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1 **Portrait of a Canine Probiotic *Bifidobacterium* – from Gut to Gut**

2

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5

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9

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11

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19 **Abstract**

20 The gastrointestinal environment is a complex interactive system involving the host, ingested  
21 dietary components, and numerous microbial species. We hypothesised that isolation and  
22 screening of *Lactobacilli* and *Bifidobacteria* adherent to healthy canine gastrointestinal tissue  
23 would yield strains with commensal activity in canines. **The aims of this study were 1) to**  
24 **isolate a bank of commensal organisms from the canine gastrointestinal tract; 2) to**  
25 **screen these novel microbial isolates for potential probiotic effects; 3) to select one**  
26 **organism from these screens and test its impact on the canine microbiota.** Lactic acid  
27 bacteria (LAB) were isolated from resected canine gastrointestinal tissue and screened *in*  
28 *vitro* for putative probiotic activities. Murine studies examined gastrointestinal transit and  
29 inhibition of *Salmonella typhimurium* translocation. One strain was progressed to a canine  
30 study where its impact on the gastrointestinal microbiota was determined. Of the 420 isolates  
31 from the canine gut, 62 strains were characterised as LAB. **Following assessment of the**  
32 **strain bank with regard to pH sensitivity, bile resistance, pathogen inhibition and**  
33 **survival following freeze drying, 4 *Lactobacillus* strains and 2 *Bifidobacteria* strains**  
34 **were selected for further examination.** *Bifidobacterium animalis* AHC7 adhered to  
35 epithelial cells, transited the murine gastrointestinal tract to high numbers and significantly  
36 reduced *Salmonella typhimurium* translocation. *Bifidobacterium animalis* AHC7  
37 consumption significantly reduced the carriage of *Clostridia*, in particular *C. difficile*, in  
38 dogs. This study describes the isolation and screening of canine-derived bacterial strains with  
39 commensal traits. The results demonstrate that *Bifidobacterium animalis* AHC7 has  
40 significant potential for improving canine gastrointestinal health.

## 41 **1. Introduction**

42 The gastrointestinal tract harbours a diverse bacterial community that, in humans, comprises  
43 more than 1000 different species, and outnumbers human somatic and germ cells tenfold  
44 (O'Hara **and** Shanahan, 2006). Under normal circumstances, commensal bacteria are an  
45 essential health asset that exert a conditioning and protective influence on intestinal structure  
46 and homeostasis. Intestinal bacteria protect against infection, and actively exchange  
47 developmental and regulatory signals with the host that prime and instruct mucosal  
48 immunity.

49 At the turn of the last century, the use of “friendly” microbes present in fermented foods for  
50 the purpose of health maintenance and disease prevention was first proposed by Metchnikoff  
51 (1907). These beliefs have been substantiated by recent research, which indicates that  
52 enhancing the beneficial components of the gut microbiota using probiotics represents a  
53 realistic therapeutic strategy in the maintenance of human health and in the treatment of  
54 various intestinal disorders (Dunne et al., 2001; O'Mahony et al., 2005; Whorwell et al.,  
55 2006; O'Hara **and** Shanahan, 2007). However, the use of probiotics in companion animals  
56 has received less attention. Indeed, it is apparent that certain strains may exert some effect on  
57 the canine microbiota (Baillon et al., 2004) while many existing probiotic strains have little or  
58 no beneficial effect on the composition of the canine microbiota (Swanson et al., 2002;  
59 Vahjen **and** Männer, 2003; Pascher et al., 2008).

60 **Some investigators have hypothesized** that commensal organisms may exert species-  
61 specific effects and therefore a successful canine probiotic organism would ideally be derived  
62 from the canine gastrointestinal tract (McCoy **and** Gilliland 2007). This is supported by a  
63 recent review of published 16s rRNA gene sequences which demonstrates that the canine  
64 microbiota is closely related to, but distinct from, the microbiota of other mammals such as  
65 humans (Ley et al., 2008). **However, while the resident microbiota may be different in**

66 composition between different species, it has never been formally demonstrated that  
67 probiotic effects are species specific. Nevertheless, the generation of a bank of lactic  
68 acid bacteria from the canine gastrointestinal tract would most likely yield microbes  
69 that would compete successfully and thrive within this environment. Therefore, the  
70 purpose of the present study was to isolate a bank of commensal organisms from the  
71 canine gastrointestinal tract and to screen these novel microbial isolates for potential  
72 probiotic effects using *in vitro* assays and murine models. In order to further validate  
73 our screening approach, we selected one commensal microbe and examined its impact  
74 on the canine microbiota following oral consumption in a clinical study.

75

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## 76 2. Materials and Methods

### 77 2.1 Isolation and identification of bacteria from healthy dogs

78 Gastrointestinal sections were obtained from canine cadavers (n=7) obtained from local  
79 veterinarians after euthanasia was performed for reasons unrelated to this study. No dog  
80 exhibited evidence of gastrointestinal disease and all gastrointestinal tissues appeared normal.  
81 The caecum, mid-colon and terminal colons were isolated, dissected and the tissue washed in  
82 Ringers solution (Oxoid, Cambridge, UK) to remove loosely adherent bacteria. **Following**  
83 **the washing step, bacteria that were still present on the tissue were termed “adherent**  
84 **bacteria” as these bacteria were not removed by gentle agitation. Tissue was**  
85 **homogenised using mechanical means (i.e. a food grade blender) and supernatants**  
86 **containing adherent bacteria were plated on selective agars. Serial 10-fold dilutions**  
87 **were performed in Ringers solution and each dilution was inoculated onto MRS agar**  
88 **(Oxoid) with 0.05% (v/v) cysteine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA)**  
89 **or Reinforced Clostridial Agar (RCA; Oxoid) with 0.05% cysteine.** All plates were  
90 incubated **anaerobically** at 37°C for 48 hours (**h**). Isolated colonies were re-streaked for 6  
91 generations in order to isolate purified individual bacterial strains. Following this  
92 purification, single strain cultures were identified using morphology, gram reaction, catalase  
93 activity, API 50CHL fermentation profiles (Biomerieux, St. Laurent, Quebec) and  
94 phosphofructoketolase test positivity.

### 96 2.2 *In vitro* assessment of bacterial isolates

97 In order to determine the ability of the LAB isolates to survive transit through the upper  
98 gastrointestinal tract we assessed strain tolerances to low pH and bile. **Forty eight** hour  
99 cultures of each strain grown in MRS broth with 0.05% (v/v) cysteine were resuspended in

100 MRS broth (Lactobacilli) or trypticase-phytone-yeast extract broth (Becton-Dickenson,  
101 Oxford, UK) for Bifidobacteria adjusted with 1N HCl to pH 2.5. Survival was measured  
102 after 0, 30, 60, 120, 180, 240, and 360 minutes by plate count method. Resistance to bile was  
103 examined using RCA plates supplemented with 0.5, 1 or 5% porcine bile (Sigma-Aldrich).

104 Bacterial strains grown **in MRS broth with 0.05% cysteine** were centrifuged and pellets  
105 were re-suspended in a cryoprotectant (18% reconstituted skim milk, 2 % sucrose). Bacteria  
106 were frozen at -20°C for 24 h and freeze dried for another 24 h. **Sterile polypropylene tubes**  
107 **were filled with each bacterial powder and wrapped in aluminium foil before being**  
108 **placed in storage.** Bacterial numbers were assessed **immediately following fermentation,**  
109 **immediately following freeze drying and following one month storage at 21°C** by plating  
110 on MRS agar (Lactobacilli) or MRS agar with cysteine (Bifidobacteria).

111 **The *in vitro* antagonistic activity against pathogenic indicator organisms was examined**  
112 **by measuring the zone of inhibition surrounding the test strain colonies.** The pathogens  
113 *E.coli* 0157H45, *Listeria monocytogenes*, *Listeria innocua* and *Salmonella typhimurium* were  
114 used as indicator organisms to test the antagonistic activities of each LAB isolate. Indicator  
115 organisms were grown in TSA (*E.coli* 0157H45 and *S. typhimurium*) or BHI (*L.*  
116 *monocytogenes* and *L. innocua*) in sloppy agar broths (0.7% w/v) and were overlaid on to the  
117 LAB plates. Cell free supernatants from the LAB strains were also tested. The supernatants  
118 were buffered with 2%  $\beta$ -glycerophosphate to inhibit the effect of acid production.

119

### 120 2.3 Sequence identification of selected probiotic strains

121 **The intergenic spacer 16-23s rDNA region was sequenced for six strains selected on the**  
122 **basis of their favourable results in the preceding assays.** Bacterial suspensions were



123 frozen at -70°C for 10 minutes, thawed and resuspended in Buffer PB from the Qiagen PCR  
124 purification kit (Qiagen, Sussex, UK). The lysate was centrifuged, washed with Buffer PE  
125 and the DNA eluted using Buffer EB. PCR was performed using the intergenic spacer (IGS)  
126 primers, IGS L: 5'-GCTGGATCACCTCCTTTC-3' and IGS R: 5'-  
127 CTGGTGCCAAGGCATCCA-3' (bifidobacteria); 5'- AGA GTT TGA T(CT) (AC) TGG  
128 CTC AG- 3' and 5'- CAC CGC TAC ACA TGG AG-3' (lactobacilli). The cycling  
129 conditions were 96°C for 1 min (1 cycle), 94°C for 30 sec, 53°C for 30 sec, 72°C for 30 sec  
130 (28 cycles) using a Hybaid thermocycler. The PCR products were cut out of the gel and the  
131 DNA purified using the Qiagen Gel extraction kit. Purified products were sequenced by  
132 LARK Technologies Inc. The resultant DNA sequence data was submitted to the NCBI  
133 standard nucleotide-to-nucleotide homology BLAST search engine  
134 (<http://www.ncbi.nlm.nih.gov/BLAST/>). The nearest match to the sequence was identified  
135 and sequences were aligned using DNASTAR MegAlign software.

136

#### 137 *2.4 Adherence to intestinal epithelial cells*

138 The human epithelial cell line, HT-29 (ATTC, Manassas, USA), were cultured in Dulbecco's  
139 modified Eagle's minimal essential medium (DMEM, GIBCO-BRL, UK), containing 25 mM  
140 glucose, 10% (v/v) heat-inactivated fetal calf serum (GIBCO-BRL) and 1%  
141 penicillin/streptomycin (Sigma-Aldrich). Following the 7 day differentiation period, the  
142 epithelial monolayers were washed with antibiotic-free DMEM. Cultures **of the six** test  
143 bacterial strains were washed twice in PBS before being resuspended in antibiotic-free  
144 DMEM at a concentration of  $1 \times 10^6$  CFU/ml. Bacterial suspensions were added to the  
145 epithelial cells for 90 minutes at 37°C and each experiment was performed in triplicate. The  
146 well characterised adherent lactobacillus strain UCC118 (Van Pijkeren et al., 2006) was

147 included as a positive control. Following incubation the epithelial monolayers were washed  
148 to remove the non-adherent cells. Epithelial cells were lysed using sterile dH<sub>2</sub>O and the  
149 number of bacterial cells remaining were quantified by plating on MRS agar (Lactobacilli) or  
150 MRS agar containing cysteine (Bifidobacteria).

151

## 152 *2.5 Gastrointestinal transit in mice*

153 A rifampicin resistant variant of **the six test probiotic cultures** were generated to facilitate  
154 uncomplicated identification of the strain in fecal cultures. These were generated by plating  
155 the bacteria on RCA plates supplemented with 0.05% cysteine and increasing concentrations  
156 of rifampicin (Sigma-Aldrich). Freeze-dried cultures of each Rif<sup>R</sup> were provided in the  
157 drinking water at a dose of 5x10<sup>8</sup> live bacterial cells per day for a period of 7 days. Faecal  
158 samples were obtained from all animals (n=8 animals per group) prior to the start of probiotic  
159 administration (day 0) and on days 1, 4 and 7 of administration and 5 days post-cessation of  
160 feeding. Faecal samples were weighed, homogenised, diluted in ringers and spread plated on  
161 RCA supplemented with 0.05% cysteine and 75µg/ml rifampicin. The plates were incubated  
162 anaerobically at 37°C for 48 h.

163

## 164 *2.6 Murine Salmonella typhimurium challenge*

165 Female Balb/C mice 6-8 weeks of age (Harlan, Oxon, UK) were housed in 12 h light/dark  
166 cycle and provided standard laboratory chow and water *ad libitum*. **All murine experiments**  
167 **were approved by the University College Cork animal ethics committee and**  
168 **experimental procedures were conducted under appropriate license from the Irish**  
169 **government.** Groups of animals (n=12 per group) were administered **one of the six test**

170 **probiotic cultures (in a freeze-dried format)** or a placebo excipient control suspended in  
171 drinking water on a daily basis for a period of 4 weeks. After 3 weeks of probiotic pre-  
172 feeding all mice were orally challenged with  $1 \times 10^7$  CFU Salmonella using a previously  
173 described method (O'Mahony et al., 2008). After 7 days all mice were euthanised by cervical  
174 dislocation and spleens and livers removed. Aliquots of tissue homogenates were serially  
175 diluted in ringers solution and spread plated on McConkey agar (Oxoid). The plates were  
176 incubated aerobically at 37°C for 24 h allowing the quantification of Salmonella load in the  
177 tissues.

178

### 179 *2.7 Influence of probiotic consumption on the canine microbiota*

180 Eleven dogs (4 females, 7 males, average age 8.4 years) were fed a commercially available  
181 Eukanuba Premium Performance diet for 6 weeks prior to enrolment in this study. In addition  
182 to the base diet, each dog received  $1.5 \times 10^9$  CFU of *Bifidobacterium animalis* AHC7 / day in a  
183 pill format for 6 weeks. Daily food intake and weekly body weights were measured during  
184 the entire experiment. Fresh faecal samples were collected before (**day -10** and **day -1**) and  
185 after probiotic feeding at weeks 4, 5, and 6. For bacterial enumeration, 10 g of feces was  
186 homogenized in 90 ml of Butterfield's Phosphate-Buffered Dilution Water and serially  
187 diluted. Spread plates were used to evaluate total anaerobes on CDC Anaerobic Blood Agar  
188 (Anaerobe Systems AS646; San Jose, CA), *Bifidobacterium spp.* on Bifid Selective Agar  
189 (Anaerobe Systems AS6423), *Lactobacillus spp.* on BBL™ LBS Agar (Becton Dickinson  
190 211327; Sparks, MD with Glacial Acetic Acid (J.T. Baker NJ 9522-33; Phillipsburg, NJ),  
191 *Bacteroides fragilis* group on Bacteroides Bile Esculin Agar (BBE) (BD 221836),  
192 *Clostridium spp.* on Clostrisel Agar (BBL Microbiology Systems 21114; Cockeysville, MD),  
193 *Clostridium difficile* on Clostridium Difficile Agar Base (Oxoid CM601; Oxoid Ltd.,

194 Basingstoke, Hampshire, England) with Clostridium Difficile Supplement (Oxoid SR96) and  
195 defibrinated horse blood (Oxoid SR50), *Escherichia coli* on Hardy ECC Chromagar (Hardy  
196 Diagnostics G137; Santa Maria, CA) and total aerobes on Difco™ Plate Count Agar (BD  
197 24790). CDC Anaerobic Blood Agar, Bifid Selective Agar, LBS Agar, BBE Agar, Clostrisel  
198 Agar, and Clostridium Difficile Agar were incubated anaerobically at 37°C for 48 h. ECC  
199 Chromagar and PCA were incubated aerobically at 37°C for 24 h and 48 h, respectively.  
200 Pour plates containing 1 ml of inoculum were used to evaluate *Clostridium perfringens* on  
201 Perfringens Agar (OPSP) (Oxoid CM0543) with Perfringens Selective Supplement A (Oxoid  
202 SR0076E), and Perfringens Selective Supplement B (Oxoid SR0077E). OPSP plates were  
203 incubated under anaerobic conditions at 37°C for 24 h. **This study was approved by the**  
204 **Procter & Gamble Pet Care Animal Care and Use Committee.**

205

#### 206 *2.10 Statistical analysis*

207 Statistical analysis of the *in vitro* results and the murine data was performed using unpaired  
208 student t-tests. Baseline microbial data in the canine study was analysed by ANOVA and  
209 GLM procedures using SAS software (SAS Institute, Cary, NC, USA).

210

### 211 3. Results

#### 212 3.1 Bacterial Isolation from the Canine Gastrointestinal Tract

213 A total of 420 isolated colonies from the canine gastrointestinal tract were purified and  
214 examined for colony morphology, gram stain appearance and catalase activity. Of these, 62  
215 strains displayed the correct colony morphology and were gram positive and catalase  
216 negative. Preliminary species identification revealed that the bank of strains isolated from the  
217 canine gut contained 30 *Leuconostoc lactis* strains, 9 *Lactobacillus acidophilus* strains, 7  
218 *Lactococcus crispatus* strains, 5 *Bifidobacteria* strains, 4 *Lactobacillus fermentum* strains and  
219 2 *Lactococcus buchneri* strains while the identity of 5 strains remained ambiguous.

220

#### 221 3.2 *In vitro* assessment of bacterial isolates

222 **The majority of the canine isolates exhibited strong tolerance to low pH conditions *in***  
223 ***vitro*. Of the 62 strains tested only 8 strains showed poor survival in an acidic**  
224 **environment. In contrast to the results observed regarding resistance to low pH**  
225 **conditions, many of the strains tested were sensitive to low concentrations of bile.**

226 **In order to identify strains that could survive technological process we determined the**  
227 **ability of each strain to survive freeze-drying and storage for one month. The majority**  
228 **of the strains grew well in the overnight cultures but 4 strains demonstrated poor**  
229 **growth prior to freeze-drying. The two strains which demonstrated superior recovery**  
230 **following freeze drying were AHCB and AHC7. Storage at room temperature (21°C)**  
231 **for one month was relatively well tolerated by most of the strains with only 4 strains**  
232 **decreasing in number by more than 1 log value (AHC3312, AHC5212, AHC5223 and**  
233 **AHCB).**

234 The majority of the strains exhibited antagonistic activity, as determined by a clear zone  
235 of inhibition surrounding the test organism, against *Salmonella typhimurium*, *E. Coli*  
236 0157:H45, *Listeria monocytogenes* and *Listeria innocua*. Seven strains exhibited minimal  
237 activity against these pathogens (AHC5121, AHC5223, AHC5333, AHC6322, AHC6341,  
238 AHCA and AHCC). Buffering of acid production by the test strains significantly  
239 decreased the zone of inhibition indicating that the mechanism of inhibition is primarily  
240 due to metabolite production.

241 From the data generated on the *in vitro* assessments described above, 6 strains were  
242 selected for further study. These strains were AHC1222, AHC3133, AHC5323,  
243 AHC6331, AHCF and AHC7.

244

### 245 3.3 Sequence identification of selected probiotic strains

246 Figure 1 illustrates the 16s ribosomal banding pattern for each of the six selected strains.

247 Sequencing of these bands and comparison with available sequences in the NCBI database

248 revealed that each of these strains were novel isolates and had not been previously described

249 (i.e. no 100% match was found). The closest available sequences identified these strains as

250 *Lactobacillus murinus/ruminus* (AHC1222, AHC3133, AHC5323 and AHC6331),

251 *Bifidobacterium globosum/pseudolongum* (AHCF) and *Bifidobacterium animalis* (AHC7).

252 The six bacterial strains were deposited with the National Collections of Industrial Food

253 and Marine Bacteria (NCIMB), Aberdeen, UK and were assigned the following

254 accession numbers – AHC1222 (NCIMB 41194); AHC3133 (NCIMB 41195); AHC5323

255 (NCIMB 41196); AHC6331 (NCIMB 41197); AHCF (NCIMB 41198); AHC7 (NCIMB

256 41199).

257

258 *3.4 Adherence to intestinal epithelial cells*

259 The six strains adhered to some degree to HT-29 cells but none of the lactobacillus strains  
260 adhered as well as the positive control organism UCC118 (**Figure 2**). Both bifidobacterial  
261 strains (AHCF and AHC7) adhered to intestinal epithelial cells to a significantly greater  
262 extent than all other strains tested.

263

264 *3.5 Gastrointestinal transit in mice*

265 All strains transited the murine gastrointestinal tract validating the *in vitro* selection criteria  
266 which suggested that these strains could survive physiologically relevant pH conditions and  
267 bile concentrations (**Figure 3**). The highest transit levels were observed for the two  
268 Bifidobacteria isolates AHCF and AHC7. None of the strains colonised the murine gut as  
269 following cessation of feeding no rifampicin resistant colonies were observed.

270

271 *3.6 Murine Salmonella typhimurium challenge*

272 Following challenge with *Salmonella typhimurium*, liver and spleen recovery of the pathogen  
273 was assessed in *Lactobacilli*, *Bifidobacteria* and placebo-fed animals. In placebo-fed  
274 animals, the mean recovery of *Salmonella typhimurium* was  $1 \times 10^9$  CFU/g and  $2 \times 10^8$  CFU/g  
275 of liver and spleen respectively. Two of the six tested probiotic organisms significantly  
276 reduced translocation of *S. typhimurium* to both liver and spleen 7 days following the initial  
277 infection (**Figure 4**). These strains were AHC7 and AHC3133.

278

## 279 3.7 Influence of probiotic consumption on the canine microbiota

280 One of the canine isolates, *Bifidobacterium animalis* AHC7, was selected on the basis of its  
281 *in vitro* and *in vivo* properties for assessment in a canine study. *Bifidobacterium animalis*  
282 AHC7 was consumed daily for 6 weeks and a comprehensive assessment of the canine fecal  
283 microbiota was performed prior to and after probiotic consumption. As there was no  
284 significant difference in bacterial counts between the two baseline determinations (**days -10**  
285 **and -1**), we averaged the two baseline counts for each micro-organism in order to  
286 **generate one baseline value for comparison to later time-points during the study period.**  
287 Total fecal *Clostridia* counts were significantly reduced at weeks 5 and 6 in the  
288 *Bifidobacterium animalis* AHC7-fed animals while assessment of individual *Clostridia*  
289 species revealed that *C. difficile* numbers, but not *C. perfringens*, was significantly reduced  
290 by week 6 (**Table 1**). *C. difficile* numbers were significantly reduced at week 6 when  
291 **compared to each baseline value individually (day -10 or day -1) or the mean baseline**  
292 **count.** The number of total aerobes, *Bacteroides*, *E. coli*, *Lactobacilli* or *Bifidobacteria* were  
293 **not affected by probiotic feeding.** Daily food intake and weekly **body weights were not**  
294 **significantly influenced by probiotic administration** (data not shown).

295



296 **4. Discussion**

297 This report describes the successful isolation and characterisation of a novel bank of strains  
298 from the canine gastrointestinal tract. Using a battery of *in vitro* and *in vivo* screening  
299 methods, the commensal *Bifidobacterium animalis* AHC7 was selected as the most suitable  
300 probiotic candidate from this strain bank. The basis for the selection of this microbe was not  
301 due to any single characteristic in particular. Indeed, *Bifidobacterium animalis* AHC7 did not  
302 rank as the most suitable strain in every assay system, however, *Bifidobacterium animalis*  
303 AHC7 was the most consistent strain in receiving a high ranking and displayed the best  
304 combination of all the putative probiotic features tested such as gastrointestinal transit,  
305 stability, epithelial adherence, *in vitro* and *in vivo* anti-microbial activity. Upon further  
306 examination in a canine feeding study, the commensal organism had a significant beneficial  
307 effect on the canine microbiota as evidenced by the reduction in total *Clostridia* levels and  
308 *Clostridium difficile* numbers.

309 Previous attempts at the isolation of commensal organisms from dogs have largely used  
310 canine feces while few attempts had been made to isolate adherent commensal bacteria  
311 directly from the canine intestinal mucosa. The fecal bacterial community is representative  
312 of the luminal contents of the large bowel but the adherent population is likely to be better  
313 adapted to the host and may provide a more efficient barrier to infection. In addition, certain  
314 commensal organisms may lose viability rapidly once shed in feces. This is supported by a  
315 previous study which was unable to isolate *Bifidobacteria* from canine feces (Greetham et al.,  
316 2002). However, multiple *Bifidobacteria* species were isolated in our study and also in  
317 another study which used intestinal samples (Kim and Adachi, 2007).

318 One of the parameters that was used in the selection of a probiotic strain for use in  
319 companion animals was the ability of a strain to survive technological processes, such as  
320 freeze-drying, and to survive storage at room temperature. This is an essential characteristic

321 for the future development of a companion animal product containing a probiotic. The poor  
322 quality control of existing pet food products which are labelled as containing probiotics was  
323 highlighted by Weese **and** Arroyo (2003). Out of the 19 commercial diets that were  
324 examined, none contained all the organisms listed and 5 contained no detectable viable  
325 bacteria. In order for a probiotic to exert a health benefit it should be delivered as a live  
326 micro-organism at a high enough dose to impact the host enteric community.

327 Consumption of *Bifidobacterium animalis* AHC7 by dogs resulted in **decreased** fecal levels  
328 of *Clostridia* and *Clostridium difficile*. The *Clostridia* species are generally regarded as  
329 opportunistic pathogens and *Clostridium difficile* is well described as the causative agent of  
330 antibiotic associated diarrhoea and pseudomembranous colitis in susceptible humans (Pepin  
331 et al., 2004; Brazier 2008). The acquisition of *C. difficile* during hospitalization of dogs is  
332 also associated with the development of diarrhea (Clooten et al., 2008). The elimination of *C.*  
333 *difficile* from the canine gut may not only improve canine gastrointestinal health but may also  
334 help reduce the risk of human infection due to owner-pet interactions. The risk for human  
335 contamination is highly significant as highlighted by one study that detected shedding of  
336 *Clostridium difficile* in 58% of visitation dogs in a healthcare setting (Lefebvre et al., 2006).

337 *Bifidobacterium animalis* AHC7 consumption did not increase the total level of  
338 Bifidobacteria detected in canine feces. **However, we have described unusually high**  
339 ***Bifidobacterium* fecal counts in this canine cohort suggesting that the selective media**  
340 **used may allow for the growth of other, non-*Bifidobacterium*, species and therefore the**  
341 **bifidobacterial count data should be treated with caution. Indeed, previous studies have**  
342 **highlighted the non-selective nature of media for enumeration of Bifidobacteria in non-**  
343 **human fecal samples (Hartemink and Rombouts, 1999).**

344 The bacterial population within the GI tract of mammals constitutes a metabolically active  
345 organ that detoxifies potentially harmful substances in the diet (Murphy et al., 2009) and acts

346 as a significant barrier to infection by exogenous pathogenic microorganisms. At present, our  
347 picture of human GI-tract ecology is far from complete, even less so for companion animals  
348 such as cats and dogs. This study adds to a growing literature on the canine microbiota by  
349 describing the isolation and screening of canine-derived bacterial strains with commensal  
350 traits. The results demonstrate that *Bifidobacterium animalis* AHC7 has significant potential  
351 for improving canine gastrointestinal health. However, the mechanism of action is uncertain  
352 and is likely to depend on individual characteristics of the strain itself and the clinical  
353 condition for which it is used.  
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424 **Legends**

425 **Table 1. Canine microbiota following *Bifidobacterium animalis* AHC7 consumption**

426 **Eleven** animals consumed the putative probiotic *Bifidobacterium animalis* AHC7 for 6 weeks  
427 and the fecal bacterial communities were assessed prior to and following feeding at weeks 4,  
428 5 and 6. Total *Clostridia* and *Clostridium difficile* numbers were significantly reduced by  
429 *Bifidobacterium animalis* AHC7 consumption. Results are expressed as the mean log CFU/g  
430 for each time-point.

431 **Figure 1. Intergenic spacer PCR products**

432 The banding patterns for the PCR products of each strain on agarose gels stained with EtBr is  
433 illustrated. Multiple products are observed for the *Lactobacillus* strains while single bands are  
434 evident for the *Bifidobacterium* strains.

435 **Figure 2. Adhesion to epithelial cells**

436 Each bacterial strain adheres to HT-29 intestinal epithelial cells while the *Bifidobacterium*  
437 strains adhere at a significantly higher level compared to the *Lactobacillus* strains (indicated  
438 by the asterix “\*”). Results are expressed as the mean % of incubated bacterial cells that  
439 adhere to the epithelial cells (n=6 different wells/strain) +/- SD.

440 **Figure 3. Murine gastrointestinal transit**

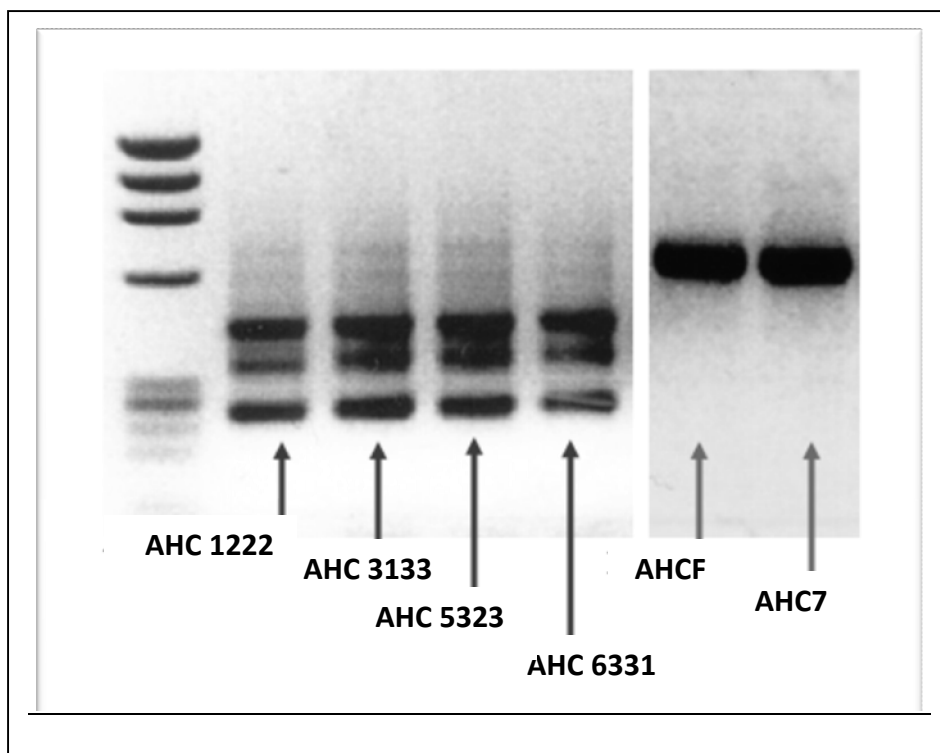
441 Rifampicin-resistant strains were consumed by mice for 7 days and fecal recovery of  
442 rifampicin-resistant colonies was performed on days 0, 1, 4, 7 and 5 days following cessation  
443 of feeding. All strains transited the murine gastrointestinal tract at a significant level during  
444 feeding but were not recovered in feces following cessation of consumption. Results are  
445 expressed as the mean CFU/gram of feces for each group of animals (n=8 animals per group).

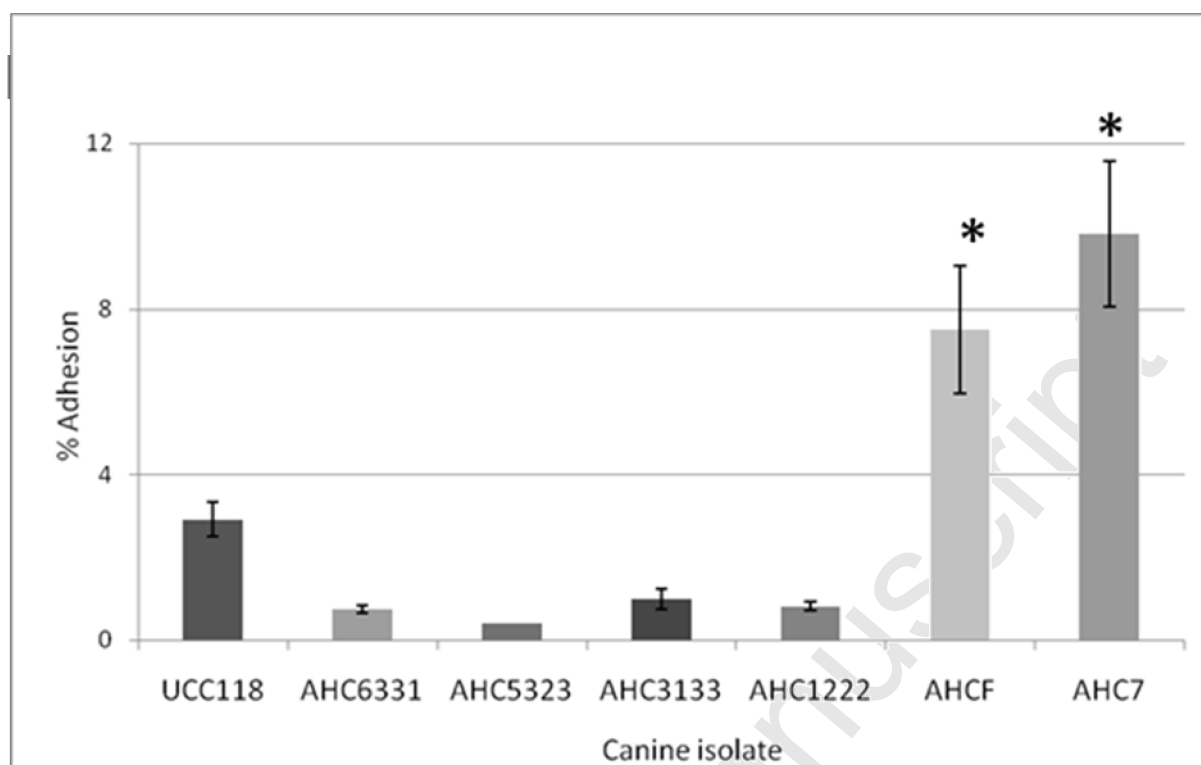


446 **Figure 4. Inhibition of *Salmonella typhimurium* translocation in a murine model**

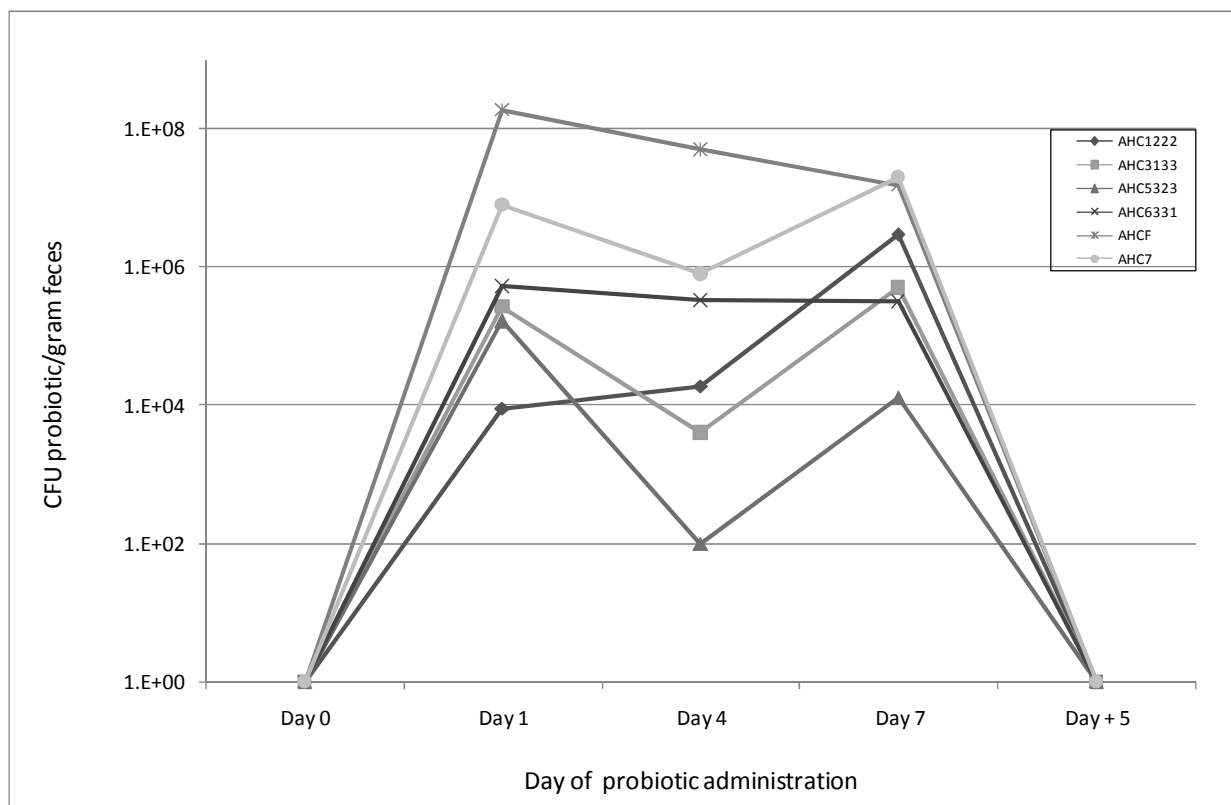
447 Translocation and survival of *Salmonella typhimurium* at systemic sites (liver and spleen)  
448 was assessed in animals that had previously consumed a *Lactobacillus* or *Bifidobacterium*  
449 strain for 3 weeks. **AHC7 and AHC3133 significantly reduced *Salmonella typhimurium***  
450 **translocation with a p-value <0.05 compared to the placebo group (\*).** Results are  
451 expressed as the mean colony forming units (cfu) recovery of *Salmonella typhimurium* from  
452 liver and spleen (n=12 animals per group).

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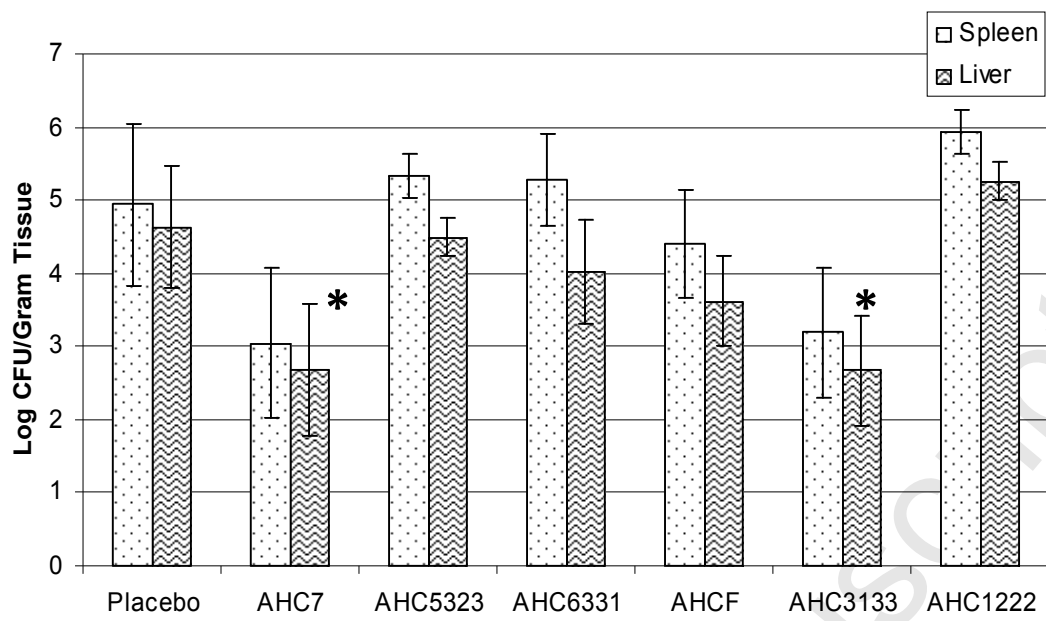




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Bacterial Strain	Baseline	Week 4	Week 5	Week 6	Pooled SEM	P-value
<b>Total Anaerobes</b>	10.44	10.36	10.11	10.21	9.64	0.23
<b>Total Aerobes</b>	9.17	8.73	8.74	8.99	8.73	0.72
<i>Bacteroides</i>	6.26	5.65	6.02	6.26	5.71	0.22
<b>Total Clostridia</b>	9.58	9.41	8.95	9.12	8.93	0.04
<i>C. perfringens</i>	7.16	7.54	7.14	7.37	6.93	0.40
<i>C. difficile</i>	7.44	6.80	7.07	5.81	6.76	0.05
<i>E. coli</i>	6.43	6.41	6.94	7.20	6.76	0.62
<b>Lactobacilli</b>	9.79	9.71	9.30	9.44	9.23	0.62
<b>Bifidobacteria</b>	10.09	10.16	9.81	10.04	9.43	0.48

**Table 4. Canine microbiota following *Bifidobacterium animalis* AHC7 consumption**