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

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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.

1. Introduction

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

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

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

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

2. Materials and Methods

2.1 Isolation and identification of bacteria from healthy dogs

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

2.2 In vitro assessment of bacterial isolates

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

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

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

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

2.3 Sequence identification of selected probiotic strains

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

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

2.4 Adherence to intestinal epithelial cells

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

included as a positive control. Following incubation the epithelial monolayers were washed to remove the non-adherent cells. Epithelial cells were lysed using sterile dH₂O and the number of bacterial cells remaining were quantified by plating on MRS agar (Lactobacilli) or MRS agar containing cysteine (Bifidobacteria).

2.5 Gastrointestinal transit in mice

A rifampicin resistant variant of **the six test probiotic cultures** were generated to facilitate uncomplicated identification of the strain in fecal cultures. These were generated by plating the bacteria on RCA plates supplemented with 0.05% cysteine and increasing concentrations of rifampicin (Sigma-Aldrich). Freeze-dried cultures of each Rif^R were provided in the drinking water at a dose of 5×10^8 live bacterial cells per day for a period of 7 days. Faecal samples were obtained from all animals (n=8 animals per group) prior to the start of probiotic administration (day 0) and on days 1, 4 and 7 of administration and 5 days post-cessation of feeding. Faecal samples were weighed, homogenised, diluted in ringers and spread plated on RCA supplemented with 0.05% cysteine and 75µg/ml rifampicin. The plates were incubated anaerobically at 37°C for 48 h.

2.6 Murine *Salmonella typhimurium* challenge

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

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

2.7 Influence of probiotic consumption on the canine microbiota

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

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

2.10 Statistical analysis

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

3. Results

3.1 Bacterial Isolation from the Canine Gastrointestinal Tract

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

3.2 In vitro assessment of bacterial isolates

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

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

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

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

3.3 Sequence identification of selected probiotic strains

Figure 1 illustrates the 16s ribosomal banding pattern for each of the six selected strains. Sequencing of these bands and comparison with available sequences in the NCBI database revealed that each of these strains were novel isolates and had not been previously described (i.e. no 100% match was found). The closest available sequences identified these strains as *Lactobacillus murinus/ruminus* (AHC1222, AHC3133, AHC5323 and AHC6331), *Bifidobacterium globosum/pseudolongum* (AHCF) and *Bifidobacterium animalis* (AHC7). The six bacterial strains were deposited with the National Collections of Industrial Food and Marine Bacteria (NCIMB), Aberdeen, UK and were assigned the following accession numbers – AHC1222 (NCIMB 41194); AHC3133 (NCIMB 41195); AHC5323 (NCIMB 41196); AHC6331 (NCIMB 41197); AHCF (NCIMB 41198); AHC7 (NCIMB 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 1×10^9 CFU/g and 2×10^8 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

3.7 Influence of probiotic consumption on the canine microbiota

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

4. Discussion

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

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

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

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

Consumption of *Bifidobacterium animalis* AHC7 by dogs resulted in **decreased** fecal levels of *Clostridia* and *Clostridium difficile*. The *Clostridia* species are generally regarded as opportunistic pathogens and *Clostridium difficile* is well described as the causative agent of antibiotic associated diarrhoea and pseudomembranous colitis in susceptible humans (Pepin et al., 2004; Brazier 2008). The acquisition of *C. difficile* during hospitalization of dogs is also associated with the development of diarrhea (Clooten et al., 2008). The elimination of *C. difficile* from the canine gut may not only improve canine gastrointestinal health but may also help reduce the risk of human infection due to owner-pet interactions. The risk for human contamination is highly significant as highlighted by one study that detected shedding of *Clostridium difficile* in 58% of visitation dogs in a healthcare setting (Lefebvre et al., 2006). *Bifidobacterium animalis* AHC7 consumption did not increase the total level of Bifidobacteria detected in canine feces. **However, we have described unusually high *Bifidobacterium* fecal counts in this canine cohort suggesting that the selective media used may allow for the growth of other, non-*Bifidobacterium*, species and therefore the bifidobacterial count data should be treated with caution. Indeed, previous studies have highlighted the non-selective nature of media for enumeration of Bifidobacteria in non-human fecal samples (Hartemink and Rombouts, 1999).**

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

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

References

- Baillon, M.L., Marshall-Jones, Z.V., Butterwick, R.F., 2004. Effects of probiotic *Lactobacillus acidophilus* strain DSM13241 in healthy adult dogs. *Am. J. Vet. Res.* 65, 338-43.
- Brazier, J.S., 2008. *Clostridium difficile*: from obscurity to superbug. *Br. J. Biomed. Sci.* 65, 39-44.
- Clooten, J., Kruth, S., Arroyo, L., Weese, J.S., 2008. Prevalence and risk factors for *Clostridium difficile* colonization in dogs and cats hospitalized in an intensive care unit. *Vet. Microbiol.* 129, 209-14.
- Dunne, C., O'Mahony, L., Murphy, L., Thornton, G., Morrissey, D., O'Halloran, S., Feeney, M., Flynn, S., Fitzgerald, G., Daly, C., Kiely, B., O'Sullivan, G.C., Shanahan, F., Collins, J. K., 2001. In vitro selection criteria for probiotic bacteria of human origin: correlation with in vivo findings. *Am. J. Clin. Nutr.* 73, 386S-392S.
- Hartemink, R., Rombouts, F.M., 1999. Comparison of media for the detection of bifidobacteria, lactobacilli and total anaerobes from faecal samples. *J. Microbiol. Methods* 36, 181-192.
- Greetham, H.L., Giffard, C., Hutson, R.A., Collins, M.D., Gibson, G.R., 2002. Bacteriology of the Labrador dog gut: a cultural and genotypic approach. *J. Appl. Microbiol.* 93, 640-646.
- Kim, S.Y., Adachi, Y., 2007. Biological and genetic classification of canine intestinal lactic acid bacteria and *bifidobacteria*. *Microbiol. Immunol.* 51, 919-928.

- Lefebvre, S.L., Waltner-Toews, D., Peregrine, A.S., Reid-Smith, R., Hodge, L., Arroyo, L.G., Weese, J.S., 2006. Prevalence of zoonotic agents in dogs visiting hospitalized people in Ontario: implications for infection control. *J. Hosp. Infect.* 62, 458-466.
- McCoy, S., Gilliland, S.E., 2007. Isolation and characterization of *Lactobacillus* species having potential for use as probiotic cultures for dogs. *J. Food Sci.* 72, M94-97.
- Metchnikoff, E., 1907. *The Prolongation of Life: Optimistic Studies*. Mitchell, C., Ed. William Heinemann, London. pp. 161–183.
- Murphy, C., Murphy, S., O'Brien, F., O'Donoghue, M., Boileau, T., Sunvold, G., Reinhart, G., Kiely, B., Shanahan, F., O'Mahony, L., 2009. Metabolic activity of probiotics- Oxalate degradation. *Vet. Microbiol.* 136(1-2), 100-107.
- O'Hara, A.M., Shanahan, F., 2006. The gut flora as a forgotten organ. *EMBO Rep.* 7, 688–693.
- O'Hara AM, Shanahan F, 2007. Mechanisms of action of probiotics in intestinal diseases. *Scientific World Journal.* 7, 31-46.
- O'Mahony, C., Scully, P., O'Mahony, D., Murphy, S., O'Brien, F., Lyons, A., Sherlock, G., MacSharry, J., Kiely, B., Shanahan, F., O'Mahony, L., 2008. Commensal-induced regulatory T cells mediate protection against pathogen-stimulated NF-kappaB activation. *PLoS Pathog.* 4, e1000112.
- O'Mahony, L., McCarthy, J., Kelly, P., Hurley, G., Luo, F., Chen, K., O'Sullivan, G.C., Kiely, B., Collins, J.K., Shanahan, F., Quigley, E.M., 2005. *Lactobacillus* and *bifidobacterium* in irritable bowel syndrome: symptom responses and relationship to cytokine profiles. *Gastroenterology* 128, 541-551.

- 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

- 421 *Bifidobacterium infantis* 35624 in women with irritable bowel syndrome. Am. J.
422 Gastroenterol. 101,1581-1590.

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

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

Figure 1. Intergenic spacer PCR products

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

Figure 2. Adhesion to epithelial cells

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

Figure 3. Murine gastrointestinal transit

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

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

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

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
<i>Bacteroides</i>	6.26	5.65	6.02	6.26	5.71	0.22
Total <i>Clostridia</i>	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
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