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

3

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## 9 Abstract

10

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

26

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

28

## 29 1. Introduction

30

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

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

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

45

## 46 **2. Material and methods**

47

### 48 *2.1 Isolation of the probiotic strain*

49

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

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

59 The taxonomy of the colonies isolated on selective RB plates was determined at the  
60 genus level by fructose-6-phosphate phosphoketolase activity(Scardovi, 1986) and confirmed  
61 by the amplification with the 16S rDNA primer set Bif164/Bif662 according to Kok et al.  
62 (1996). Attribution to the species of RB and LAMVAB colonies was obtained by automated  
63 Ribotyping device, RiboPrinter Microbial Characterisation System (Qualicon Inc.,  
64 Wilmington, DE, USA) as described by Massi et al. (2004). The speciation was confirmed by  
65 rDNA sequence analysis (Massi et al., 2004).

66 After speciation, it was decided to use one of the isolated strains (LA4) belonging to the  
67 species *L. animalis* in a feeding trial with adult dogs because it was the most predominant  
68 *Lactobacillus* strain recovered in the faeces of the sampled healthy dogs and for its high  
69 biomass yield. *L. animalis* LA4 was grown on the following complex medium (CM): phytone,  
70 10 g/L; casein hydrolysate, 10 g/L; sodium acetate, 2.5 g/L; yeast extract, 10 g/L; Tween 80,  
71 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,  
72 0.5 g/L; Glucose 20 g/L; pH adjusted to 6.8. The LA4 strain was subcultured in Lactobacilli  
73 MRS broth containing 0.5 g/L L-cysteine · HCl and anaerobically incubated at 37°C for 24 h.  
74 Cells from the MRS cultures were inoculated (5% v/v) into CM medium and incubated  
75 anaerobically at 37°C for 48 h. After incubation, the biomass was harvested by centrifugation,  
76 resuspended in preservation Suspending Fluid (skim milk 50 g/L; lactose 30 g/L; yeast  
77 extract 50 g/L; ascorbic acid, 5 g/L), frozen at -80°C and freeze-dried. The freeze-dried  
78 probiotic contained about 10<sup>9</sup> CFU/g and was free of contaminations.

79

## 80 2.2 *In vitro* experiment

81

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

97

### 98 2.3 *In vivo* experiment

99

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

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

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

111

#### 112 *2.4 Chemical, microbiological and statistical analysis*

113

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

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

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



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

133

### 134 **3. Results**

135

#### 136 *3.1 In vitro experiment*

137

138 Bacterial counts in faecal cultures are reported in Figure 1. Enterococci were  
139 significantly reduced and lactobacilli increased ( $P < 0.05$ ) by LA4 throughout the study. After  
140 24 h, *C. perfringens* counts were significantly reduced by LA4 ( $P < 0.05$ ). Faecal pH and  
141 ammonia and SCFA concentrations are reported in Table 1. After 8 h, ammonia was  
142 significantly reduced (4.10 vs 9.68 mmol/L;  $P < 0.001$ ) by LA4. At 4 h, lactic acid  
143 concentration was significantly increased by LA4 (1.44 vs 1.16 mmol/L;  $P < 0.05$ ).

144

#### 145 *3.2 In vivo experiment*

146

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

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

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

157

#### 158 **4. Discussion and Conclusion**

159

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

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

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

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

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

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

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

203

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256 **Table 1.** pH values and ammonia and short-chain fatty acids (mmol/L) concentrations in dog faecal cultures added (Lac +) or not  
 257 (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           |

258 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 1) 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 ( $P < 0.001$ ).





