# *Bacillus subtilis* and *Bacillus licheniformis* reduce faecal protein catabolites concentration and odor in dogs

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# Abstract

Background: Direct-fed microbials (DFM), as Bacillus subtilis and Bacillus licheniformis, may improve gut functionality of the host by favoring non-pathogenic bacteria and reducing the formation of putrefactive compounds. The aim of this study was to assess the nutrients digestibility, faecal characteristics and products of intestinal fermentation in dogs fed diets with Bacillus subtilis and Bacillus licheniformis. Sixteen adult beagle dogs randomly distributed were used. Every eight dogs were fed with the control diet or the diet to which 62.5 g DFM (B. subtilis and B. licheniformis)/ton were added. Diets were provided during a twenty days adaptation period, followed by five days of total faecal collection. Nutrients digestibility and metabolizable energy of the diets, and faecal characteristics and products of dogs' intestinal fermentation were assessed.

Results: There were no differences in nutrients digestibility ($P > 0.05$). The DFM supplementation, however, improved the faecal score and resulted in less fetid faeces ($P < 0.001$). The DFM inclusion reduced ($P < 0.05$) the biogenic amines concentration: putrescine, spermidine and cadaverine, and the concentration of phenols and quinoline.

Conclusions: The use of B. subtillis and B. licheniformis as DFM reduce the concentration of nitrogen fermentation products in the faeces and faecal odor, but the digestibility of nutrients is not altered in dogs.


# Background

Many complete dog foods have a high protein amount in their formulation, which can result in a high concentration of undigested nitrogen compounds in the large intestine, leading to the formation of putrefactive compounds. These compounds include ammonia, biogenic amines, branched chain fatty acids, sulphidric gas, phenols and indoles [1]. Some of these catabolites can negatively affect intestinal functionality, contributing to inflammatory processes as colitis and colon carcinogenesis, as well as worsening the faecal odor of dogs [2, 1]. Furthermore, the increase in non-digestible proteins flow that reach the colon provide fermentation substrates to organisms with pathogenic potential, such as species of *Clostridium, Salmonella* and *Escherichia* [3, 4], and can also contribute to dysbiosis, an imbalance of the intestinal microbiota.

Thus, the search for nutritional strategies as the use of direct-fed microbials (DFM) to improve dog's intestinal functionality and faecal quality is extremely relevant. The DFM has been defined by the US Food and Drug Administration as the feed product containing the source of live naturally existing microbes, as some bacteria of genus *Bacillus.* When supplemented in the diet, these microorganisms may favor non-pathogenic bacteria [5, 6].

Bacteria of the *Bacillus* genus, as the facultative anaerobic species *B. subtillis* and *B. licheniformis,* have the advantage of sporulation. This characteristic makes them more viable in the food and resistant to the gastric acidic pH [7, 8, 6].*B. subtilis* and *B. licheniformis* are commonly found as spores in the soil. The

spores are dehydrated and when exposed to appropriate nutrients and moisture will germinate in the small intestine, resuming their cell vegetative growth [9]. After being excreted, these organisms can sporulate again in the faeces [7, 10].

Studies have shown that *B. subtilis* and *B. licheniformis* can improve faecal odor and reduce gas formation in the intestine of dogs [11], prevent necrotic enteritis in broilers [12], and reduce diarrhea in piglets [13]. As they are viable after excretion, these organisms degrade organic matter in the faeces, reducing ammonia production [4] and also potentially reduce faecal odor. Given the above, the aim of the present study was to evaluate the diet digestibility, faecal characteristics and intestinal fermentation products in dogs supplemented or not with the DFM *Bacillus subtilis* and *Bacillus licheniformis.*

# Results

All dogs remained healthy throughout the experiment. No episodes of vomiting or diarrhea was observed. No differences in DM intake (Control = 201.2 + 4.33 g/day and DFM = 204.1 + 5.34 g/day) and on body weight (Control =10.2 ± 1.04 kg and DFM = 10.5 ± 1.08 kg were observed between treatments (P >0.05).

The inclusion of DFM with *Bacillus subtilis* and *Bacillus licheniformis* in extruded dog diets did not alter ($P$ >0.05) the nutrients ATTD and diets ME (Table 1), as well as faecal DM, ammonia, faecal pH and sialic acid (Table 2), and SCFA and BCFA concentration in the faeces (Table 3). The faecal score, however, was increased and the DFM inclusion resulted in less fetid faeces both in fresh samples and 6 hours after defecation ($P$ <0.05, Table 4 and Figure 1). According to 82% of the evaluators, fresh faeces of dogs consuming the diet with DFM were less fetid than those from animals consuming the control diet. In a similar way, 78% of evaluators considered that 6 hours after defecation the faeces of dogs consuming DFM were less fetid than those in the control group ($P$ <0.05, Figure 1). Concentration of biogenic amines: *putrescine,* spermidine and cadaverine, and also of phenols and quinoline were reduced ($P$ <0.05) with the DFM inclusion (Tables 5 and 6).

**Table 1** Means of ATTD and ME of diets with or without DFM.

| Item | Control | DFM | SEM | *P-value* |
|---|---|---|---|---|
| ATTD (%) | | | | |
| Dry matter | 79.1 | 78.8 | 0.31 | 0.739 |
| Organic matter | 84.1 | 83.8 | 0.25 | 0.754 |
| Crude protein | 79.9 | 78.8 | 0.56 | 0.281 |
| Ether extract | 86.5 | 85.6 | 0.42 | 0.297 |
| ME (kcal/kg) | 4034.9 | 4062.4 | 10.49 | 0.199 |

*ATTD* Coefficients of total tract apparent digestibility, *ME* Metabolizable energy

*DFM* Direct-fed microbials (62.5 mg/kg of diet of a mixture of $3.66x10^7$ cfu/kg *Bacillus subtilis* and $3.66x10^7$ cfu/kg *Bacillus licheniformis*)

**Table 2** Means of faecal characteristics of dogs fed diets with or without DFM.

| Item | Control | DFM | SEM | *P-value* |
|---|---|---|---|---|
| Fresh faeces pH | 6.32 | 6.50 | 0.058 | 0.128 |
| After 6 hours pH | 6.32 | 6.31 | 0.035 | 0.933 |
| Fresh feces humidity (%) | 70.04 | 69.12 | 0.563 | 0.434 |
| After 6 hours humidity (%) | 67.39 | 67.36 | 0.707 | 0.981 |
| Fresh faeces ammonia (%) | 0.05 | 0.05 | 0.001 | 0.834 |
| After 6 hours ammonia (%) | 0.07 | 0.06 | 0.001 | 0.897 |
| Faecal production | 0.13 | 0.12 | 0.005 | 0.379 |
| Sialic acid (µmol/g) | 2.46 | 2.47 | 0.04 | 0.943 |

*DFM* Direct-fed microbials (62.5 mg/kg of diet of a mixture of $3.66x10^7$ cfu/kg *Bacillus subtilis* and $3.66x10^7$ cfu/kg *Bacillus licheniformis*)

**Table 3** Means of SCFA and BCFA in faeces of dogs fed diets with or without DFM.

| Item | Control | Probiotic | SEM | *P-value* |
|---|---|---|---|---|
| SCFA (µmol/g) | | | | |
| Acetic | 31.21 | 33.79 | 1.118 | 0.263 |
| Propionic | 27.66 | 27.16 | 1.205 | 0.845 |
| Butyric | 4.78 | 3.94 | 0.316 | 0.193 |
| Total SCFA | 64.33 | 64.89 | 1.899 | 0.887 |
| BCFA (µmol/g) | | | | |
| Isobutyric | 0.79 | 0.91 | 0.035 | 0.090 |
| Isovaleric | 0.78 | 0.67 | 0.044 | 0.198 |
| Valeric | 0.29 | 0.31 | 0.011 | 0.415 |
| Total BCFA | 1.94 | 1.87 | 0.065 | 0.640 |

*SCFA* short chain fatty acids, *BCFA* branched chain fatty acids.

*DFM* Direct-fed microbials (62.5 mg/kg of diet of a mixture of $3.66x10^7$ cfu/kg *Bacillus subtilis* and $3.66x10^7$ cfu/kg *Bacillus licheniformis*)

**Table 4** Faecal score and odor medians and interquartile of dogs fed diets with or without DFM.

| Item | Control | DFM | *P-value* |
|---|---|---|---|
| Faecal score | 4 (3/4) | 4 (4/4) | <0.001 |
| Odor fresh faeces | 2 (2.0/2.0) | 1 (1.0/1.0) | <0.001 |
| Odor after 6 h | 2 (2.0/2.0) | 1 (1.0/1.0) | <0.001 |

*Faecal score* 1 (liquid stools) to 5 (dry stools), *Faecal odor* 1 (less fetid than control), 2 (same as control) and 3 (more fetid than control).

*DFM* Direct-fed microbials (62.5 mg/kg of diet of a mixture of $3.66x10^7$ cfu/kg *Bacillus subtilis* and $3.66x10^7$ cfu/kg *Bacillus licheniformis*)

**Table 5** Means of biogenic amines (mg/kg) in the faeces of dogs fed diets with or without DFM.

| Item | Control | DFM | SEM | *P-value* |
|---|---|---|---|---|
| Tiramine | 48.36 | 46.22 | 10.770 | 0.443 |
| Putrescine | 71.24 | 47.51 | 9.321 | 0.025 |
| Cadaverine | 129.87 | 88.63 | 18.935 | 0.050 |
| Histamine | 22.93 | 23.89 | 5.280 | 0.448 |
| Serotonin | 3.20 | 2.39 | 0.381 | 0.057 |
| Agmatine | 0.00 | 0.00 | 0.000 | 1.000 |
| Spermidine | 26.77 | 19.94 | 2.451 | 0.015 |
| Phenylethynamine | 0.55 | 0.92 | 0.253 | 0.147 |
| Tryptamine | 0.51 | 0.22 | 0.344 | 0.272 |
| Total amines | 303.42 | 209.90 | 38.301 | 0.031 |

*DFM* Direct-fed microbials (62.5 mg/kg of diet of a mixture of $3.66x10^7$ cfu/kg *Bacillus subtilis* and $3.66x10^7$ cfu/kg *Bacillus licheniformis*)

**Table 6** Mean percentage of peak areas of more abundant volatile organic compounds present in faeces of dogs.

| | Item | Control | DFM | SEM | *P-value* |
|---|---|---|---|---|---|
| Fresh faeces | Phenols | 37.18 | 18.96 | 3.13 | <0.001 |
| | Quinoline | 14.57 | 3.06 | 3.14 | <0.001 |
| | Indoles | 63.32 | 67.08 | 2.98 | 0.548 |
| After 6 hours | Phenols | 33.48 | 34.58 | 2.86 | 0.855 |
| | Quinoline | 7.68 | 7.33 | 1.85 | 0.927 |
| | Indoles | 58.83 | 58.04 | 3.98 | 0.929 |

*DFM* Direct-fed microbials (62.5 mg/kg of diet of a mixture of $3.66x10^7$ cfu/kg *Bacillus subtilis* and $3.66x10^7$ cfu/kg *Bacillus licheniformis*)

## Discussion

The dietary supplementation of DFM resulted in positive indicators of improvement in intestinal functionality in the animals, as a reduction in the concentration of protein fermentation compounds in the faeces and, consequently, less faecal odor.

The absence of DFM effects on the digestibility of diet components was similar to those observed by [14, 15]. The authors found no difference in the digestibility of diets for dogs containing $7.5 \times 10^6$ cfu of *Bacillus* CIP 5832 and $2 \times 10^7$ cfu of *Lactobacillus acidophilus.*

Besides nutrients digestibility, faecal characteristics should also be taken into consideration in the evaluation of foods for dogs. Faecal characteristics can be a reflex of intestinal functionality and have become more relevant for pet owners that look for dog foods that will lead to low faecal odor and better consistency.

When compared to the control diet, the effect of DFM was reflected by improvement in faecal consistency. These results were also reported by [6], using a 0.01% supplement of *Bacillus subtilis* (C–3102) and [11], using 0.5g/100g *Bacillus subtilis* (C–3102) in dogs. Similarly, when using a probiotic containing *Bacillus subtilis* and *Bacillus licheniformis,* [13] reported a reduction of diarrhea in piglets.

Maintenance of intestinal eubiosis plus SCFA production in the colon can explain the increase in faecal consistency [16] with the use of probiotics in the diet. SCFA production by intestinal microorganisms and their absorption by the colonocytes stimulate water and electrolytes absorption, and also increase the absorption rate of sodium, responsible for most of the water absorbed in the intestinal lumen [17]. Despite this, SCFA concentration in the faeces was not altered in the present study with the use of DFM. This may be due to the rapid absorption rate of SCFA by the intestinal mucosa [18, 1].

In the same manner, no changes were observed in the dogs faecal pH, in agreement with what has been reported by other authors [1, 6, 19]. On the other hand, [5] observed a reduction in faecal pH of dogs fed with *Lactobacillus* spp. in the diet. The absence of alteration in faecal pH of dogs fed with *Bacillus* spp. can be due to the limited ability to produce lactic acid of the *Bacillus* genus species when compared to *Lactobacillus* [6].

Catabolites of amino acid fermentation are considered to be main odoriferous components of faeces and can have negative influences on the intestinal functionality due to their toxicity and by favoring the survival of bacteria with pathogenic potential [20, 21, 22]. Several putrefactive compounds can be produced from the fermentation of undigested amino acids by deamination, deamination-decarboxylation or carboxylation [23], being the major groups ammonia, biogenic amines, BCFA, indoles, phenols and volatile compounds containing sulphur [23, 22].

In the present study, DFM supplementation decreased the concentration of putrefactive compounds potentially toxic for the intestinal mucosa when present in high concentrations. The compounds that were reduced were: putrescine, spermidine and cadaverine, phenols and quinoline, which resulted in faeces with less odor, both when fresh and 6 hours after defecation.

The amines putrescine, spermidine and cadaverine are produced from the decarboxylation of amino acids ornithine, methionine and lysine, respectively [24]. Phenols and indoles are produced from the fermentation of amino acids, tyrosine and tryptophan, respectively [25]. As these mechanisms are mediated by enzymes produced mainly by intestinal bacteria with pathogenic potential, it is possible that the DFM influenced the intestinal microbiota, favoring the non-pathogenic bacterial population.

The fact that the odor was still low 6 hours after defecation (simulating what usually happens in the home environment) indicates that *B. subtillis* and *B. licheniformis* have a continuous action on faeces after excretion. According to [4], spore-forming *Bacillus* species produce substances that are antagonists to the development of organisms with pathogenic potential (generally proteolytic) and produce enzymes that degrade OM present in the excreta, reducing ammonia production. These effects can occur both in the gut and in the faeces. Still according to these authors, bacteria of the *Bacillus* genus have shown major results in sanitizing poultry and swine waste.

Another mechanism which may have contributed to the intestinal microbiota balance would be the immunomodulatory action of *B. subtillis* and *B. licheniformis* in the gut [26], reducing the establishment of organisms with pathogenic potential, protecting the villi and the absorption surface against irritating toxins [27], such as biogenic amines, phenols and indoles. In a study with broilers, [12] observed that the use of *Bacillus licheniformis* in the diet helped to prevent necrotic enteritis.

Considering all this information, dietary supplementation with DFM *B. subtilis* and *B. licheniformis* have potential beneficial effects on gut functionality of dogs and even in other species. The major limitation of the present study was that was not measured faecal microbiota of dogs. Thus, it is suggested that further studies evaluating the faecal microbiota of dogs supplemented with these DFM species are required to confirm their possible effects on intestinal functionality and modulation of the intestinal microbiota of dogs.

# Conclusions

Inclusion of $3.66x10^7$ cfu/kg of feed of *Bacillus subtilis* and $3.66x10^7$ cfu/kg of feed of *Bacillus licheniformis* in extruded dog diets improves faeces consistency and odor. It also reduces faecal concentration of compounds produced in protein catabolism as putrescine, spermidine, cadaverine, phenols and quinoline, demonstrating possible beneficial effects on dogs intestinal functionality.

# Methods

### Animals and housing

Sixteen adult intact beagle dogs were used (eight males and eight females), average body weight being $10.3 \pm 1.07$ kg and four years of age. All animals underwent previous clinical and physical examinations, were vaccinated, dewormed, and individually housed in

covered brickwork kennels (5 meters long x 2 meters wide), containing a bed and free access to fresh water. The environment temperature ranged from 16 °C to 28 °C with a 12-h light–dark cycle (light 6 am–6 pm). All animals were brought to Research laboratory on canine nutrition of the Federal University of Parana (Curitiba, PR, Brazil) from Maiorca Kennel (Colombo, PR, Brazil), since they have 3-4 months old.

During the most of diet adaptation period (until 16$^{th}$ day) dogs have free access to outdoor area under supervision during 2h per day. Among days 17-25$^{th}$ dogs were individually housed at the kennels to allow fecal collection. All dogs received extra attention and kennel enrichment during this period. Dogs will be donated when they complete 6 years old. The use of animals for this study was approved by the Ethics Committee on Animal Use from the Sector of Agrarian Sciences, Federal University of Paraná, Curitiba, PR, Brazil (012/2019).

## Experimental diets

The same commercial diet for adult dogs was divided into two parts and used in the experimental treatments. One part (eight dogs, four males and four females) was used in the control treatment, with no DFM, and the other part (eight dogs, four males and four females) was used as the test treatment, containing 62.5 mg/kg of diet of a mixture of *Bacillus subtilis* (3.66x10$^7$ cfu/kg of diet) and *Bacillus licheniformis* (3.66x10$^7$ cfu/kg of diet) as DFM (PureGro®, DSM, Heerlen Netherlands). The diet had the following composition: poultry viscera meal, meat meal, corn, soybean meal, poultry fat, swine liver hydrolysate, sodium chloride, citric acid, antioxidants (BHT, BHA), propionic acid, vitamin A, vitamin D3, vitamin E, vitamin B1, vitamin B6, vitamin B12, vitamin K3, nicotinic acid, folic acid, biotin, calcium pantothenate, zinc sulfate, calcium iodate, sodium selenite, copper sulfate, iron sulfate, manganese sulfate and zinc oxide. The experimental chemical composition of the diets is shown in (Table 7).

The DFM was diluted in poultry viscera oil and used on top of the test diet. The same amount of oil, without DFM, was used on the control treatment, ensuring that the diets were isonutritive.

**Table 7** Analysed chemical composition of the experimental diets based on dry matter (%).

| Item | Control | DFM |
|---|---|---|
| Dry matter | 91.77 | 91.51 |
| Crude protein | 21.75 | 21.12 |
| Ether extract in acid hydrolysis | 9.22 | 9.04 |
| Ash | 7.02 | 7.00 |
| Calcium | 1.18 | 1.23 |
| Phosphorus | 0.89 | 0.91 |

*DFM* Direct-fed microbials (62.5 mg/kg of diet of a mixture of $3.66x10^7$ cfu/kg *Bacillus subtilis* and $3.66x10^7$ cfu/kg *Bacillus licheniformis*)

## Experimental procedures

The digestibility assay followed the total faeces collection method as recommended by Association of American Feed Control Official [28]. The diets were provided during a twenty days adaptation period, followed by five days of total faeces collection, resulting in a mixture of faeces from each animal.

The food was provided twice a day (8:30 a.m. and 4:00 p.m.), the amounts being sufficient to meet the animal's metabolizable energy (ME) according to [29], where: ME (kcal/day) = 130 x Body weight$^{0.75}$. Water was provided *ad libitum*. The faeces were collected and weighed at least two times per day and stored in individual plastic containers previously identified, covered and stored in a freezer (-14°C) to be analyzed later.

At the end of the collection period, the faeces of each replicate were thawed at room temperature and homogenized separately, forming a composite sample of each animal. Faeces were dried in a forced ventilation oven (320-SE, Fanem, São Paulo, Brazil) at 55ºC during 48 hours or until reaching constant weight. Diets and faeces were ground to 1.0 mm in a hammer mill (Arthur H. Thomas Co., Philadelphia, PA, USA), using 1.0-mm wire mesh

sieves for the bromatological testing (in duplicate and with repetitions when the variation was higher than 5%).

The amounts of dry matter at 105ºC (DM105), crude protein (CP, method 954.01), ether extract in acid hydrolysis (EEAH, method 954.02) and ash (942.05) were determined in both diets and faeces according to Association of the Official Analytical Chemists [30]. The amount of gross energy (GE) was established using a calorimetric pump (Parr Instrument Co., model 1261, Moline, IL, USA), and organic matter (OM) was calculated by the difference between 100 – Ash.

The faecal odor was evaluated and scored on the 25th day of the experimental period. Faeces from three animals per treatment were randomly collected, homogenized and the same amounts (5.0 g) were placed in plastic containers of the same size, covered with plastic film with holes (same number and size). The containers were classified as: A (control diet) and B (diet with DFM), so the participants would not have information about treatment. The sensorial analysis was performed by 50 evaluators with fresh faeces (up to 30 minutes after defecation) and also six hours after defecation, with different people at each point in time. In the evaluation, sample B with DFM was compared to A (control diet), using a scoring system: 1 = better odor than control (less fetid); 2 = same as control; 3 = worse than control (more fetid).

Faecal pH and ammonia concentration were analyzed in faeces collected up to 15 minutes after defecation. Faecal pH was measured in digital pH meter (331, Politeste Instrumentos de Teste Ltda, São Paulo, SP, Brazil), using 3.0 g of fresh faeces diluted in 30 mL distilled water. The ammonia concentration was determined according to the method described by [32].

Fresh faeces collected up to 15 minutes after defecation were used to determine SCFA and BCFA. A properly labelled plastic container with a lid was used to weigh 10 g of faeces that were mixes with 30 mL 16% formic acid. This mixture was homogenized and stored at 4°C for 3 to 5 days. Before the analysis, these solutions were centrifuged at 5000 rpm (2K15 centrifuge, Sigma, Osterodeam Hans, Germany) during 15 minutes. At the end, the supernatant was separated and centrifuged. Each sample underwent three centrifugations and at the end of the last one, part of the supernatant was transferred to a

properly identified eppendorff for subsequent freezing. Later on, the samples were thawed and centrifuged again at 14000 rpm during 15 minutes (Rotanta 460 Robotic, Hettich, Tuttlingen, Germany). Faecal SCFA and BCFA were determined by gas chromatography (Shimadzu®, model GC-2014, Kyoto, Japan), using a 30 m long and 0.32 mm wide glass column (Agilent Tecnologias, HP INNO cera-19091N, Santa Clara, USA). Nitrogen was used as the carrier gas at a 3.18 mL/min flow rate. Working temperatures were 200°C at injection, 240°C in the column (at a 20°C/min rate), and 250°C in the flame ionization detector.

Phenols and indoles were analyzed by chromatography, a GCMS2010 Plus gas chromatographer (Shimadzu®), coupled to a TQ8040 mass spectrometer with an AC 5000 autosampler and a split-splitless injector. Chromatographic separations were obtained in the SH-Rtx-5MS (30 m x 0.25 mm x 0.25 µm - Shimadzu®) column with a 1.0 mL $min^{-1}$ flow rate, and helium as the drag gas at 5.0 rate. The transfer line and ionization source temperatures were maintained at 40°C and 220°C, respectively, the 1 L injection volume in the split mode (1:10 rate). The GC oven temperature was maintained at 220°C (5 min), with 40°C $min^{-1}$ increase to 280°C (5 min). Total analysis time was 31 minutes and the mass spectrometer operated in the full scan modes (m/z = 40 to 400) and selective ion monitoring (SIM), electron ionization at 70 eV. GCMSsolution® was the software used in the data analysis.

For the sialic acid determination, faeces were lyophilized (Alpha 1-4 LO plus, Christ, Osterodeam Hans, Germany) and analyzed according to the method described by [33]. Biogenic amines were analyzed according to the method described by [34] in fresh faeces, collected up to 15 minutes after defecation.

The DMf, consistency score, faecal odor, pH, ammonia, phenols and indoles were also analyzed in the same samples 6 hours after defecation. For the analysis performed 6 hours after defecation, faeces were maintained at room temperature (average 24.5ºC and 84% relative humidity of air), in the shade during 6 hours.

## Calculations and Statistical analyses

Based on the laboratory results, the coefficients of total tract apparent digestibility (ATTD) and the diets metabolizable energy (ME) were calculated according to [28]:

ATTD% = (g of nutrient intake – g of nutrient excretion)/g of nutrient intake x 100.

ME (kcal/g) = {kcal/g GE intake – kcal/g GE faecal excretion – [(g CP intake – g CP faecal excretion) x 1.25kcal/g]} / g of feed intake.

The experiment had a completely randomized design with two treatments, each one with eight replicates, except for faecal odor that had 50 replicates. Each dog was considered an experimental unit. The Shapiro-Wilk test was used to determine normality of the data and the homoscedasticity of variances was analysed by Bartlett's test. When these assumptions were met, the t-Student's test was used at a 5% significance level. The non-parametric data were analyzed by Mann-Whitney-Wilcoxon test ($P$ <0.05). Frequency of faecal odor scores was analyzed by the chi-square test ($P$ <0.05).

# Abbreviations

DFM: Direct-fed microbials; ME: metabolizable energy; CP: crude protein; EEAH: ether extract in acid hydrolysis; DM: dry matter; DMf: faecal dry matter; GE: amount of gross energy; OM: organic matter; SCFA: short chain fatty acids; BCFA: branched chain fatty acids; ATTD: coefficients of total tract apparent digestibility.

# Declarations

*Ethical approval*

The experiment was approved by the Ethics Committee on Animal Use from the Sector of Agrarian Sciences, Federal University of Paraná, Curitiba, PR, Brazil, under protocol number 012/2019.

*Consent for publication*

Not applicable.

*Availability of data and materials*

All data generated or analyzed during this study will be available from the corresponding author on reasonable request and with permission of DSM.

*Competing interests*

The authors declare that they have no competing interests.

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*Authors contributions*

APF, LCB, SGO and AM designed and supervised the study, carried out data analyses and reviewed the manuscript. DCL, TSB and CMMS conducted the research and carried out laboratory analyses. DCL and TSB interpreted data and wrote the draft of the manuscript. LCB, assisted in the translation of the manuscript. All authors have read and approved the final manuscript.

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# Figures

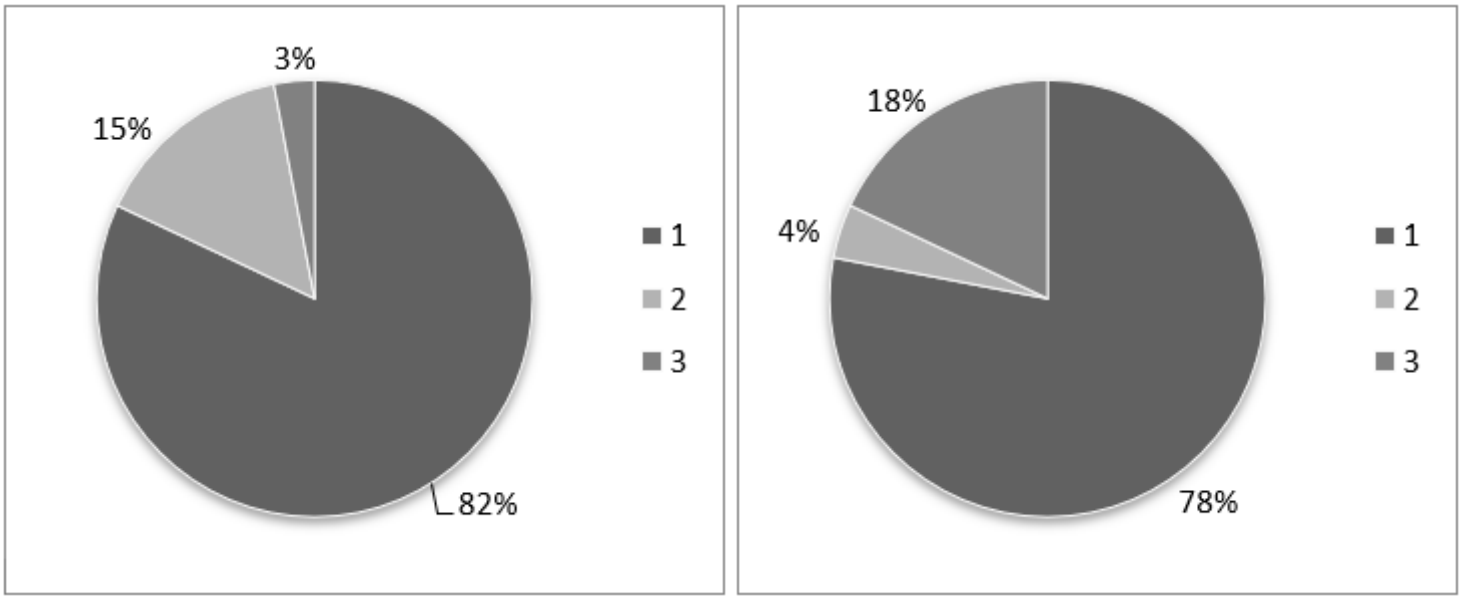


**Figure 1**

Points frequency of faecal odor scores attributed by evaluators (n = 50). 1 = less fetid odor; 2 = same odor and 3 = more fetid odor, as compared to faeces of dogs fed the control diet.

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