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**Effect of a *Lactobacillus animalis* strain on composition and metabolism of the intestinal  
microflora in adult dogs**

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## Abstract

Probiotics are microorganisms that are added to food to exert beneficial effects on the host. Aim of the present study was the in vitro and in vivo evaluation of the effect of *Lactobacillus animalis* LA4 (isolated from the faeces of a healthy adult dog) on composition and metabolism of dog intestinal microflora. When added to dog faecal cultures, LA4 reduced enterococci and increased lactobacilli counts throughout the study, whereas *C. perfringens* counts were significantly reduced at 24 h. After 8 h of incubation, LA4 reduced ammonia and increased lactic acid concentrations. For the in vivo study, nine adult dogs received the freeze-dried preparation of *L. animalis* LA4 for 10 days. On day 11, faecal lactobacilli were higher than at trial start (6.99 vs. 3.35 Log CFU/g of faeces) and faecal enterococci showed a trend towards a numerical reduction ( $P = 0.08$ ). *L. animalis* LA4 was recovered in all faecal samples collected on day 11 and in four samples at day 15. The present results show that LA4 was able to survive gastrointestinal passage and transitorily colonize the dog intestine where, based on the in vitro results, it could positively influence composition and metabolism of the intestinal microflora. These results suggest that *L. animalis* LA4 can be considered as a potential probiotic for dogs.

**Keywords:** dog, intestinal microflora, lactobacillus, probiotics

## 1. Introduction

Probiotics are microorganisms that are added to food to exert beneficial effects on the host. Schrezenmeier and De Vrese (2001) defined a probiotic as “A preparation or product

containing viable, defined micro-organisms in sufficient numbers, which alter the microflora of the host intestine and by that exert beneficial health effects on the host”.

Composition of dog intestinal microflora shows a large individual variability and may also depend on diet composition. While protein-rich diets increase the number of *Clostridium perfringens*, rations that contain fermentable carbohydrates lead to higher numbers of lactic acid bacteria (LAB) and bifidobacteria (Zentek, 2000). Furthermore, the normal composition of dog intestinal microflora can be altered by stressful conditions, such as weaning, dietary changes, gastrointestinal infections, and oral administration of antibiotics. For this reason, the feeding of probiotics to dogs may be a valuable tool to improve animal health and reduce the risk of gastrointestinal disorders. Aim of the present study was the in vitro and in vivo evaluation of the effect of a *Lactobacillus sp.* strain, isolated from faeces of an adult dog, on composition and metabolism of dog intestinal microflora.

## 2. Material and methods

### 2.1 Isolation of the probiotic strain

The faeces of eight healthy adult dogs (household dogs, different breeds, fed different commercial dry diets and living in different environments; between 1 and 3 years of age) were frozen at  $-18^{\circ}\text{C}$  within 20 min after excretion. Within 10 days from collection, faeces were homogenized and serially diluted in Wilkins-Chalgren Anaerobe Broth (WCAB, Oxoid LTD, Basingstoke, Hampshire, UK) added with L-cysteine  $\cdot$  HCl (0.5 g/L). Dilutions were plated on Raffinose Bifidobacterium Agar (RB Agar; Hartemink et al., 1996) and LAMVAB Agar (Hartemink et al., 1997), for bifidobacteria and lactobacilli counts, respectively. Plates were

incubated in an anaerobic cabinet (Anaerobic System, Forma Scientific Co., Marietta, USA) under a N<sub>2</sub> 85%, CO<sub>2</sub> 10%, H<sub>2</sub> 5% atmosphere at 37°C for 48 h.

The taxonomy of the colonies isolated on selective RB plates was determined at the genus level by fructose-6-phosphate phosphoketolase activity(Scardovi, 1986) and confirmed by the amplification with the 16S rDNA primer set Bif164/Bif662 according to Kok et al. (1996). Attribution to the species of RB and LAMVAB colonies was obtained by automated Ribotyping device, RiboPrinter Microbial Characterisation System (Qualicon Inc., Wilmington, DE, USA) as described by Massi et al. (2004). The speciation was confirmed by rDNA sequence analysis (Massi et al., 2004).

After speciation, it was decided to use one of the isolated strains (LA4) belonging to the species *L. animalis* in a feeding trial with adult dogs because it was the most predominant *Lactobacillus* strain recovered in the faeces of the sampled healthy dogs and for its high biomass yield. *L. animalis* LA4 was grown on the following complex medium (CM): phytone, 10 g/L; casein hydrolysate, 10 g/L; sodium acetate, 2.5 g/L; yeast extract, 10 g/L; Tween 80, 1 g/L; L-cysteine · HCl, 0.5 g/L; MnSO<sub>4</sub> · 7H<sub>2</sub>O, 7 mg/L; KH<sub>2</sub>PO<sub>4</sub>, 0.15 g/L; MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.5 g/L; Glucose 20 g/L; pH adjusted to 6.8. The LA4 strain was subcultured in Lactobacilli MRS broth containing 0.5 g/L L-cysteine · HCl and anaerobically incubated at 37°C for 24 h. Cells from the MRS cultures were inoculated (5% v/v) into CM medium and incubated anaerobically at 37°C for 48 h. After incubation, the biomass was harvested by centrifugation, resuspended in preservation Suspending Fluid (skim milk 50 g/L; lactose 30 g/L; yeast extract 50 g/L; ascorbic acid, 5 g/L), frozen at -80°C and freeze-dried. The freeze-dried probiotic contained about 10<sup>9</sup> CFU/g and was free of contaminations.

## 2.2 In vitro experiment

Fresh faeces from two adult healthy dogs were resuspended 1% (w/v) in pre-reduced WCAB. Faecal suspension was used to inoculate (1% v/v) 100 mL anaerobic serum bottles containing 80 ml of Dog Faecal Extract Medium (Benno and Mitsuoka, 1992). Faecal cultures were inoculated (1% v/v) with freeze-dried LA4 resuspended in WCAB medium at the final concentration of  $10^8$  CFU/ml or, as a negative control, with the same volume of WCAB medium. Each bottle received the addition of 1 g of pre-digested commercial dry food for dogs suspended in 10 ml of physiological solution. Dry food was digested using the two-step procedure (4 h incubation with HCl and pepsin followed by 4 h with pancreatin) proposed by Vervaeke et al. (1989) modified by adding gastric lipase (10 g/L; Amano Lipase F-AP15, Amano Enzyme Inc., Nagoya, Japan) to the HCl-pepsin solution and bile salts (25 g/L; Cholic acid-Deoxycholic acid sodium salt mixture, Fluka, USA) to the pancreatin solution. After *in vitro* digestion, the undigested fraction contained 12% crude protein, 2% ether extract, and only traces of starch. Faecal cultures were incubated at 39°C in anaerobiosis and samples were collected for analyses at 0, 4, 8 and 24 h. All preparations were done in an anaerobic cabinet.

### 2.3 *In vivo* experiment

The *in vivo* experiment has been done with client-owned dogs. The experimental protocol was reviewed and approved by the Ethical Committee of the University of Bologna.

Sixteen adult healthy dogs (household dogs, different breeds, fed different commercial dry diets and living in different environments; between 1 and 3 years of age) that had followed a pre/probiotic-free diet for 1 month were screened for faecal lactobacilli and bifidobacteria contents. Nine dogs having lactobacilli counts lower than  $10^4$  CFU per g of faeces were selected to assess the *in vivo* effect of *L. animalis* LA4. Dogs were fed the same

commercial dry diet (the same one used for the in vitro study) and received for 10 d a single oral daily dose of 0.5 g of the freeze-dried probiotic. Faecal samples were collected immediately after excretion and frozen the day before probiotic administration started (day 0) and again 1 and 5 d after withdrawal of the probiotic (day 11 and 15, respectively).

#### 2.4 Chemical, microbiological and statistical analysis

Analyses of the commercial dry diet after in vitro digestion were performed according to AOAC standard methods (AOAC, 2000). Ammonia in faecal cultures and homogenized faeces samples was measured using a commercial kit (Urea/BUN – Color, BioSystems S.A., Barcelona, Spain). Short-chain fatty acids (SCFA) in faecal cultures and homogenized faeces samples were analyzed by gas chromatography (Biagi et al., 2006).

Within 10 days from collection, faecal specimens were homogenized, serially diluted with anaerobe half-strength WCAB and plated in triplicate onto selective media: MacConkey Agar (Merck, Darmstadt, Germany) for coliforms, OPSP Agar (Oxoid, Basingstoke, UK) for *C. perfringens*, LAMVAB Agar for lactobacilli, Azide Maltose Agar (Biolife, Milano, Italy) for enterococci, and RB Agar for bifidobacteria. All media were kept  $\geq 24$  h in the anaerobic chamber before use. MacConkey Agar and Azide Maltose Agar plates were incubated aerobically at 37°C for 24 and 48 h, respectively; all other media were incubated anaerobically at 37°C for 48-72 h. The same selective media (with the only exception of RB Agar) were used for the bacterial counts from the faecal cultures samples. rDNA sequence of representative colonies on LAMVAB was determined for strain-level identification.

Data from the in vitro trial were analyzed using the Student-Newman-Keuls test. In the in vivo trial, results at Day 0, 11 and 15 were analyzed by one-way ANOVA with time as the



main factor; differences among means of groups were analyzed using the Student-Newman-Keuls test. Differences were considered statistically significant at  $P < 0.05$ .

### 3. Results

#### 3.1 *In vitro* experiment

Bacterial counts in faecal cultures are reported in Figure 1. Enterococci were significantly reduced and lactobacilli increased ( $P < 0.05$ ) by LA4 throughout the study. After 24 h, *C. perfringens* counts were significantly reduced by LA4 ( $P < 0.05$ ). Faecal pH and ammonia and SCFA concentrations are reported in Table 1. After 8 h, ammonia was significantly reduced (4.10 vs 9.68 mmol/L;  $P < 0.001$ ) by LA4. At 4 h, lactic acid concentration was significantly increased by LA4 (1.44 vs 1.16 mmol/L;  $P < 0.05$ ).

#### 3.2 *In vivo* experiment

Among the 16 sampled dogs, LAB faecal counts were higher than  $10^5$  CFU/g in three subjects and than  $10^6$  CFU/g in four (data not shown). On the contrary, the majority (9 out of 16) of the dogs had a LAB count of  $10^4$  CFU/g or lower .

All dogs remained in good health during the administration of *L. animalis* LA4. Faecal microbial counts are shown in Figure 2. On day 11, lactobacilli faecal counts were significantly higher than at trial start (6.99 vs. 3.35 Log CFU/g of faeces;  $P < 0.001$ ). The strain LA4 was recovered in all faecal samples collected on day 11 and in faeces of four dogs out of nine at day 15. Ammonia and SCFA faecal concentrations were not influenced by LA4

(data not shown in tables) and averaged 44.1, 71.5, 42.1, 2.13, and 16.2 mmol/L for ammonia, acetic acid, propionic acid, iso-butyric acid, and *n*-butyric acid, respectively.

#### 4. Discussion and Conclusion

The lactobacilli faecal counts of the 16 dogs screened for the selection of the animals to be used in the in vivo study confirmed that a very high variability exists within the canine population. The dogs were housed in different environments and fed different commercial dry diets. Nonetheless, it is known that the intestinal microflora of dogs shows big differences even if they are similarly housed and fed identical diets (Suchodolski et al., 2005).

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

The competitive exclusion of pathogens is a well-known beneficial effect of LAB probiotics (Rolfe, 2000). In the present in vitro study, addition of LA4 reduced enterococci counts throughout the study and *C. perfringens* at 24 h. When fed to adult dogs, LA4 did not significantly influence faecal counts of *C. perfringens*, coliforms and enterococci and this finding might be explained by the relative low number of animals used in this study and the high individual variability. However, enterococci and *C. perfringens* showed a trend towards a numerical reduction, and the reduction of faecal enterococci came close to a significant difference ( $P = 0.08$ ). In another study, when a *L. acidophilus* strain was fed to dogs (Baillon et al., 2004), clostridia faecal counts were reduced. Because enterotoxigenic *C. perfringens*

can be responsible of diarrhoea in dogs (Weese et al., 2001), LAB probiotics might help reduce the incidence in dogs of *C. perfringens* enteric disease and environmental shedding. The latter might be important for dog-owners, because *C. perfringens* enterotoxin has been associated with diarrhoea and food poisoning in humans (Mpamugo et al., 1995).

In vitro, LA4 reduced ammonia concentration by 58% after 8 h of incubation. This finding could be the consequence of the partial inhibition of proteolytic bacteria by LA4, as suggested by the reduction of *C. perfringens* observed at 24 h. Similarly, during a 24 h in vitro study with swine cecal chyme (Piva et al., 2005), a *L. brevis* strain reduced ammonia after 8 h but not after 24 h of incubation. Both studies suggest that LAB strains can reduce ammonia concentrations but that this effect disappears when energy sources such as fermentable carbohydrates are depleted. In our study, the dry food did not contain after enzymatic digestion energy sources available to LAB bacteria. Results from other studies (Tzortzis et al., 2003; Piva et al., 2005) confirm that ammonia reduction is more effective when LAB strains are associated with a non-digestible disaccharide (NDO).

Changes in dog faecal microflora were not reflected by differences in faecal concentrations of ammonia and SCFA. It is known (Stevens and Hume, 1998) that concentration of bacterial metabolites that are able to cross the intestinal mucosa can vary while digesta move along the intestine. As such, faeces might not reflect the changes in the concentration of ammonia and SCFA that LA4 might have induced in the hindgut.

The present results show that *L. animalis* LA4 was able to survive gastrointestinal passage and transitorily colonize the dog intestine where, based on the in vitro results, it could positively influence composition and metabolism of the intestinal microflora. These results suggest that LA4 can be considered as a potential probiotic for dogs.

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**Table 1.** pH values and ammonia and short-chain fatty acids (mmol/L) concentrations in dog faecal cultures added (Lac +) or not (Lac -) with *Lactobacillus animalis* LA4.

	4 h				8 h				24 h			
	Lac -	Lac +	Pooled	t-test <i>P</i>	Lac -	Lac +	Pooled	t-test <i>P</i>	Lac -	Lac +	Pooled	t-test <i>P</i>
	SEM				SEM				SEM			
pH	6.14	6.13	0.012	0.656	6.12	6.07	0.025	0.086	5.73	5.59	0.137	0.352
Ammonia	7.74	8.41	0.895	0.482	9.68	4.10	0.693	< 0.001	6.73	9.91	1.768	0.122
Acetic acid	16.1	17.3	1.532	0.453	18.6	18.3	1.414	0.855	21.3	21.5	1.148	0.813
Propionic acid	5.33	5.58	0.540	0.659	5.74	5.82	0.437	0.857	11.3	11.4	1.898	0.973
iso-Butyric acid	0.27	0.28	0.021	0.502	0.26	0.26	0.018	0.990	0.27	0.31	0.026	0.227
n-Butyric acid	1.24	1.24	0.117	0.984	1.22	1.22	0.096	0.977	2.55	2.25	0.412	0.492
iso-Valeric acid	0.25	0.25	0.015	0.736	0.22	0.22	0.014	0.805	0.22	0.25	0.020	0.209
Lactic acid	1.16	1.44	0.108	0.040	1.57	1.67	0.194	0.639	0.98	1.18	0.182	0.325
n-Valeric acid	0.10	0.08	0.012	0.134	0.07	0.07	0.004	0.964	0.07	0.06	0.005	0.649
Total SCFA	24.4	26.2	2.199	0.455	27.7	27.6	2.069	0.963	36.7	37.0	3.102	0.925

Values are the mean of four replicates.

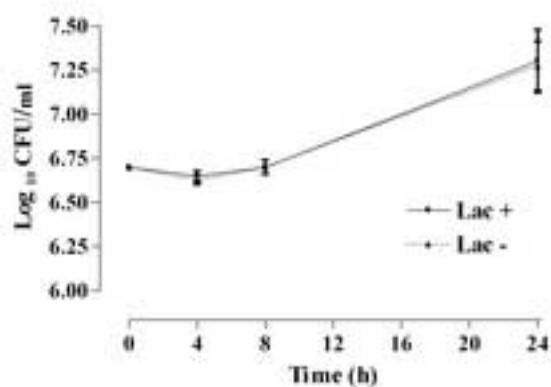
259 **Fig. 1.** Counts (log CFU/mL) of viable coliforms, enterococci, *Clostridium perfringens*,  
260 bifidobacteria, and lactobacilli in dog faecal cultures added (Lac +) or not (Lac -) with  
261 *Lactobacillus animalis* LA4. Values are the mean of four replicates  $\pm$  SEM.

262

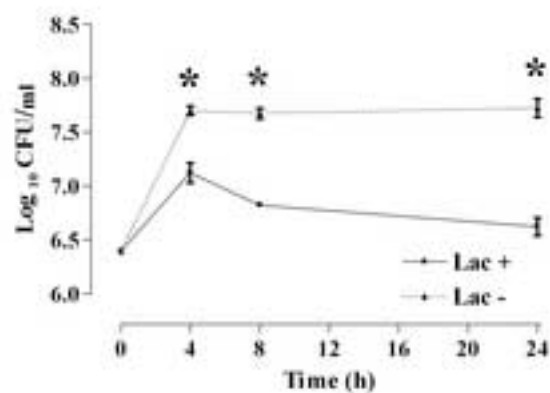
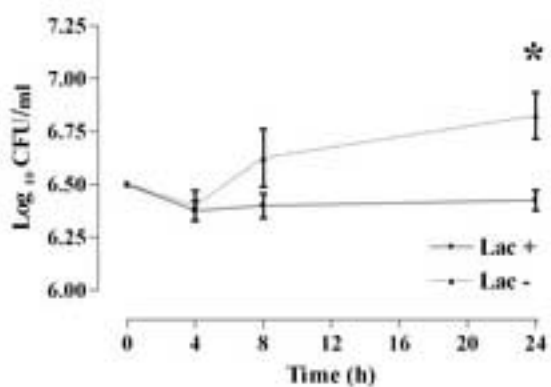
263 **Fig. 2.** Counts of viable bacteria in the faeces of nine dogs before (Day 0) and 1 (Day 11) and 5  
264 d (Day 15) after a 10 d administration of *Lactobacillus animalis* LA4 (log CFU/g); values are  
265 means  $\pm$  SEM; within a bacterial population, means without a common superscript letter differ  
266 ( $P < 0.001$ ).



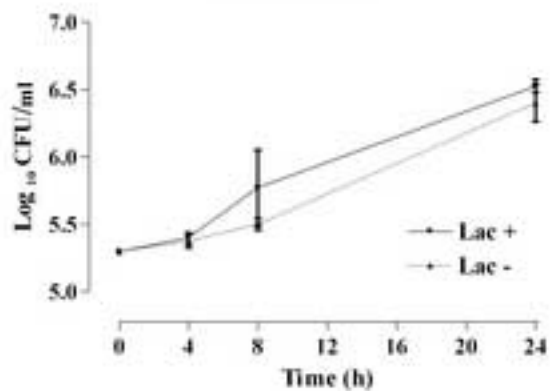
Coliforms



Enterococci

*Clostridium perfringens*

Bifidobacteria



Lactobacilli

