Portrait of a Canine Probiotic – from Gut to Gut

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

2

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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.

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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**

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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.**
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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.

95

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. **Forthy eight** hour
99 cultures of each strain grown in MRS broth with 0.05% (v/v) cysteine were resuspended in

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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 inocua* 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. inocua*) 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% β-glycerophosphate to inhibit the **e**ffect 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

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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 $1x10^6$ 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

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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_2O 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^R were provided in the
157 drinking water at a dose of $5x10^8$ 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**

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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 $1x10^7$ 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 $1.5x10^9$ 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.,

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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).

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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).**

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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 inocua.* 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).**

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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 $1x10^9$ CFU/g and $2x10^8$ 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.

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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).

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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

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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 pseudomembraneous 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

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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.
354

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355 **References**

356 Baillon, M.L., Marshall-Jones, Z.V., Butterwick, R.F., 2004. Effects of probiotic
357 *Lactobacillus acidophilus* strain DSM13241 in healthy adult dogs. Am. J. Vet. Res. 65, 338-
358 43.

359 Brazier, J.S., 2008. *Clostridium difficile*: from obscurity to superbug. Br. J. Biomed.
360 Sci. 65, 39-44.

361 Clooten, J., Kruth, S., Arroyo, L., Weese, J.S., 2008. Prevalence and risk factors for
362 *Clostridium difficile* colonization in dogs and cats hospitalized in an intensive care unit. Vet.
363 Microbiol. 129, 209-14.

364 Dunne, C., O'Mahony, L., Murphy, L., Thornton, G., Morrissey, D., O'Halloran, S.,
365 Feeney, M., Flynn, S., Fitzgerald, G., Daly, C., Kiely, B., O'Sullivan, G.C., Shanahan, F.,
366 Collins, J. K., 2001. In vitro selection criteria for probiotic bacteria of human origin:
367 correlation with in vivo findings. Am. J. Clin. Nutr. 73, 386S–392S.

368 Hartemink, R., Rombouts, F.M., 1999. Comparison of media for the detection of
369 bifidobacteria, lactobacilli and total anaerobes from faecal samples. J. Microbiol. Methods
370 36, 181-192.

371 Greetham, H.L., Giffard, C., Hutson, R.A., Collins, M.D., Gibson, G.R., 2002.
372 Bacteriology of the Labrador dog gut: a cultural and genotypic approach. J. Appl. Microbiol.
373 93, 640-646.

374 Kim, S.Y., Adachi, Y., 2007. Biological and genetic classification of canine intestinal
375 lactic acid bacteria and *bifidobacteria*. Microbiol. Immunol. 51, 919-928.

Accepted Manuscript

ACCEPTED MANUSCRIPT

376 Lefebvre, S.L., Waltner-Toews, D., Peregrine, A.S., Reid-Smith, R., Hodge, L.,
377 Arroyo, L.G., Weese, J.S., 2006. Prevalence of zoonotic agents in dogs visiting hospitalized
378 people in Ontario: implications for infection control. J. Hosp. Infect. 62, 458-466.

379 McCoy, S., Gilliland, S.E., 2007. Isolation and characterization of *Lactobacillus*
380 species having potential for use as probiotic cultures for dogs. J. Food Sci. 72, M94-97.

381 Metchnikoff, E., 1907. The Prolongation of Life: Optimistic Studies. Mitchell, C., Ed.
382 William Heinemann, London. pp. 161–183.

383 Murphy, C., Murphy, S., O'Brien, F., O'Donoghue, M., Boileau, T., Sunvold, G.,
384 Reinhart, G., Kiely, B., Shanahan, F., O'Mahony, L., 2009. Metabolic activity of probiotics-
385 Oxalate degradation. Vet. Microbiol. 136(1-2), 100-107.

386 O'Hara, A.M., Shanahan, F., 2006. The gut flora as a forgotten organ. EMBO Rep. 7,
387 688–693.

388 O'Hara AM, Shanahan F, 2007. Mechanisms of action of probiotics in intestinal
389 diseases. Scientific World Journal. 7, 31-46.

390 O'Mahony, C., Scully, P., O'Mahony, D., Murphy, S., O'Brien, F., Lyons, A.,
391 Sherlock, G., MacSharry, J., Kiely, B., Shanahan, F., O'Mahony, L., 2008. Commensal-
392 induced regulatory T cells mediate protection against pathogen-stimulated NF-kappaB
393 activation. PLoS Pathog. 4, e1000112.

394 O'Mahony, L., McCarthy, J., Kelly, P., Hurley, G., Luo, F., Chen, K., O'Sullivan,
395 G.C., Kiely, B., Collins, J.K., Shanahan, F., Quigley, E.M., 2005. *Lactobacillus* and
396 *bifidobacterium* in irritable bowel syndrome: symptom responses and relationship to cytokine
397 profiles. Gastroenterology 128, 541-551.

Accepted Manuscript

ACCEPTED MANUSCRIPT

398 Pascher, M., Hellweg, P., Khol-Parisini, A., Zentek, J., 2008. Effects of a probiotic
399 *Lactobacillus acidophilus* strain on feed tolerance in dogs with non-specific dietary
400 sensitivity. Arch Anim Nutr. 62, 107-116.

401 Pepin, J., Valiquette, L., Alary, M. E., Villemure, P., Pelletier, A., Forget, K., Pepin,
402 K. & Chouinard, D., 2004. *Clostridium difficile* associated diarrhea in a region of Quebec
403 from 1991 to 2003: a changing pattern of disease severity. CMAJ 171, 466–472.

404 Ley, R.E., Lozupone, C.A., Hamady, M., Knight, R., Gordon, J.I., 2008. Worlds
405 within worlds: evolution of the vertebrate gut microbiota. Nat. Rev. Microbiol. 6, 776-788.

406 Swanson, K.S., Grieshop, C.M., Flickinger, E.A., Bauer, L.L., Chow, J., Wolf, B.W.,
407 Garleb, K.A., Fahey, G.C. Jr, 2002. Fructooligosaccharides and *Lactobacillus acidophilus*
408 modify gut microbial populations, total tract nutrient digestibilities and fecal protein
409 catabolite concentrations in healthy adult dogs. J. Nutr. 132, 3721-3731.

410 Vahjen, W., Männer, K., 2003. The effect of a probiotic *Enterococcus faecium*
411 product in diets of healthy dogs on bacteriological counts of *Salmonella* spp., *Campylobacter*
412 spp. and *Clostridium* spp. in faeces. Arch Tierernahr. 57, 229-233.

413 Van Pijkeren, J.P., Canchaya, C., Ryan, K.A., Li, Y., Claesson, M.J., Sheil, B.,
414 Steidler, L., O'Mahony, L., Fitzgerald, G.F., van Sinderen, D., O'Toole, P.W., 2006.
415 Comparative and functional analysis of sortase-dependent proteins in the predicted secretome
416 of *Lactobacillus salivarius* UCC118. Appl. Environ. Microbiol. 72, 4143-4153.

417 Weese, J.S., Arroyo, L., 2003. Bacteriological evaluation of dog and cat diets that
418 claim to contain probiotics. Can. Vet. J. 44, 212-216.

419 Whorwell, P.J., Altringer, L., Morel, J., Bond, Y., Charbonneau, D., O'Mahony, L.,
420 Kiely, B., Shanahan, F., Quigley, E.M., 2006. Efficacy of an encapsulated probiotic

Accepted Manuscript

ACCEPTED MANUSCRIPT

421 *Bifidobacterium infantis* 35624 in women with irritable bowel syndrome. Am. J.

422 Gastroenterol. 101,1581-1590.

423

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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).

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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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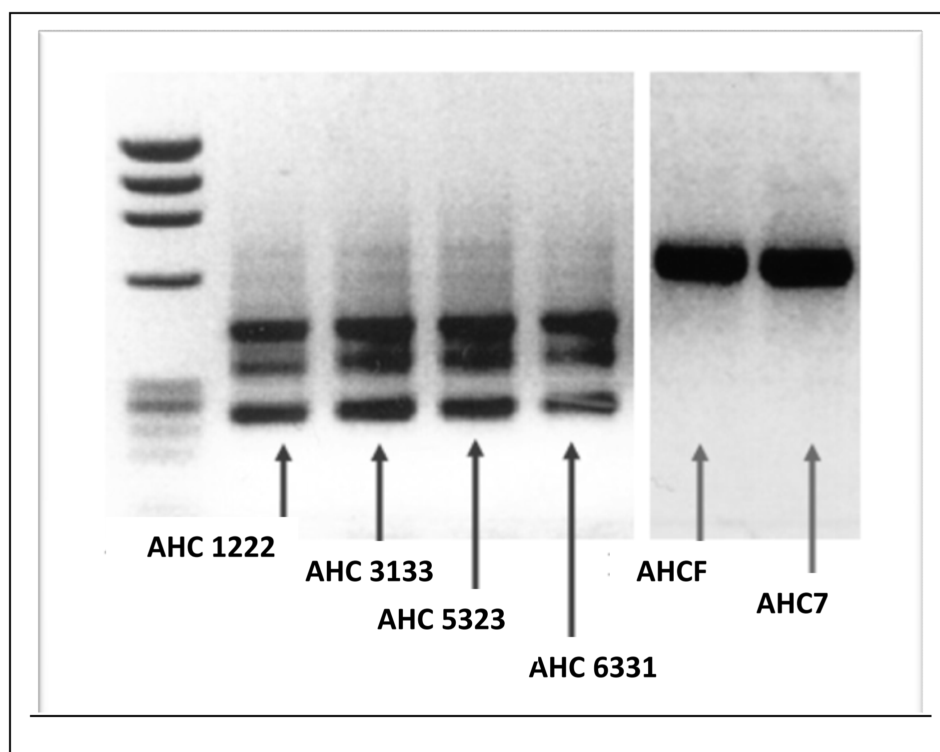

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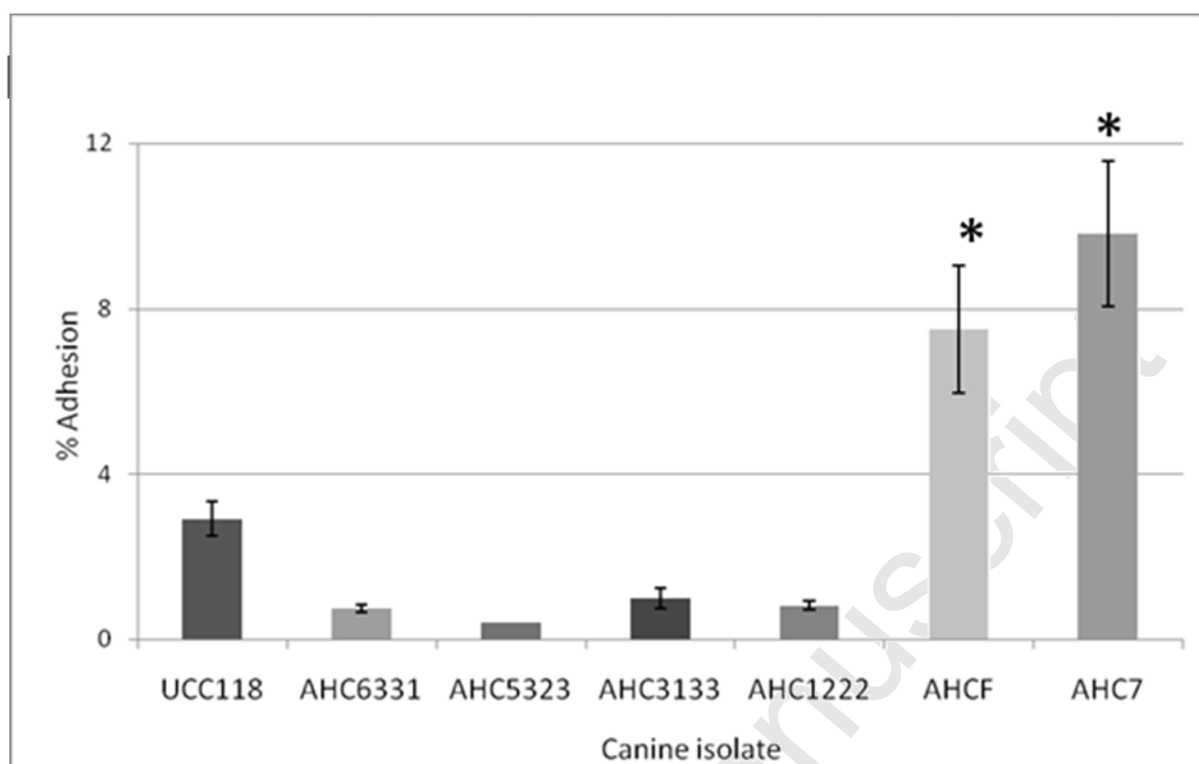

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Figure 3

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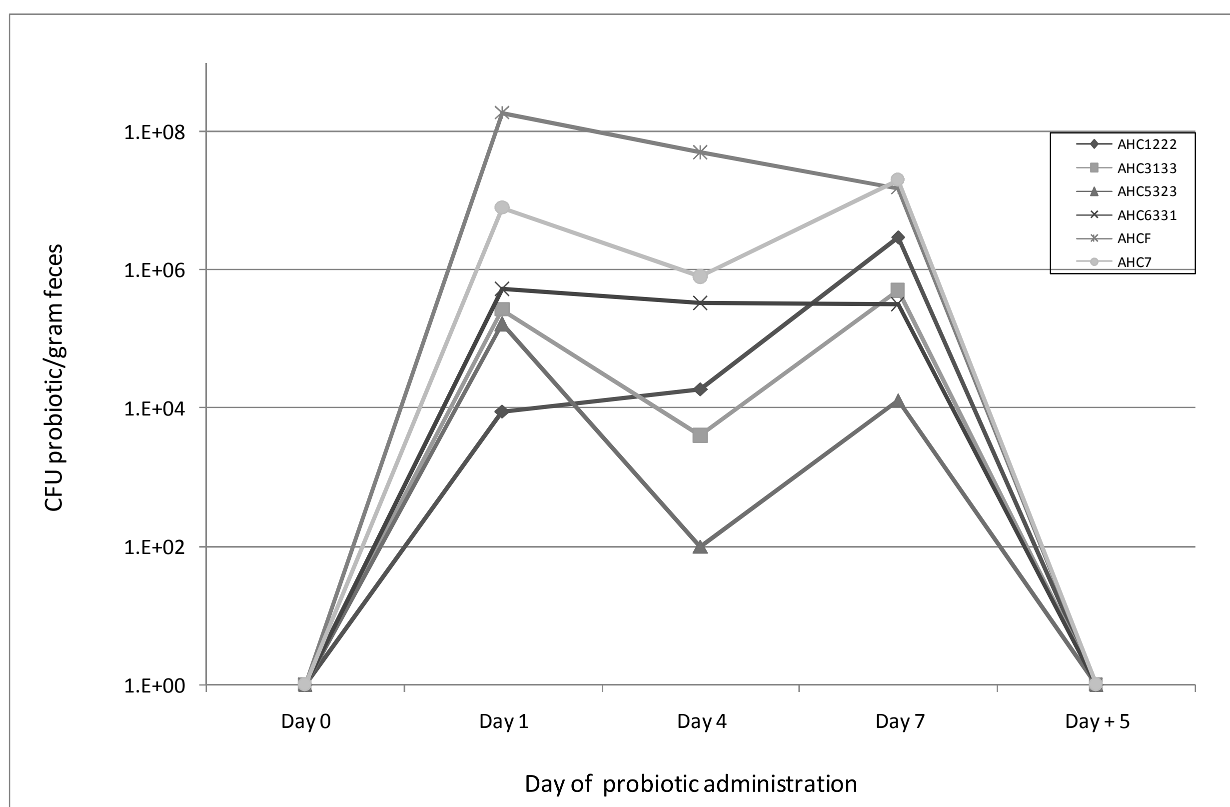

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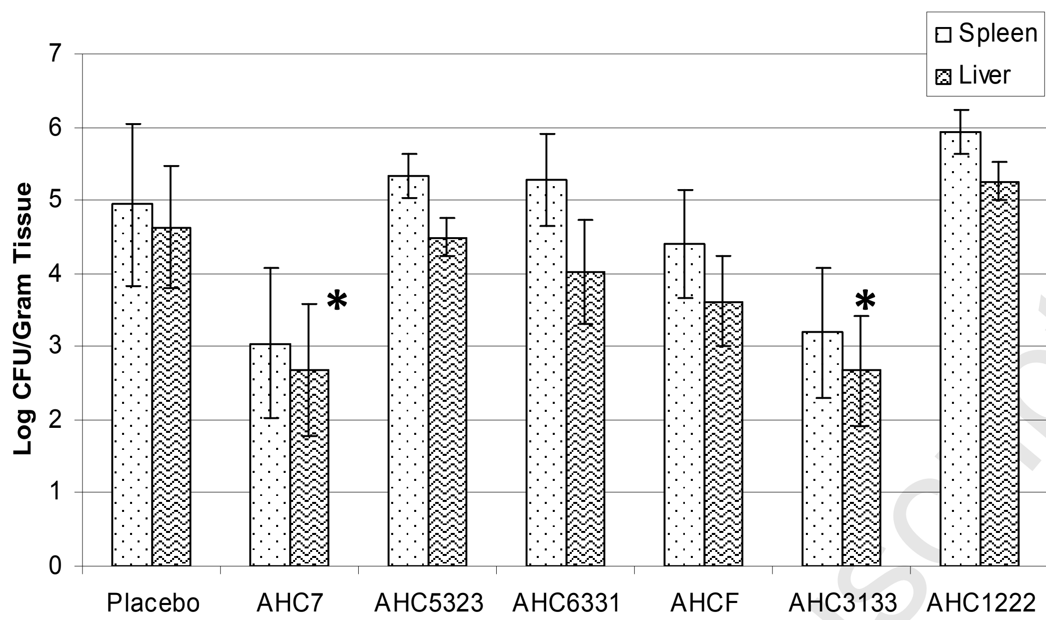

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Bacterial Strain	Baseline	Week 4	Week 5	Week 6	Pooled SEM	P-value
Total Anaerobes	10.44	10.36	10.11	10.21	9.64	0.23
Total Aerobes	9.17	8.73	8.74	8.99	8.73	0.72
Bacteroides	6.26	5.65	6.02	6.26	5.71	0.22
Total *Clostridia*	9.58	9.41	8.95	9.12	8.93	0.04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

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

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