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Article

Effects of *Limosilactobacillus reuteri* NBF 1 DSM 32203 on Body Condition Score, Fecal Parameters and Intestinal Microbiota of Healthy Dachshund and Chihuahua Dogs

Benedetta Belà [1,*], Daniele Di Simone [2], Giulia Pignataro [1], Isa Fusaro [1] and Alessandro Gramenzi [1]

[1] Department of Veterinary Medicine, University of Teramo, Piano d'Accio, 64100 Teramo, Italy; gpignataro@unite.it; ifusaro@unite.it; agramenzi@unite.it

[2] Department of Economics and Finance, University of Bari, Largo Abbazia Santa Scolastica, 53, 70124 Bari, Italy; daniele.disimone93@gmail.com

* Correspondence: bbela@unite.it

Abstract: In recent years, the interest in the well-being of companion animals has grown more and more. After discovering the central role of the intestinal microbiota, various nutritional strategies (prebiotics, probiotics and postbiotics) have been evaluated to modulate its composition. However, to date, there are still few studies that analyze the effects of a specific probiotic and even fewer are the studies that evaluate how this strain can have different effects depending on the breed and size of the animal. In this study was investigated the effect of a specific probiotic strain, Limosilactobacillus reuteri NBF 1 DSM 32203, on the fecal parameters and intestinal microbiota of healthy small-sized adult dogs, specifically Chihuahuas and Dachshunds. The results obtained have highlighted how this probiotic is able to improve fecal parameters, reducing fecal humidity with the formation of more compact feces, significantly improving the fecal score. Furthermore, L. reuteri NBF 1 DSM 32203 is able to modulate the microbiota of Chihuahuas and Dachshunds by increasing the number of beneficial bacterial species such as lactobacilli and decreasing that of potentially pathogenic ones such as coliforms.

Keywords: Body Condition Score; Fecal Score; intestinal microbiota; *L. reuteri*; probiotics; dogs

1. Introduction

The cuddles, loyalty, companionship and unconditional affection they can give make dogs and cats increasingly present in homes all over the world; furthermore, several studies have shown how the presence of a pet also improves the mental and physical health of humans [1-3]. Thanks also to a better quality of life, owners treat these animals as real members of the family, paying close attention to their well-being. Following the discovery of the importance and great influence that the intestinal microbiota can have on animal health, nutritional strategies aimed at maintaining a more balanced intestinal microflora are increasingly. It is well known that probiotics can modulate the composition of the canine and feline intestinal microbiota, promoting the intestinal colonization of beneficial bacterial species able to release metabolites that strengthen the integrity of the intestinal barrier, making the environment more hostile to potentially harmful bacteria. However, it is important to remember that when we talk about probiotics, we are referring to live microorganisms that belong not only to different bacterial phyla but also to different bacterial classes, orders, families, genera and species; it is essential to know this because recent studies have highlighted how bacteria belonging to the same species can perform different functions within the organism based on the specific strain to which they belong, the well-known concept of 'strain-specificity'. Considering what has been illustrated so far, it is better to evaluate the effects of a specific probiotic strain and not of a specific species, in fact, even if bacteria belonging to the same species will have many common characteristics,

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it is better to analyze the relative strains to have a more accurate and detailed information about their properties. However, among the species of bacteria that, during evolution, have managed to colonize the intestinal tract, adapting to coexist in a mutualistic way with the organism, we find *Lactobacillus reuteri* currently known as *Limosilactobacillus reuteri*. "Limose" from the latin "*limosus*" means slimy and indicates the ability of this bacterial species to produce exopolysaccharides (EPS). The new classification provides a better environmental and functional view and a better understanding of the differences within lactobacilli. Host-adapted bacteria, for example, are more competitive than those that do not have a common evolutionary history with the host, which is interesting for the effectiveness of a product in fighting pathogens. This reclassification of lactobacilli is a unique opportunity to better explain the world of bacteria, to better understand the specificities of new groups and to develop more targeted products and, ultimately, for greater efficacy. EPS are important food and pharmaceutical additives with beneficial antioxidant, antitumor, and immunological effects for human health. However, EPS are difficult to produce due to complex conditions in fermentation. EPS produced by *L. reuteri* are important for biofilm formation and adhesion of *L. reuteri* to epithelial surfaces; EPS synthesized by *L. reuteri* can inhibit the adhesion of *E. coli* [4]. In addition, they can reduce the gene expression of proinflammatory cytokines induced by *Escherichia coli* infection (including IL-1b and IL-6). Other *in vivo* experiments [5] showed similar results: EPS produced by *L. reuteri* prevent diarrhea in bacterial infection by reducing the adhesion of *E. coli*. Furthermore, EPS can act also as a prebiotic. The present study is the last of three research conducted to evaluate the efficacy of a specific strain of *L. reuteri*, *L. reuteri* NBF 1 DSM 32203, on the intestinal microbiota and fecal parameters of healthy adult dogs. In the two previously published research were evaluated the effects of *L. reuteri* NBF 1 DSM 32203 on the intestinal microbiota of medium-large sized dogs (Golden Retriever) and subjects with a predisposition to intestinal diseases like French Bulldogs. We thought it was important to evaluate the effectiveness of this probiotic on small dogs such as Chihuahuas and Dachshunds; in fact, analyzing the growth curves of large and small dogs, those of large dogs show a greater slope underlying how these animals undergo a notable increase in their body weight during the first weeks of life while small breeds show a growth curve with a lesser slope and a moderate increase. These observations suggest that the effort that large breed dogs make during the early stages of growth, when their intestinal microbiota is still unstable, may compromise its integrity in later adulthood by influencing its composition and making it more susceptible to potentially harmful agents; the fact that large dogs live less than small dogs could also be connected to this process. For this reason, it is important to analyze the effect of the same probiotic strain on dogs of different sizes to see if the probiotic can have different effects depending on the intestinal ecosystem it will colonize. *L. reuteri* NBF 1 DSM 32203 can improve fecal quality parameters in healthy Golden Retriever dogs promoting an increase in the amount of *Lactobacillus* spp., a beneficial bacterial group able to favor a balanced intestinal ecosystem [7]. Similar results were reported by another study that analyzed the effects of the same probiotic strain on the intestinal microbiota of French bulldogs [8], also in this case, *L. reuteri* NBF 1 DSM 32203 was able to improve the Fecal Score decreasing the fecal humidity with a production of more consistent and well-formed feces indicator of an adequate intestinal function, in addition, the probiotic enhanced the amount of lactobacilli causing a small reduction in the total coliforms. Except for these two studies, in literature, there are no publications on the effects of *L. reuteri* NBF 1 DSM 32203 on the intestinal microbiota and fecal parameters of adult dogs. Zhao *et al*. (2023) [6] investigated the effects of another strain of *L. reuteri*, specifically *Limosilactobacillus reuteri* ZJF036, on growth performance and gut microbiota of young Beagle dogs showing that this specific probiotic strain is not able to influence the daily weight gain, but it is able to decrease Chao1 index and ACE index increasing the relative abundance of *Firmicutes* and *Fusobacteria*. Obviously, this study is not comparable with those conducted on adult animals because, in young dogs the intestinal microbiota has yet to fully stabilize.

Aim of the Study

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The aim of the present study is to analyze the effects of the specific probiotic strain *Limosilactobacillus reuteri* NBF 1 DSM 32203 on the body Weight, Body Condition Score, fecal quality parameters and intestinal microbiota of healthy small breed dogs like Dachshunds and Chihuahuas.

2. Materials and Methods

2.1. Ethic Statement

The research was conducted according to the directive 2010/63/EU; the study did not imply any form of animal suffering or health risk, since it focused on the administration of a natural substance.

2.2. Animals and Study Design

Sixty healthy Chihuahua (*n*=30) and Dachshund dogs (*n*=30), 26 males (12 Chihuahuas + 14 Dachshunds = 26) and 34 non-pregnant females (18 Chihuahuas + 16 Dachshunds = 34), age> 1 year were involved in the study and randomly assigned to the control group (CTR; *n*= 30: 15 Chihuahuas + 15 Dachshunds) and experimental group (LACTO; *n*= 30: 15 Chihuahuas + 15 Dachshunds). Animals were divided randomly (SurveyMonkey Excel) into the two groups and the group assignment was organized according to kennel management standard procedure. The experimental group was supplemented with *Limosilactobacillus reuteri* NBF 1 DSM 32203 while the control group did not receive the probiotic and was supplemented by maltodextrin used as placebo. The two dog breeds were analyzed individually to evaluate any differences inherent to the breed itself or to the diet administered. Table 1 reports age, sex and body weight of each dog of the two breeds belonging to the control and experimental groups. Cleaning and disinfecting procedures of the single fences were carried out and the animals have been individually stabulated. Moreover, the animals were evaluated daily by a veterinarian for any health and welfare concerns throughout the experimental period. The study lasted a total of 49 days: 35 days of study and 14 days of acclimatization.

Table 1.

Dogs (CTR group)	Age (months)	Sex	Body weight (Kg)	Breed
1	14.8	M	2.60	Chihuahua
2	23.6	M	2.70	Chihuahua
3	23.6	M	2.50	Chihuahua
4	30.4	M	2.80	Chihuahua
5	14.8	M	2.90	Chihuahua
6	14.8	M	2.60	Chihuahua
7	30.4	F	1.80	Chihuahua
8	30.4	F	1.90	Chihuahua
9	23.6	F	1.70	Chihuahua
10	23.6	F	1.60	Chihuahua
11	14.8	F	1.80	Chihuahua
12	14.8	F	1.80	Chihuahua
13	23.6	F	1.80	Chihuahua
14	30.4	F	1.70	Chihuahua
15	30.4	F	1.60	Chihuahua
16	26.2	M	8.40	Dachshund
17	27.4	M	8.60	Dachshund
18	26.8	M	8.40	Dachshund
19	28.9	M	8.80	Dachshund
20	30.2	M	8.70	Dachshund
21	26.9	M	8.80	Dachshund
22	34.5	M	8.60	Dachshund
23	36.2	F	7.50	Dachshund
24	34.9	F	7.60	Dachshund

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25	33.5	F	7.70	Dachshund
26	34.7	F	7.50	Dachshund
27	32.8	F	7.60	Dachshund
28	32.2	F	7.80	Dachshund
29	29.9	F	7.90	Dachshund
30	30.3	F	7.50	Dachshund
Dogs (LACTO group)	**Age (months)**	**Sex**	**Body weight (Kg)**	**Breed**
31	30.4	M	2.90	Chihuahua
32	30.4	M	2.80	Chihuahua
33	23.6	M	2.70	Chihuahua
34	14.8	M	2.90	Chihuahua
35	14.8	M	2.90	Chihuahua
36	23.6	M	2.80	Chihuahua
37	23.6	F	1.70	Chihuahua
38	23.6	F	1.70	Chihuahua
39	23.6	F	1.60	Chihuahua
40	14.8	F	1.80	Chihuahua
41	14.8	F	1.90	Chihuahua
42	30.4	F	1.80	Chihuahua
43	30.4	F	1.70	Chihuahua
44	30.4	F	1.70	Chihuahua
45	14.80	F	1.80	Chihuahua
46	28.8	M	8.80	Dachshund
47	26.6	M	8.90	Dachshund
48	25.4	M	8.70	Dachshund
49	24.5	M	8.70	Dachshund
50	29.7	M	8.60	Dachshund
51	27.8	M	8.50	Dachshund
52	30.5	M	8.80	Dachshund
53	33.2	F	7.50	Dachshund
54	35.6	F	7.50	Dachshund
55	34.2	F	7.60	Dachshund
56	31.7	F	7.70	Dachshund
57	34.5	F	7.80	Dachshund
58	34.2	F	7.70	Dachshund
59	33.3	F	7.60	Dachshund
60	31.5	F	7.90	Dachshund

2.3. Feed Supplement and Diet

All dogs had free access to potable water and were fed with two different commercial petfood: Chihuahua dogs consumed Royal Canin mini adult extruded feed while the dachshund dogs ate Eukanuba adult small breed food. For the entire duration of the study, feed was administered to each animal once a day based on the energy requirements of each dog (adult dogs: 110 kcal x $BW^{0.75}$ kg) [9]; table 2 reports the analytical components of each petfood administered. Dogs belonging to the experimental group received commercial feed with the addition of 10 g/100 kg of *Limosilactobacillus reuteri* NBF 1 DSM 32203, corresponding to 5 x 10^9 colony-forming units (CFU)/kg food; the process to obtain the right probiotic amount consisted in using 50 g of the feed additive (standard concentration ≥ 1.0 x 10^{11} CFU/g), pre-mixed in laboratory with 9950 g of maltodextrins. Then, a total of 20 g of this pre-mixture was daily added to each 980 g of commercial feed in the bowl. The control group received the commercial diet, with the addition of 20 g of maltodextrin in 980 g of dog feed (placebo).

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A. Royal Canin mini adult extruded feed

Table 2. Analytical components of Royal Canin mini adult extruded feed (A) and Eukanuba adult small breed food (B).

Parameter	Amount (%)
Crude protein	27.00
Crude fats	16.00
Raw fiber	1.30
Raw ash	6.80
Metabolizable energy	*3920 Kcal/Kg*

Composition: dehydrated poultry protein, wheat flour, maize flour, animal fats, maize, wheat gluten, barley, maize gluten, wheat, hydrolysed animal proteins, minerals, beet pulp, soya oil, yeast products, fructooligosaccharides, fish oil, Schizochytrium sp. algae oil.

Nutritional additives: Vitamin A: 15700 IU, Vitamin D3: 1000 IU, Iron (3b103): 36 mg, Iodine (3b201, 3b202): 3.6 mg, Copper (3b405, 3b406): 11 mg, Manganese (3b502, 3b504): 47 mg, Zinc (3b603, 3b605, 3b606): 133 mg, Selenium (3b801, 3b811, 3b812): 0.09 mg - Preservatives - Antioxidants.

- Eukanuba adult small breed food

Parameter	Amount (%)
Crude protein	30.00
Crude fats	18.00
Raw fiber	2.00
Raw ash	6.70
Metabolizable energy	*3923 Kcal/Kg*

Composition: dehydrated chicken and turkey (28% of which chicken 17%, natural source of glucosamine, chondroitin sulphate and taurine), fresh chicken (15%), corn, wheat, poultry fat, oats, barley, millet, beet pulp (3.5%), chicken gravy, fish meal (natural source of Omega 3 fatty acids), minerals (including sodium hexametaphosphate 0.35%), dehydrated whole eggs, fructo-oligosaccharides (0.38%, natural prebiotics).

Nutritional additives: Vitamin A (47,749 IU/kg), Vitamin C (60 mg/kg), Vitamin D3 (1,584 IU/kg), Vitamin E (265 mg/kg), Beta-Carotene (5.2 mg/kg), L-Carnitine (50 mg/kg), Copper (9 mg/kg), Iodine (1.1 mg/kg), Manganese (4 mg/kg), Zinc (100 mg/kg), Selenium (0.01 mg/kg). Antioxidants: tocopherol-rich vegetable oil extracts (86 mg/kg); flavourings: organic rosemary extract (46 mg/kg), tea extract (23 mg/kg).

2.4. Data Collection

Body Weight (BW), Body condition Score (BCS), Fecal Score (FS) and Fecal Moisture (FM) were recorded at the beginning of the experiment (day 0= T0), after one week (T1), after 2 weeks (T2), after 3 weeks (T3), after 4 weeks (T4) an at the end of the study (T5) according to the American Animal Hospital Association (AAHA) Nutritional Assessment Guidelines for Dogs and Cats [10]. The Body Weight of each dog was measured by the same operator at the same time (morning, before feed administration), with the same instrument while the body composition assessment was carried out by visual examination and palpation of the animal on a scale between 1 and 9 where a score of 4 or 5 is reflecting the ideal body condition [11]. A 7-point scoring chart was used for the analysis of the fecal score according to the Nestle Purina Fecal Scoring System while for the analysis of fecal moisture (FM) the fecal samples were collected from each dog at T0, T1, T2, T3, T4 and T5 and then stored at +4°C until they are brought to the laboratory, where they are stored at −20°C. 5–10 g of stool was weighed and dried in an oven at a temperature of 105°C–110°C for 20–24 hours, cooled down in a desiccator for another 20–24 hours, after which the Fecal Moisture content was calculated as lost weight after desiccation. In addition, a microbiological analysis was conducted to identify and quantify specific intestinal bacterial species, one gram of fresh stool was used to carry out this analysis

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at four specific time points: T0, T1, T3 and T5. Briefly, fresh stool was diluted in sterile saline solution with a ratio of 1:10 and then vortexed for two minutes to have a homogeneous suspension which was plated on different culture media for total bacterial counts and identification; for *Escherichia coli* and total coliforms was used the eosin methylene blue agar (Oxoid, Italy) with an incubation of 24 hours at 37°C. *E. coli* colonies show growth with a green metallic reflex, while coliforms show growth with blue, red, or uncolored colonies. For lactobacilli identification was used the Man Rogosa and Sharpe agar (Oxoid) and the plates were incubated for 48 hours at 37°C under anaerobic condition. All the analysis was performed in duplicate.

2.4. Statistical Analysis

For the statistical analysis a Mixed Model with repeated measurements has been used, which allows to estimate the parameters considering both random effect and fixed effect [12]. The model has been estimated as the following:

$y_{i,j,k} = \mu + S_i + G_j + T_k + G_j * T_k + e_{i,j,k}$

where y = dependent variable (FM, FS, BW, BCS, LB, COLI); μ = overall mean; Si = fixed effect of the ith sex (I = 1, 2); Gj = fixed effect of the jth group (j = 1, 2); Tk = fixed effect of the kth time (k = 0, 5) and ei,j,k = error. The software used was R Core Team (2020), R: A language and environment for statistical computing [13] and for the different analysis was used the Mixed Model [14] and the Least Squares [15]. Time was used as repeated measurement and therefore each subject has been analyzed in every different temporal instant. The autoregressive covariance structure was used. Least Square Means were estimated, and they have been statistically tested using Student's t test (with Tukey p-value adjustment). To be able to describe the goodness of the fit of the mixed model, we used the R squared described by Nakagawa *et al*. [16]. No outliers and missing data were found. Table 6 reports the power of the t-test at time 5 (T5).

3. Results

The results of the study show how *L. reuteri* NBF 1 DSM 32203 supplementation has no influence on the weight (Table 3) and body condition (Table 4) of Chihuahua and Dachshund adult healthy dogs; in fact, the body weight of each dog remained almost constant throughout the entire duration of the study.

Table 3. Effect of *Limosilactobacillus reuteri* NBF 1 DSM 32203 supplementation on body weight of Chihuahua (A) and Dachshund (B) adult healthy dogs.

(A) Effect of *Limosilactobacillus reuteri* on body weight (LS Mean ± SE) of Chihuahua dogs			
Time	**CTR group**	**LACTO group**	***p*-value**
Overall	2.20±0.05	2.23±0.05	0.306
T0	2.22±0.08	2.28±0.08	0.850
T1	2.21±0.08	2.21±0.08	1.000
T2	2.21±0.08	2.22±0.08	1.000
T3	2.17±0.08	2.23±0.08	0.850
T4	2.20±0.08	2.19±0.08	1.000
T5	2.19±0.08	2.27±0.08	0.510
(B) Effect of *Limosilactobacillus reuteri* on body weight (LS Mean ± SE) of Dachshund dogs			
Time	**CTR group**	**LACTO group**	***p*-value**
Overall	8.08±0.06	8.13±0.06	0.160
T0	8.13±0.10	8.19±0.10	0.960
T1	8.09±0.10	8.13±0.10	0.990
T2	8.10±0.10	8.08±0.10	1.000
T3	8.08±0.10	8.10±0.10	1.000
T4	8.01±0.10	8.11±0.10	0.612
T5	8.05±0.10	8.17±0.10	0.340

Table 4. Effect of *Limosilactobacillus reuteri* NBF 1 DSM 32203 supplementation on body condition score of Chihuahua (A) and Dachshund (B) adult healthy dogs.

(A) Effect of *Limosilactobacillus reuteri* on body condition score (LS Mean ± SE) of Chihuahua dogs			
Time	**CTR group**	**LACTO group**	***p*-value**
Overall	4.73±0.010	4.69±0.010	0.483
T0	4.71±0.24	4.65±0.24	1.000
T1	4.75±0.24	4.71±0.24	1.000
T2	4.71±0.24	4.71±0.24	1.000
T3	4.78±0.24	4.68±0.24	0.990
T4	4.71±0.24	4.68±0.24	1.000
T5	4.71±0.24	4.68±0.24	1.000
(B) Effect of *Limosilactobacillus reuteri* on body condition score (LS Mean ± SE) of Dachshund dogs			
Time	**CTR group**	**LACTO group**	***p*-value**
Overall	4.68±0.10	4.72±0.10	0.660
T0	4.64±0.24	4.70±0.24	1.000
T1	4.70±0.24	4.74±0.24	1.000
T2	4.70±0.24	4.70±0.24	1.000
T3	4.67±0.24	4.77±0.24	1.000
T4	4.67±0.24	4.70±0.24	1.000
T5	4.67±0.24	4.70±0.24	1.000

However, it is very interesting to see how fecal hmoisture (Table 5) decreased in the group of dogs treated with the probiotic *L. reuteri* NBF 1 DSM 32203 compared to the control one; similar results were reported in both Chihuahua and Dachshund dogs. At the end of the 35 days of study, Chihuahuas supplemented with *L. reuteri* NBF 1 DSM 32203 showed a decrease in fecal moisture of 0.2 points compared to T0 (beginning of the study), while the dachshund dogs showed a decrease of 0.24 points (Table 5).

Table 5. Effect of *Limosilactobacillus reuteri* NBF 1 DSM 32203 supplementation on fecal moisture of Chihuahua (A) and Dachshund (B) adult healthy dogs: results of mixed models showing least squares means ± SE in the CTR (control group) and LACTO (experimental group) dogs, for the six individual sampling times and overall, throughout the study.

(A) Effect of *Limosilactobacillus reuteri* on fecal moisture (LS Mean ± SE) of Chihuahua dogs			
Time	**CTR group**	**LACTO group**	***p*-value**
Overall	0.68±0.01	0.60±0.01	<0.001
T0	0.68±0.02	0.67±0.02	0.950
T1	0.71±0.02	0.69±0.02	0.410
T2	0.68±0.02	0.63±0.02	<0.001
T3	0.68±0.02	0.58±0.02	<0.001
T4	0.67±0.02	0.52±0.02	<0.001
T5	0.69±0.02	0.48±0.02	<0.001
(B) Effect of *Limosilactobacillus reuteri* on fecal moisture (LS Mean ± SE) of Dachshund dogs			
Time	**CTR group**	**LACTO group**	***p*-value**
Overall	0.68±0.01	0.57±0.01	<0.001
T0	0.68±0.02	0.67±0.02	0.960
T1	0.68±0.02	0.64±0.02	0.001
T2	0.67±0.02	0.60±0.02	<0.001
T3	0.67±0.02	0.56±0.02	<0.001
T4	0.68±0.02	0.50±0.02	<0.001
T5	0.69±0.02	0.43±0.02	<0.001

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Another interesting effect to consider is the improved fecal score reported in dogs that received the probiotic under study. Starting from the second week of study (T2), the Chihuahua group treated with *L. reuteri* NBF 1 DSM 32203 reported a Fecal score value of 2.09 (Table 6), very close to the value considered optimal, i.e. 2. The dachshund dogs reported the same excellent score starting from the fourth week of study (T4), while in the respective control group no similar improvement was observed (Table 6).

Table 6. Effect of *Limosilactobacillus reuteri* NBF 1 DSM 32203 supplementation on the fecal score of Chihuahua (A) and Dachshund (B) adult healthy dogs: results of mixed models showing least squares means ± SE in CTR (control group) and LACTO (experimental group) dogs, for the six individual sampling times and overall, throughout the study.

(A) Effect of *Limosilactobacillus reuteri* on fecal score (LS Mean ± SE) of Chihuahua dogs			
Time	**CTR group**	**LACTO group**	***p*-value**
Overall	2.55±0.08	2.07±0.08	<0.001
T0	2.26±0.30	2.66±0.30	0.980
T1	2.26±0.30	2.26±0.20	0.037
T2	2.49±0.22	2.09±0.18	0.001
T3	2.62±0.18	1.99±0.18	<0.001
T4	2.46±0.18	1.79±0.18	<0.001
T5	2.62±0.18	1.62±0.18	<0.001
(B) Effect of *Limosilactobacillus reuteri* on fecal score (LS Mean ± SE) of Dachshund dogs			
Time	**CTR group**	**LACTO group**	***p*-value**
Overall	2.91±0.15	2.31±0.15	<0.001
T0	2.93±0.25	2.96±0.25	1.000
T1	2.86±0.25	2.56±0.25	0.300
T2	3.00±0.25	2.23±0.25	<0.001
T3	2.86±0.25	2.10±0.25	<0.001
T4	2.86±0.25	2.00±0.25	<0.001
T5	2.93±0.25	2.00±0.25	<0.001

As regards the microbiological analysis, the trend of lactobacilli is very similar between Chihuahua and Dachshund dogs supplemented with *L. reuteri* NBF 1 DSM 32203; in both groups a clear increase of this bacterial genus is observed starting from the third week of the study and continuing to increase until the end of the experiment (Table 7, Figure 1). Specifically, in the group of Chihuahuas treated with *L. reuteri* NBF 1 DSM 32203, a significant increase in lactobacilli was recorded, going from an initial concentration of 4.72 ± 0.06 CFU/ml to 5.63 ± 0.06 CFU/ml after 35 days of study. As for the Dachshunds, the LACTO group went from an initial concentration of 4.80 ± 0.06 CFU/ml to reach a concentration of 5.62 ± 0.06 CFU/ml at the end of the experiment (Table 7).

Table 7. Effect of *Limosilactobacillus reuteri* NBF 1 DSM 32203 supplementation, expressed as log CFU/g, on the total amount of Lactobacilli present in the intestinal microflora of Chihuahua (A) and Dachshund (B) adult healthy dogs: results of mixed models showing least squares means ± SE in CTR (control group) and LACTO (experimental group) dogs, for the four individual sampling times and overall, throughout the study.

(A) Effect of *Limosilactobacillus reuteri* on total amount of Lactobacilli (LS Mean ± SE) in Chihuahua dogs			
Time	**CTR group**	**LACTO group**	***p*-value**
Overall	4.70±0.04	5.11±0.04	<0.001
T0	4.67±0.06	4.72±0.06	0.780
T1	4.71±0.06	4.89±0.06	<0.001
T3	4.70±0.06	5.22±0.06	<0.001
T5	4.71±0.06	5.63±0.06	<0.001

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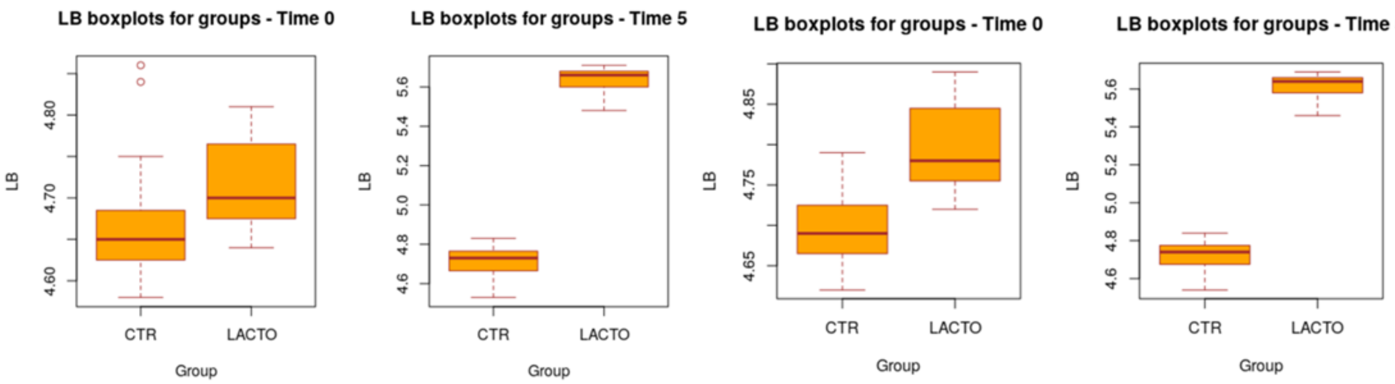
(B) Effect of *Limosilactobacillus reuteri* on total amount of Lactobacilli (LS Mean ± SE) in Dachshund dogs			
Time	**CTR group**	**LACTO group**	***p*-value**
Overall	4.72±0.04	5.15±0.04	<0.001
T0	4.71±0.06	4.80±0.06	0.102
T1	4.73±0.06	4.96±0.06	<0.001
T3	4.72±0.06	5.24±0.06	<0.001
T5	4.72±0.06	5.62±0.06	<0.001



(A) Chihuahua dogs. (B) Dachshund dogs

Figure 1. Box plot showing the effect of *Limosilactobacillus reuteri* NBF 1 DSM 32203 supplementation on total Lactobacilli count (LB) in Chihuahua (A) and Dachshund (B) healthy adult dogs during the overall period. CTR, control group; LACTO, experimental group.

In addition, total coliforms decreased in the probiotic-treated groups compared to the control ones: Chihuahua dogs reported an initial concentration of 4.65±0.06 CFU/ml and a final concentration of 3.94±0.06 CFU/ml , while the Dachshunds showed an initial concentration of 4.55±0.06 CFU/ml and a concentration of 3.96±0.06 CFU/ml at the end of the study (Table 8, Figure 2).

Table 8. Effect of *Limosilactobacillus reuteri* NBF 1 DSM 32203 supplementation, expressed as log CFU/g, on the amount of *E. coli* present in the intestinal microflora of Chihuahua (A) and Dachshund (B) adult healthy dogs: results of mixed models showing least squares means ± SE in CTR (control group) and LACTO (experimental group) dogs, for the four individual sampling times and overall, throughout the study.

(A) Effect of *Limosilactobacillus reuteri* on total amount of *E. coli* (LS Mean ± SE) in Chihuahua dogs			
Time	**CTR group**	**LACTO group**	***p*-value**
Overall	4.70±0.04	4.31±0.04	<0.001
T0	4.73±0.08	4.65±0.08	0.514
T1	4.66±0.08	4.45±0.08	<0.001
T3	4.70±0.08	4.21±0.08	<0.001
T5	4.71±0.08	3.94±0.08	<0.001
(B) Effect of *Limosilactobacillus reuteri* on total amount of *E. coli* (LS Mean ± SE) in Dachshund dogs			
Time	**CTR group**	**LACTO group**	***p*-value**
Overall	4.64±0.04	4.28±0.05	<0.001
T0	4.60±0.08	4.55±0.08	0.912
T1	4.57±0.08	4.40±0.08	0.004
T3	4.72±0.08	4.23±0.08	<0.001
T5	4.68±0.08	3.96±0.08	<0.001

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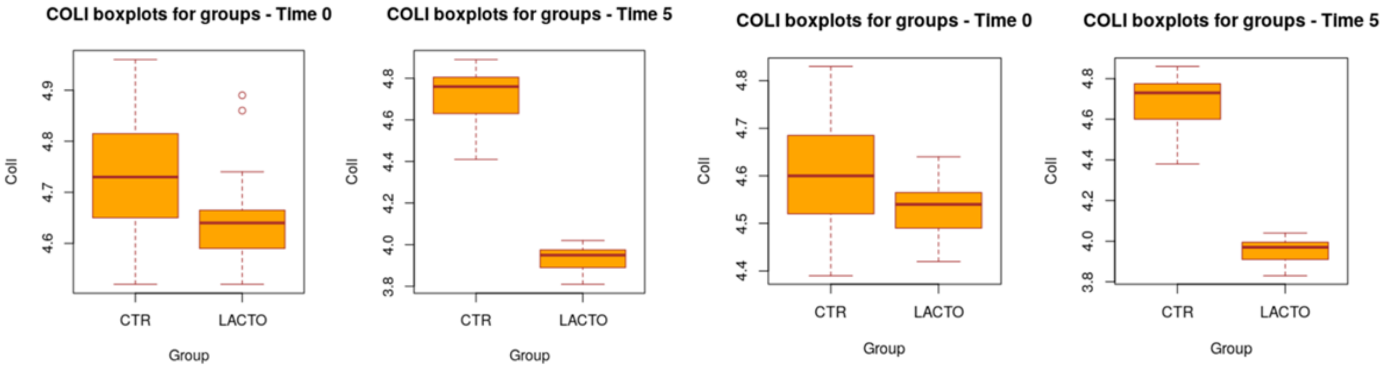

(A) Chihuahua dogs (B) Dachshund dogs

Figure 2. Box plot showing the effect of *Limosilactobacillus reuteri* NBF 1 DSM 32203 supplementation on total coliforms (Coli) in Chihuahua (A) and Dachshund (B) healthy adult dogs during the overall period. CTR, control group; LACTO, experimental group.

4. Discussion

The beneficial effects deriving from a probiotic administration are now well known, however to date very few studies have focused on the effects of a specific probiotic strain. In fact, a specific probiotic strain can exert a different action compared to other strains belonging to the same bacterial group, it is not a coincidence that today we speak of 'strain -specificity'. In addition, in literature very few studies analyzed the effects of the bacterial species *L. reuteri* on dog and no study has yet evaluated how the effect of these probiotics may vary according to the size of the animal. Today there are more than 400 dog breeds with high variability in size and weight starting from small dogs like Chihuahuas that can weigh 1 kg up to the 100 kg of a St. Bernard [17]. Digestion is a very important factor for canine health; this process involves physicochemical, mechanical, and microbial mechanisms. In recent years, the intestinal microbiota has gained relevance in dog nutrition and health. However, all the substances used to modulate the intestinal microbiota such as prebiotics, probiotics, postbiotics and other nutraceutical products should consider all the aspects concerning canine digestion and absorption. Recently, different petfood manufacturers produce specific feeds for certain dog breeds based on their size and predispositions [18–21]. To date, the effect that a dog's size may have on the digestion and absorption of substances remains little investigated. An interesting review published by Deschamps *et al*. [22] found only 163 studies that focused on the digestive differences that may exist in dogs of different sizes; among these studies small dogs were involved in only 7 publications, medium dogs in 71 and large dogs in 9, the other research has focused on the study of a single breed. It is easy to understand that to date the studies performed on the different digestive peculiarities between dogs of different sizes are very few and we still have little information on the matter. About the gastrointestinal motility and the transit time the studies performed so far do not show consistent results [23, 24] suggesting that gastric emptying time is more related to food consistency than animal size [25]. Even regarding the small intestinal transit time, to date it does not appear to be related to the weight of the animal [26]. However, Hernot *et al*. [27] demonstrated a longer large intestinal transit time in large dogs than in small dogs and a correlation between the large intestinal transit time and the canine size. The length of the large intestine but also its area and volume increases with the size of the animal, this seems to be closely associated with a higher colonic transit time influencing not only the absorption of water and nutrients but also the intestinal microbiota composition and fecal humidity. However, Guard and Suchodolski [28] highlighted that although the intestinal microbiota composition varies greatly between dogs, the functions performed by these microorganisms remain always the same and are unchanged in dogs of different sizes. However, among the most important metabolites released by intestinal microbes we find short-chain fatty acids and it appears that their fecal concentration increases as the animal's weight increases [29]; in fact, a longer large intestinal transit time can be related to a higher microbial fermentation. Excessive bacterial fermentation can create problems in water absorption due to the

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excessive production of SCFAs with high osmotic power. To what has been described so far, we should add the increased intestinal permeability observed in large breed dogs with a luminal retention of water and electrolytes [30]. Large dogs appear to be more delicate from an intestinal point of view than small dogs and considering this evidence it is appropriate to consider that the same probiotic strain may have different effects on dogs of different sizes as it finds a different intestinal ecosystem. This is why our research group wanted to evaluate the effects of the same probiotic strain on dogs of different breeds and sizes. In the present study we focused on the effects of *Limosilactobacillus reuteri* NBF 1 DSM 32203 on small dogs, observing how it manages to make the feces more consistent and formed, reducing fecal humidity, reaching a fecal score equal or very close to 2, considered optimal. The improvement in the fecal parameters is an indication of a correct digestive process and adequate intestinal absorption. Furthermore, as expected, the administration of the probiotic caused an increase in total lactobacilli, beneficial bacteria for intestinal health, and a significant reduction in coliforms, which are potentially harmful bacterial species. In previous studies we conducted on Golden Retrievers and French Bulldogs we found the same power of *L. reuteri* NBF 1 DSM 32203 to increase lactobacilli, however the reduction of coliforms seemed to be less evident than that observed in Chihuahuas and Dachshunds [7, 8]. Studies conducted so far would suggest a greater ability of *L. reuteri* NBF 1 DSM 32203 to reduce the concentrations of potentially pathogenic bacterial species such as coliforms in small animals compared to large ones, perhaps due to the different intestinal ecosystem they colonize.

5. Conclusions

As previously mentioned, this study is the last in a series of 3 experiments performed to evaluate the efficacy of the probiotic *Limosilactobacillus reuteri* NBF 1 DSM 32203 on body condition, fecal parameters and intestinal microbiota of healthy adult dogs of different sizes. The result obtained from the present study suggest how *L. reuteri* NBF 1 DSM 32203 can improve the fecal parameters of small dogs like Chihuahua and Dachshund managing to modulate the composition of the intestinal microbiota with an increase in beneficial bacterial species such as lactobacilli and a decrease in potentially harmful ones such as *E. coli*. Furthermore, this study showed the greater ability of *L. reuteri* to reduce total coliforms in small dogs compared to large dogs.

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