protease in the diets of dogs and cats increased the digestibility coefficient of CP. It didn't negatively affect metabolic, and blood biomarkers related to animal health. Serum protein catabolism increased in dogs, probably due to the increased digestibility of dietary protein.

Ethics Committee

The ethics committee approved the project on the use of animals in UDESC research, protocol number 7874200223. The experiment followed the rules of the National Council for the Control of Animal Experimentation.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Appendix

Supplementary Material 1. Blood count of dogs fed with exogenous protease enzymes.

	CONTROL	PROTEASE	SEM	P-value: Treat × Day
Erythrocytes (×10^6 µL)				0.92
d 1	7.93	7.10	0.09	
d 40	7.79	7.69	0.11	
Hematocrit (%)				0.95
d 1	45.2	46.8	0.85	
d 40	51.5	51.2	0.57	
Hemoglobin (g/dL)				0.94
d 1	16.9	17.1	0.22	
d 40	19.0	18.7	0.28	
Leukocytes (×10^3 µL)				0.26
d 1	3.98	4.07	0.35	
d 40	4.27	3.77	0.42	
Monocytes (×10^3 µL)				0.85
d 1	0.62	0.55	0.15	
d 40	0.35	0.37	0.20	
Lymphocytes (×10^3 µL)				0.52
d 1	2.20	2.37	0.32	
d 40	2.49	2.10	0.36	
Granulocytes (×10^3 µL)				0.77
d 1	1.16	1.15	0.25	
d 40	1.43	1.31	0.31	
Platelets (×10^3 µL)				0.60
d 1	210	253	14.3	
d 40	254	252	8.12	

[1]Treatments were: CONTROL treatment containing extruded commercial meat feed for adult dogs + 40 g/kg soybean oil. PROTEASE treatment containing extruded commercial meat food for adult dogs + 40 g/kg of soybean oil + protease enzyme at a 250 g/ton dose. OBS: There was no significant difference between treatments for blood count variables (P > 0.05).

Supplementary Material 2. Hemogram of cats fed with exogenous protease enzymes.

Group	Leukocytes	Granulocytes	Lymphocytes	Monocytes	Erythrocytes	Hematocrit	Hemoglobin	Platelets
A	4.63	2.12	1.70	1.49	5.04	32.2	6.47	230
B	5.87	3.04	2.15	2.50	5.45	35.2	6.43	242
C	4.53	2.51	1.48	1.96	5.94	33.4	6.75	231
D	5.34	2.51	1.72	1.77	5.56	34.4	7.20	296
SEM	0.19	0.17	0.17	0.48	0.06	0.35	0.05	16.9
P-value	0.74	0.71	0.54	0.35	0.89	0.68	0.82	0.28

Note 1: There was no significant difference between treatments for blood count variables (P > 0.05).