Effects of *Akkermansia muciniphila* Supplementation on Markers of Intestinal Permeability in Dogs Following Antibiotic Treatment

THESIS

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Abstract

Diarrhea and other sequelae of gastrointestinal hyperpermeability are common complications of antibiotic therapy. *Akkermansia muciniphila* is a mucin-degrading bacterium, positively associated with gastrointestinal epithelial health and decreased permeability. The objectives of this study were to measure effects of oral *Akkermansia* administration on markers of gastrointestinal permeability following antibiotic administration.

Eight healthy, purpose-bred dogs were randomized to receive either *Akkermansia* (10^9 CFU/kg; N=4) or vehicle (N=4) for 6 days following a 7-day course of metronidazole. After a 20-day washout, dogs were crossed-over to the alternate treatment. After an additional 20-day washout, the experiment was repeated with amoxicillin-clavulanate. Fecal *Akkermansia* qPCR and plasma concentrations (measured by ELISA) of cytokeratin-18, lipopolysaccharide, and glucagon-like peptides (GLP-1, GLP-2) were measured at baseline (T0), post-antibiotic (T1), and post-treatment (vehicle or *Akkermansia*; T2). For each antibiotic, absolute or delta concentrations were compared between time-points using paired samples t test.

*Akkermansia* was detected in feces in 7/8 dogs following supplementation (T2) but not at T0 or T1. Delta (T2-T1) cytokeratin-18 after metronidazole was significantly lower on vehicle (-0.27 ng/ml) versus *Akkermansia* (2.4 ng/ml; p=0.03). Cytokeratin-18 concentrations tended to decrease from T0 to T1 on amoxicillin-clavulanate (p=0.05). Post-prandial GLP-1 concentrations (38.2 pM) were higher than pre-prandial (15.5 pM) concentrations. No adverse side-effects or other significant biomarker alterations were noted.
*Akkermansia muciniphila* PCR detection suggested successful gastrointestinal transit following oral supplementation in dogs, with an effect on gastrointestinal epithelium based on plasma cytokeratin-18 alterations. Further study is needed to determine impact in dogs with naturally-occurring disease.
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Chapter 1: Gastrointestinal (GIT) Microbiome and GIT Homeostasis

1.1 Microbiome
The environment of the gastrointestinal tract (GIT) is composed of a diverse microbiome, which develops early in life under the influence of environmental, dietary, and genetic factors from a sterile environment to one consisting primarily of anaerobes.\(^1\) During development and once the microbiome is stabilized, both commensal and pathogenic microorganisms, along with their interactions, impact the overall microenvironment. The resultant steady-state microenvironment or alternations in steady-state from microbial organisms and their products (e.g. pH, fatty acid production) interact with the host through the local immune system and enteroendocrine signaling. These local effects, influenced by diet composition and inter-microbial interactions, affect the primary GIT microorganism population and impact GIT motility, permeability, and nutrient absorption, thereby affecting the health status of the GIT.\(^2\) Furthermore, extra-intestinal factors [alterations in the systemic immune system (e.g. IgA deficiency), endocrine influences] can alter the GIT microbiome, thereby impacting GIT motility, permeability, and nutrient absorption.\(^1,3,4\)

The normal microbiome in adult dogs and cats is primarily composed of organisms from the phyla Actinobacteria, Bacteroides, Bifidobacteria, Firmicutes, Fusobacteria, and Proteobacteria; phyla proportions differ along the GIT.\(^5\)\(^{-}\)\(^{10}\) While major phyla are relatively conserved, significant intra- and inter-individual variability in phyla proportions occurs over time, with less inter-individual variability observed in cats.\(^7,11\) Despite similarity in phyla, significant differences exist
in the microorganism population at lower levels (i.e. genus and species); the importance of these
differences in relation to gastrointestinal health and disease have not been fully clarified. It is
proposed that alterations in the normal microbiome composition contribute to acute and chronic
enteropathies and affect the host both locally and outside the GIT, in part due to alterations in
microbial by-product formation (e.g. increased serum D-lactate concentrations in cats with GIT
disease).6,12-16

1.2 Enteroendocrine Cells and GIT Epithelial Cell Barrier
Glucagon-like peptide 2 (GLP-2) is a 33-amino acid, peptide enteroendocrine hormone derived
from proglucagon. It is a member of the glucagon superfamily, with amino acid sequence
homology to other enteroendocrine hormones, including secretin, glucagon, glucagon-like peptide
1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), exendin-3/4, growth hormone-
releasing factor, and vasoactive intestinal polypeptide (VIP). Actions of GLP-2 complement and
counteract the actions of other members of the glucagon superfamily. Significant amino acid
homology exists among most evaluated species, with 88% homology between humans and
dogs.17

GLP-2 production and release
GLP-2 release occurs primarily from ileal and colonic enteroendocrine L-cells.17,18 Co-secretion
occurs with GLP-1, allowing extrapolation of GLP-2 control from more extensive literature. The
localization of these production sites has been established by decreased blood concentrations
following surgical removal of the ileum and colon and subsequent intestinal dysfunction.17
GLP-2 is secreted into the circulation in its 33-amino acid form but exists in this form for a limited amount of time, with an approximately 7-minute half-life in humans. Circulating dipeptidylpeptidase IV (DPP-IV) rapidly degrades the 33-amino acid sequence to GLP-2 (3-33) at its N-terminal region.\(^\text{17,18}\) Half-life in humans for the GLP-2 (3-33) form is longer, at approximately 27 minutes. This relatively short-half is important, as the shortened amino acid sequence has no significant gastrointestinal impact at physiologic concentrations but may, through competitive receptor action, have impact at supraphysiologic doses. Inhibition of DPP-IV results in greater local GLP-2 impact, and species differences in basal DPP-IV concentration may affect overall GLP-2 action.\(^\text{17}\) GLP-2 is excreted through the kidneys, implying that renal function could impact systemic GLP-2 concentrations.\(^\text{17,18}\)

**Stimulation of secretion**

Secretion of GLP-2 is influenced by local alimentary, paracrine, endocrine, and nervous input. Local stimulation of secretion occurs with meal ingestion and provides the main stimulatory signal. Studies in dogs and rodents have demonstrated increased blood concentrations of glucagon hormones following enteral feeding and direct infusion of nutrients (i.e. glucose) into the intestines. These effects have not been replicated with intravenous supplementation, supporting direct local regulation. Furthermore, normal blood concentrations require that enteral intake comprise certain percentages of total nutrient support. Form of nutrient intake may also modify the degree of stimulation, with greater stimulation by liquid diets, carbohydrates, and fats. If GLP-2 secretion mirrors that of GLP-1, fat may provide greater stimulation than simple carbohydrates (i.e. glucose). Elemental components may also be important, as GLP-1 secretion is influenced by sodium (Na)-glucose co-transport (e.g. glucose, galactose, 3-O-methylglucose), monounsaturated fatty acids, and peptones, but not other sugars (e.g. fructose), saturated fatty
acids, or individual amino acids. As these affects are primarily described in relation to GLP-1, stimulatory mechanisms are extrapolated to GLP-2. Direct evidence for short-chain fatty acid (SCFA) and enteral fiber stimulation has been described for GLP-2. ¹⁷

Additional local control results from proximal stimulation of the intestinal endocrine and nervous system. Specifically, K-cells in the duodenum release GIP in response to enteral nutrition, and the enteric nervous system is activated through the enteric reflex arc. Likely, other neurotransmitters (e.g. calcitonin gene-related peptide, neuromedin B) also play a neuronal stimulatory role. Activation of the enteric nervous system increases blood concentrations of distally secreted enteroendocrine hormones prior to direct nutritional input in the ileum and colon. These mechanisms have been demonstrated for GLP-1 and extrapolated to GLP-2. ¹⁷

Local action and control of GLP-2
Most of GLP-2’s biologic effect occurs within the gastrointestinal tract, with limited systemic influences. Primarily, GLP-2 locally impacts intestinal structure. GLP-2 results in intestinal mucosal growth, defined by greater intestinal weight, increased intestinal DNA and protein proportions, and amplified crypt depth and villus height. ¹⁷ A rodent study explored these effects with continuous intravenous infusion of GLP-2 at varying concentrations for 14 days. Post-infusion intestinal biopsy demonstrated increased intestinal DNA and protein content; increases were dose-dependent and maximized at an approximate concentration of 130% control levels, with decreased DNA and protein content at higher doses. ¹⁹ Mice injected subcutaneously for 10 days with GLP-2 or a degradation-resistant GLP-2 analog had increased intestinal weight at study completion, with an amplified impact by the degradation-resistant analog. Both mucosal thickness and villus height were increased in this study, but no impact on crypt depth was seen.
Individual epithelial cell morphology was also altered with treatment; specifically, cells were longer and narrower in treated mice than controls.\textsuperscript{20}

Mediation of increased mucosal weight occurs through epithelial cell proliferation in conjunction with prevention of epithelial apoptosis. Both proliferation of mature crypt cells, as well as stem cells, are influenced through \(\beta\)-catenin, a protein involved in gene transcription signaling. The proportion of dividing cells increases, as cells remain in an active replication cycle. Intestinally-derived insulin-like growth factor-1 (IGF-1) appears to facilitate GLP-2 proliferative effects, but the exact process is unknown. Some studies have demonstrated that rodents without IGF-I have decreased to absent response to GLP-2 treatment; IGF-1 influence on GLP-2 action appears mediated through activation of \(\beta\)-catenin.\textsuperscript{18} However, GLP-2 may not be completely dependent on IGF-1, as effects of treatment were seen in mice missing IGF-1 in other studies. These studies suggest a role for a member of the tyrosine kinase receptor family, ErbB. Treatment of mice with GLP-2 promoted expression of ErbB ligands in the small intestine and colon. Furthermore, GLP-2 affected multiple ligands, which the authors suggested indicated coordinated mechanisms for signal induction. Mice treated with an ErbB inhibitor concurrently with GLP-2 did not display intestinal mucosal growth relative to controls.\textsuperscript{21} IGF-2 plays a lesser role but appears to facilitate GLP-2-mediated epithelial crypt cell fission, resulting in increased intestinal crypt numbers.\textsuperscript{18}

Growth of colonic mucosal epithelium may also be mediated by keratinocyte growth factor (KGF). KGF likely also facilitates goblet cell delineation within the colon. Although KGF is found throughout the gastrointestinal tract, rodents missing KGF had decreased colonic response to GLP-2, while small intestinal response was unaltered.\textsuperscript{18} Impact on mucosal structure has been demonstrated through experimentally administered GLP-2 and comparison of mucosal changes with physiologic blood GLP-2 concentrations. Local impact is limited to the intestinal tract, with
no impact on pancreatic secretion. The lack of pancreatic influence is in contrast to other proglucagon-derived hormones.\textsuperscript{17}

Aside from paracrine and neural influences, changes in the gastrointestinal microbiome may stimulate GLP-2 secretion. Mice administered prebiotics had increased plasma GLP-2 concentrations. These mice also had an increase in specific bacteria numbers, leading to the conclusion that certain gastrointestinal microbiota may interact with the local environment, leading to GLP-2 stimulation.\textsuperscript{22}

Other, less defined, actions of GLP-2 within the intestinal tract include suppressive actions, such as decreased gastric acid secretion, slowed gastrointestinal motility, as well as metabolic actions, such as increased intestinal blood flow.\textsuperscript{17} Most knowledge of GLP-2 influence on gastrointestinal motility has been extrapolated from oncologic studies of neuroendocrine tumors. Gastrointestinal emptying was prolonged, and side-effects from tumor-related hypermotility were improved with GLP-2 supplementation.\textsuperscript{17} Intestinal blood flow has been evaluated in pigs receiving total parenteral nutrition (TPN), and amplified blood flow was thought to be mediated through increased local nitric oxide concentrations.\textsuperscript{17,18} The impact on mucosal blood flow was concentration-dependent in research settings and appeared facilitated by endothelial nitric oxide synthase (eNOS).\textsuperscript{18}

Although primary effects occur within the gastrointestinal tract, GLP-2 actions are systemically relevant, as GLP-2 augments intestinal nutrient uptake and up-regulates enzymatic nutrient processing systems, specifically hydrolases. Rodent studies have demonstrated amplified sodium/glucose co-transporter (SGLT-1) expression and activity as one mechanism of greater
nutrient (specifically glucose and galactose) uptake. These findings have not been consistent among all studies, with some demonstrating no glucose uptake above baseline concentrations. In a study evaluating continuous intravenous infusion of GLP-2 in healthy rats, absorption of glycine and galactose were increased in comparison to control rats. Effect was dose-dependent with greater absorption seen at higher infusion concentrations. Additionally, increased capacity for amino acid and lipid absorption has been demonstrated via similar mechanisms.

Mucosal growth limits abnormal intestinal hyperpermeability. Concurrently, both intercellular and transmembrane permeability are reduced, and epithelial repair is enhanced following mucosal injury. Rodent models have demonstrated diminished movement of small particles between epithelial cells and decreased endocytosis of larger compounds. Mice were injected subcutaneously with GLP-2 or a degradation-resistant GLP-2 analog for 10 days. Both treatments decreased paracellular passage of Cr-EDTA, while only the degradation-resistant analog decreased paracellular Na⁺ movement. Serving as a large-molecule model, decreased transepithelial movement of horseradish peroxidase (HRP) was noted, with more substantial reduction in analog-treated mice. The impact of the degradation-resistant analog was evaluated at intervals of 4 hours and 48-hours post injections. Treatment demonstrated time-dependency on the above effects, with ionic and small molecule movement decreased by 4 hours, cellular morphology changes at 48 hours, and no impact on mucosal weight or thickness at either time-frame. Mice with increased plasma GLP-2 concentrations secondary to prebiotic administration and concurrent changes in gastrointestinal microbiota demonstrated decreased measures of gastrointestinal permeability, including decreased blood LPS and diminished dextran permeability. Additionally, these mice had increased occludin (tight junction plasma-membrane protein) mRNA, suggesting a mechanism for improved gastrointestinal barrier function.
Improvement in barrier function was not observed when a GLP-2 antagonist was administered concurrently.22 This decreased permeability could provide a mechanism to address pathologic conditions, such as endotoxemia and gastrointestinal-mediated systemic inflammation, which are associated with altered intestinal permeability. As metabolic changes occurred more rapidly than structural changes,17,20 more immediate therapeutic effects may occur than have been demonstrated in research evaluating histopathology. It is important to note that impacts of GLP-2 on an individual are likely related both to age (i.e. maturity of the gastrointestinal tract) and the health status of the gastrointestinal tract.18

*Systemic actions of GLP-2*

There is some evidence to support GLP-2 as a mediator of appetite stimulation, with suppression of hunger seen at higher blood concentrations. These effects have primarily been documented following experimental infusions of GLP-2. It may also serve as a central appetite mediator, as receptors located in the hypothalamus respond to direct cerebral ventricular infusion of GLP-2, resulting in appetite suppression.17

*Influences of GLP-2 in pathologic conditions*

Several studies have demonstrated the role of GLP-2 stimulation in bowel adaptation following intestinal resection.17,18 In these situations, secretion of GLP-2 is thought to occur secondary to fermentation of SCFA, as well as abnormal nutrient, specifically fat and carbohydrate, malabsorption. Current theory is that malabsorbed nutrients reach intestinal L-cells in increased concentrations, resulting in direct stimulatory effect. Providing direct sugar supplementation in the intestines results in adaptive intestinal changes, concurrent with increased blood GLP-2 (and GLP-1) concentrations without concurrent increases in other enteroendocrine hormones. Similar
intestinal mucosal trophic changes have been documented in humans with short bowel syndrome when treated with GLP-2. Evidence for systemic impact outside the gastrointestinal tract is conflicting, with some studies documenting overall increased body weight and mass and others documenting no effect.\(^{17}\)

Due to the potential impact on intestinal permeability, GLP-2 has been evaluated in human and rodent models of IBD and intestinal mucosal damage.\(^{17,18}\) Humans with IBD had higher blood concentrations of GLP-2, with limited evidence of complete amino acid sequence breakdown; authors suggested this as a protective and potentially naturally therapeutic mechanism. Local gastrointestinal inflammation in rodents, however, showed lower mucosal levels of GLP-2 in association with pathologic tissue changes. Treatment with GLP-2 reduced negative structural pathology.\(^{17}\) Epithelial repair properties appear mediated through vasoactive intestinal peptide (VIP) in rodent IBD models.\(^{18}\) Furthermore, non-steroidal anti-inflammatory drug (NSAID)-induced colitis and reperfusion injury were associated with decreased mortality and attenuated local and systemic damage with GLP-2 treatment.\(^{17}\) There is also evidence for a protective role of GLP-2 in situations with anticipated mucosal damage, such as total parenteral nutrition and chemotherapeutic-induced gastrointestinal toxicity. Some anti-inflammatory properties are observed without simultaneous mucosal hypertrophy, suggesting discrete mechanisms.\(^{18}\)

1.3 Biomarkers of GIT Permeability

**Cytokeratin-18**

Differential cytoplasmic filament expression allows discrimination of cell types, with cytokeratin expressed by epithelial cells. Specifically, cytokeratin-18 (CK-18) is a relatively large component of the small intestinal and colonic epithelial cell cytoskeleton, though not specific for these
epithelial cell types. As an intracellular component, serum CK-18 is a biomarker for epithelial cell damage, and serum concentrations are increased in human chemotherapeutic patients experiencing GIT epithelial cell toxicity. Larger increases were demonstrated in patients with dose-limiting toxicity than without.

\textit{Lipopolysaccharide}

Lipopolysaccharides (LPS) comprise the outer surface membrane of gram-negative bacteria, which encompass a large percentage of the GIT microbiome. The endotoxin, or Lipid A component, triggers the immune response against this outer membrane component. In patients with attenuated GIT barrier function, systemic LPS absorption and penetration of antigens occurs, stimulating an abnormal inflammatory response to otherwise harmless antigens. Bacterial translocation from the GIT to the blood stream and subsequent systemic distribution has been described in both macroscopic (e.g. ulcerative colitis) and microscopic (e.g. lymphocytic colitis) primary GIT diseases, as well as diseases secondarily affecting GIT permeability (e.g. acute pancreatitis, cirrhotic liver disease). This creates an inflammatory cycle, whereby systemic endotoxemia worsens GIT hyper-permeability, potentially mediated through an iNOS-related pathway. Within the blood stream, LPS complexes with lipopolysaccharide-binding protein (LBP), triggering cytokine production and monocyte activation.

Lipopolysaccharide concentrations mirror disease extent and severity scores in human IBD and correlate with both active and inactive disease states, as well as clinical relapse. While LPS has been more commonly measured in association with GIT hyperpermeability, LBP may be a more reliable biomarker, with a half-life of 24-48 hours versus 1-3 hours, respectively, in
humans. Improved serum LBP concentrations above LPS concentrations have been demonstrated following IBD treatment.
Chapter 2: Probiotics in Small Animal Veterinary Medicine

Probiotics are “live microorganisms that, when consumed in adequate amounts, have the potential to confer a beneficial health effect.” Proposed mechanisms by which probiotics work include displacement of pathogenic microorganisms, production of antimicrobial by-products, improvement in GIT epithelial barrier function, improvement in micronutrient absorption, and modulation of the enteric and innate immune response. Even minor product alterations (e.g. microorganism strain) can determine whether or not these benefits are realized. The investigation of probiotic microorganisms and their application in health and disease is on-going in veterinary medicine.

General considerations

As information accumulates regarding probiotics and their effects, it is important to understand some practical aspects regarding formulation and labeling of probiotics which make direct comparisons and interpretation of research studies challenging. Regulation of commercial veterinary and human probiotics vary based on country (e.g. United States vs European Union) and the product’s labeled use [e.g. supplements vs. food vs biologics (i.e. products intended to treat or cure disease)], leading to common labeling inconsistencies. Products containing live organisms inherently pose challenges relative to manufacturing stability, contamination, and long-term storage, leading to actual microorganism concentrations ranging from 0.008-215% compared to label claims. In one study, all eight veterinary products that were evaluated contained individual microorganism concentrations < 2% of label claims, as well as unlisted,
potentially pathogenic genera (e.g. *Staphylococcus*, *Pediococcus*). Additional studies have documented similar findings, including more recent investigations into veterinary probiotic content. Other recent studies have demonstrated adequate bacterial growth on culture, even if still less than label claim. Importantly, lack of growth may be related to the fastidious nature of some microorganisms, such as the strict anaerobe *Bifidobacterium*, rather than actual lack of product inclusion or manufacturing issues.

When comparing studies that evaluate commercial probiotic products, it is important to note that the manufacturing processes can affect the ability of bacteria to express desirable traits. Therefore, two probiotics of the same microorganism strain and concentration could have different effects on the GIT microbiome because they were manufactured differently. For example, probiotic culture media and microorganism viability of various *Lactobacillus* spp. impacted probiotic efficacy in pathogen exclusion. Heat-inactivated *Lactobacillus* displayed greater in vitro pathogen exclusion for *Enterococcus canis* and *Clostridium perfringens* than viable forms, while viable forms were more efficacious against *Salmonella typhimurium* on some media. Additionally, the in vitro exclusion of the above pathogens differed when the probiotic was culture in soy-based vs broth media. Growth characteristics of *Lactobacillus acidophilus* and *Bifidobacterium* spp. are affected by co-culture with oligosaccharides; extrusion and drying can negatively impact *Bacillus* spore viability when incorporated into a food product (>99% spore loss) and when applied as a powder coating (60% expected spore concentrations). *Bacillus*, *Lactobacillus* spp., and *Bifidobacterium* spp. tolerated storage in food for 12 months, but this cannot be extrapolated to other species. These inter-species interactions are crucial when considering combination products formulated with multiple microorganisms or as synbiotics (i.e. prebiotic and probiotic combinations). Prebiotics are substrates for
microorganism fermentation, which can be tailored to a specific microorganism’s growth needs.\textsuperscript{49} Inclusion of a prebiotic may select for enhanced activity of the probiotic microorganism, as well as those bacteria within the GIT. \textit{Lactobacillus acidophilus} demonstrated superior growth when cultured with fructooligosaccharides (FOS), while \textit{Bifidobacterium} spp. demonstrated superior growth on galactooligosaccharides (GOS).\textsuperscript{47} Therefore, effects of synbiotic products cannot be extrapolated to the effect of the microorganism alone or vice versa.

The ability of enteric pathogens to cause clinical disease depends, in part, on their ability to penetrate the GIT biofilm and adhere to intestinal mucosa.\textsuperscript{50} Likewise, penetration and adherence are important for biological effect of many probiotic bacteria. Similarly, interaction with mucosal immune cells, as well as the ability to interfere with adherence and proliferation of pathogenic bacteria, is important. For example, several bacterial species express mucous-binding proteins or fimbriae, allowing direct adhesion. However, many host-microbial interactions rely on GIT recognition of microorganism-associated molecular patterns and interaction with GIT cells, including dendritic cells. Effects include up-regulated cytokine expression and mucous secretion, leading to changes in GIT homeostasis.\textsuperscript{33,51} As such, probiotic effects may be further modulated by the ability of the probiotic organism to adhere to GIT mucosa and by its tolerance of the GIT environment (e.g. bile concentration, acidity). Probiotic microorganisms that are able to adhere may have improved pathogen exclusion or displacement than those which only alter cytokine profiles or GIT secretions. There are standard laboratory test criteria, which may be used to evaluate microorganisms and probiotic products for these properties (e.g. incubation in solutions of varying acidities and bile concentrations, bile salt hydrolase activity, in-vitro mucosal adherence testing).\textsuperscript{40,52}
Goals of probiotic supplementation vary, but some measures of clinical effect include improvement in fecal score, decreased duration and severity of clinical signs, decreased pathogenic microorganism fecal counts, and decreased systemic inflammatory markers (e.g. inflammatory cytokines). Probiotic bacteria have been evaluated in various in vitro studies. In-vitro studies are useful in generating hypotheses for in vivo studies. Otherwise their clinical applicability is very limited due to lack of ability to evaluate the impact of microbial by-product formation, microorganism interaction, cellular signaling, and environmental influences. Effects of specific probiotic bacteria have been evaluated in various in vitro studies, and a summary of these studies is listed in Table 1. (Appendix A. Table 1)

Furthermore, the effect of a probiotic in a certain clinical context is likely unique to that context. One cannot extrapolate, for example, the effect of a probiotic in diseased dogs from studies on healthy dogs or from one disease to the other. Because of the importance of the clinical context, studies on the use of probiotics should ideally define the study population clearly (including diet and diet history, microbiome before dosing, etc.) and describe the probiotic fully (including exact strain, dose, dosage regimen, etc.). It should be noted that most existing studies are limited in numbers of individuals included, the extent of population characterization, appropriate characterization of underlying disease, control of potential confounders (diets and other environmental effects), and in comprehensive examination of potential outcome measures. Also, many studies are uncontrolled or use inappropriate or incomplete controls. Therefore, drawing conclusions about probiotic efficacy is impossible based on current literature.
Evidence for use of probiotics in healthy dogs and cats

While studies in healthy dogs and cats frequently report on seemingly positive effects of probiotics, the implication to disease states is unknown. In healthy animals, there are many potential benefits to probiotics that could be explored (e.g. improved fecal consistency, immune modulation for immune-mediated diseases), but to date, most of these applications have not been explored. Importantly, no study has found major negative side-effects in healthy animals, suggesting relative safety and allowing further investigation. Tables 2 and 3 summarize in vivo studies on probiotics in healthy dogs and cats, respectively. (Appendix A. Table 2; Appendix A. Table 3)

Probiotics are often studied in healthy animals to assess the ability of the microorganism to survive transit in the GIT and persist after cessation of treatment in fecal material, either via culture or PCR. It is important to note that PCR identification of DNA does not imply viability but rather that the supplemented microorganism was present in the probiotic and recognizable DNA sequences survived GIT passage. The importance of post-treatment persistence of the microorganism itself is unclear because there could also be a persistent effect after the supplemented microorganism can no longer be detected. One study in dogs found altered Lactobacillus strain diversity (assessed by denaturing gradient gel electrophoresis [DGGE]) after cessation of probiotic treatment. The fact that this effect was demonstrated when the supplemented bacteria was no longer detected implies continued impact.53

Many of the studies in healthy dogs and cats report on changes in bacteria populations that are typically considered pathogens, but these microorganisms were present in healthy animals. Therefore, the presence of these presumed pathogenic organisms might not necessarily cause
clinical disease. Some authors have also presumed that decreased microbial diversity is a benefit of probiotic supplementation and evidence of restoration of a “healthy” or stable bacterial population.\textsuperscript{54} However, decreased microbial diversity has been demonstrated in disease states, such as inflammatory bowel disease (IBD) and following antibiotic therapy.\textsuperscript{55-57} The significance of shifts in microbiota populations in healthy animals and whether they have any clinical impact is unknown. Maintenance of a healthy bacterial end-product metabolome, which could be identical despite variations in microbiota, might be of more importance than maintenance of the microbiota themselves.

While most studies in healthy animals aim to document viability of probiotic species in the product itself and post-treatment in feces, in vitro studies have documented effects of non-viable organisms, which may be greater under some circumstances than viable organisms.\textsuperscript{58} Therefore, lack of viability or persistence does not imply lack of effect, especially when extrapolating data from healthy patients to a clinical population.

\textit{Evidence for use in dogs and cats with gastrointestinal illness}

Table 4 summarizes the studies on probiotics in dogs and cats with gastrointestinal disease. (Appendix A. Table 4)

\textit{Acute enteropathy}

Two studies have observed sled dogs with exercise-induced diarrhea. In one study, dogs in routine training who were treated with a synbiotic (\textit{Enterococcus faecium} SF68, \textit{Bacillus coagulans}, \textit{L. acidophilus}, fructooligosaccharides, mannooligosaccharides and B vitamins) had decreased fecal microbiota biodiversity, with no change in fecal (short-chain fatty acid) SCFA
Fatty acid composition of fecal material is evaluated as a marker of colonic health because SCFA, such as butyrate, are an important energy source for colonic epithelium. Also in that study, dogs’ fecal scores, scales denoting fecal consistency as a surrogate of moisture content, improved relative to baseline, and the treated dogs experienced fewer days of diarrhea in comparison to control dogs treated with a vehicle (microcrystalline cellulose). While this study shows a positive effect of the tested product, no conclusion can be drawn regarding an independent role of the probiotic component. Furthermore, importance of changes in GIT microbiota biodiversity in the absence of metabolome changes is unknown. In an unpublished study that compared the effect of *E. faecium* SF68 to a vehicle in training sled dogs, the probiotic treated group showed faster clinical improvement, fewer diarrhea episodes, and resolution of clinical signs by day five of treatment. Diarrhea did not resolve over the seven-day study course in the placebo group. Unfortunately, this study did not evaluate the microbiome or fecal biochemistry.

In a shelter setting, treatment with *E. faecium* SF68 resulted in a lower percentage of cats experiencing diarrhea longer than two days compared to cats treated with the vehicle alone. A similar comparison could not be accomplished in dogs of this study because too few had diarrhea for more than two days in either the probiotic or placebo group. This study was also limited in that the clinical outcome was determined on a dynamic population (animals were treated for various lengths of time depending on how long they remained in the shelter), and the results were confounded by the presence of GIT parasites (15% cats/17% dogs). Another study performed by the manufacturer and presented only in abstract form compared the effect of same probiotic product as above (*E. faecium* SF68) in combination with metronidazole to metronidazole alone in shelter dogs. Dogs treated with the probiotic and metronidazole had a more rapid improvement
in fecal scores (2.8 vs 4.4 days, respectively), but fecal score at study completion was not different. A synergistic effect between the probiotic and the antibiotic could not be excluded in this study because there was no comparison to a group treated with the probiotic alone. Taken together, these studies do not provide evidence for the use of this probiotic in a shelter setting in dogs and only weak evidence for its use in cats. Some studies on the effects of *E. faecium* SF68 have not been published in a peer-review format, precluding full evaluation.

Dogs treated with *Bifidobacterium animalis* (canine isolate AHC7) prior to and during kenneling had fewer stress-induced diarrhea episodes, improved fecal scores and increased counts of *Bifidobacteria* (in fecal cultures) compared to untreated controls. These effects were dose dependent. Fecal *Clostridium perfringens* culture counts were unchanged. Although dogs originated from different environments, an attempt was made to decrease variability by a two-week acclimation period and a standard diet. Administration of the same *B. animalis* isolate at a higher dose twice daily in cocoa butter treats resulted in a shorter duration of clinical signs in dogs suffering from acute idiopathic diarrhea (3.9 vs 6.6 days). Dogs were enrolled if they developed diarrhea over a three-month time period. In the context of probiotic effect, it is important to note that dogs (N= unknown) were allowed to receive metronidazole at the veterinarian’s discretion. Fewer dogs with probiotic treatment were treated with metronidazole (38.5 vs 50%); however, metronidazole was usually given when multiple dogs had an outbreak in the same area, and adding metronidazole was a subjective decision. These limitations prevent a meaningful comparison because it is unknown whether baseline illness severity was equal between groups. No fecal microbiome analysis was performed to allow comparison to the dogs undergoing kenneling stress.
A commercial combination product of *Lactobacillus farcininis* (porcine isolate), *Pediococcus acidilactici*, *Bacillus subtilis* (soil isolate), *Bacillus licheniformis* (soil isolate), and *L acidophilus* MA 64/4E (human isolate) has been studied in dogs with acute diarrhea of various etiologies. Compared to placebo, administration of the commercial product three times per day at double the recommended dose, resulted in a tendency toward shorter duration of acute diarrhea (1.3 vs 2.2 days), with no impact on vomiting duration or combined clinical signs. The results of this study are confounded by differences in presenting complaint, severity of disease and potentially by concurrent unstandardized manipulation of diet making it impossible to assess whether the difference between group was truly a result of probiotic administration.  

In an experimental model of antibiotic-induced diarrhea in otherwise healthy dogs, treatment with the yeast *Saccharomyces boulardii* (1000 mg/d) after diarrhea onset was associated with shorter duration of clinical signs (2.9 vs 6.5 days, in treated vs untreated dogs, respectively) and faster return to baseline of fecal SCFA concentrations. Dogs that received the probiotic concurrently with antibiotics never developed diarrhea and had no change in fecal SCFA concentrations. There was no difference between groups and time points (before, at diarrhea onset, at resolution, and one week past resolution) in cultures of *Escherichia coli, Shigella, Salmonella, Campylobacter*, smears for *C. perfringens* spores and *Clostridium difficile* toxin; no toxins or pathogens were isolated at any point.

Overall, studies in acute diarrhea in dogs and cats present weak and scattered evidence for the exclusive use of probiotics, and mostly in the setting of ‘preventable’ stress-induced diarrhea (shelter and kenneling, extreme physical exercise, or antibiotic-induced). Evidence for use of probiotics in naturally occurring acute diarrhea in dogs and cats is lacking.
Chronic enteropathy

Evidence for efficacy of probiotics in dogs with chronic *Giardia* infection is limited. A subset of shelter dogs with diarrhea of at least two days’ duration with *Giardia* that were treated with *E. faecium* SF68, in combination with metronidazole (25 mg/kg q12h), had normal fecal consistency by study completion in comparison to 43% *Giardia*-positive dogs treated with metronidazole alone.66 Severity of clinical signs related to *Giardia* infection versus comorbidities was unknown, and the number of dogs with *Giardia* was small. As mentioned above, the independent effect of the probiotic could not be assessed in this study because of the lack of a probiotic-treated group (without metronidazole). In a study of dogs with chronic *Giardia* infection (diagnosed by direct fecal IFA), treatment with *E. faecium* SF68 over six weeks had no advantage over a placebo powder in terms of clinical response, improvement in fecal shedding, or change in immune indicators (fecal IgA or blood leukocyte phagocytic activity). No fecal microbiome evaluation was performed in this study.68

Several studies have evaluated dogs with food-responsive enteropathies. In two studies, the addition of a probiotic combination (*L. acidophilus NCC2628/NCC2766, L. johnsonii NCC2767* combination; *E. faecium*, fructooligosaccharides, Gum Arabic) to an elimination diet resulted in similar improvement in clinical signs compared to treatment with an elimination diet alone.69,70 Both treatment groups had a similar decrease in *Enterobacteria* counts (fecal culture)55 and a similar change in histopathologic markers of inflammation (IL-1β, IL-18).70 Authors, therefore, concluded a large impact of the standardized elimination diet. In contrast, dogs with idiopathic IBD had similar, though slower, response to treatment with a probiotic (*Lactobacillus, Bifidobacteria*, and *Streptococcus*) compared to dogs treated with metronidazole (20 mg/kg
q12h) and prednisone (1 mg/kg q24h) (10.6 vs 4.8 days). However, in this study, baseline clinical scores were lower (suggesting milder disease) in the group treated with probiotics. Mucosal TGF-beta increased in both groups, with greater in increase probiotic-treated dogs. Mucosal CD3+ T cells decreased and FoxP3+ regulatory T cells increased in probiotic treated dogs. Fecal quantitative PCR targeting *Faecalibacterium* spp. and *Turicibacter* spp. demonstrated lower concentrations of these bacteria in the probiotic group compared to healthy controls at baseline, with *Faecalibacterium* spp. increasing in the probiotic group, suggesting a positive modulation of the microbiome. However, using qPCR, the investigators narrowed the assessment of the entire fecal microbiota to these two genera. A broader assessment of the microbiome, which might have revealed other, potentially negative, effects was not reported. Differences in histopathologic findings between dogs with food-responsive and idiopathic enteropathies in these studies could reflect differences in response to probiotic treatment or differences in the underlying disease mechanism. While these results are encouraging for dogs with IBD, it is important to emphasize that the probiotic combination in these studies contained probiotics and prebiotics, and the isolated effect of either was not examined. Overall, there is currently no firm evidence that probiotics are effective in treating chronic diarrhea in dogs, especially not in more severe IBD cases. Taken as a whole, however, the fact that probiotics can affect both the host and the GIT microbiota in dogs with IBD suggest that further investigation is warranted.

Treatment of six German Shorthair Pointers with chronically poor fecal scores with *L. acidophilus* (DSM 13241) following a 12-week period of diet acclimation, reduced frequency of defecation and improved fecal consistency during supplementation and four weeks after cessation of treatment. Fecal dry matter (i.e. water-free percent of fecal material; an objective measure of
fecal consistency) was only increased during treatment. Fecal samples were assessed for \( C. \) perfringens, \( Escherichia \) spp., \( Lactobacilli \), \( Bifidobacteria \) in combination with fluorescent in situ hybridization (FISH) and demonstrated no significant changes. There were trends toward increased \( Lactobacilli \) and \( Bifidobacteria \) spp. and decreased \( C. \) perfringens and \( Escherichia \) spp. in treated dogs, and a trend towards more constant microbiome population, (as assessed by FISH), which led the authors to suggest that the treatment led to stabilization in the GIT populations. However, the use of fecal microbiota by FISH, limited the assessment of the microbiota to only few species. Broader investigation of the entire microbiota with large-scale DNA sequencing was not reported in this study. This was also a study on a small number of dogs, and further investigation will be needed to elucidate the significance of these positive trends.

In privately-owned cats with undefined chronic (>3 weeks) diarrhea, stool firmness increased in 72% of cats treated with synbiotic combination (\( E. \) faecium NCIMB 30183, \( B. \) bifidum NCIMB 30179, \( E. \) thermophiles NCIMB 30189, \( L. \) delbrueckii NCIMB 30186, \( L. \) casei NCIMB 30188, \( L. \) plantarum, NCIMB 30187, \( L. \) acidophilus NCIMB 30184 with fructooligosaccharides and arabinogalactans) when a capsule was opened and mixed in food. However, this was an uncontrolled study in which cats received other treatments and diet changes, and response to treatment was subjectively evaluated by owners. In a controlled study (published as an abstract, not published in a peer-review format), cats with undefined chronic diarrhea experienced decreased frequency of severe diarrhea when fed an \( E. \) faecium SF68 probiotic.

Overall, limited comparisons can be made between probiotics and their use in acute and chronic gastrointestinal illness. In most studies, the underlying disease processes were unknown, precluding specific therapeutic targets and the use of directed evaluation markers. Furthermore,
studies in sick pets often focus practically on resolution of clinical signs, lacking evaluation of the baseline GIT microbiome and how it changes with treatment, potentially overlooking subtle but important changes. In any given disease, when defined, only one or two probiotic species were evaluated, and most studies were underpowered or confounded by response to traditional therapy. As in healthy animals, no adverse effects were reported with probiotic supplementation, allowing consideration for future studies.

**Evidence for use in puppies and kittens**

Table 5 summarizes studies on probiotics in puppies and kittens. (Appendix A. Table 5)

The impact of probiotic administration on the GIT microbiota, health, and immunity, as well as clinical signs, in puppies and kittens has been evaluated in a few studies. A non-peer reviewed study demonstrated increased fecal *Bifidobacteria* and *Lactobacillus* spp., typically considered beneficial GIT bacteria, in puppies treated with *E. faecium* SF68\(^a\) (administered in food) from three weeks to one year of age.\(^a, i\) However, there was no difference between treated and untreated puppies in fecal *E. coli*, *Campylobacter*, or *Salmonella* and no clinical benefit was demonstrated.\(^i\) In contrast, *E. faecium* SF68 was inconsistently detected via culture and subsequent PCR in the feces of pathogen-free kittens while treated with the same commercial product\(^a\) from 7-27 weeks of age; there was no difference in fecal quality and detection of fecal *Clostridium* enterotoxin (ELISA) among the kittens treated with probiotics compared to untreated kittens.\(^75\) A different *E. faecium* product\(^j\) had positive effects in healthy research puppies treated from 2-5 days of age; it was associated with improved nutrient digestibility (based on fecal analysis) in large breed puppies and improved daily weight gain in small breed puppies in comparison to control dogs.\(^76\) In contrast, *E. faecium* SF68\(^a\) had no effect on weight gain in kittens.\(^75\) Puppies and kittens
treated with this commercial product from 8 - 52 and 7-27 weeks of age, respectively, demonstrated enhanced immune responses to vaccination. They received vaccinations at weeks 1 and 4 of the trial. Although there was a trend toward increased fecal IgA (specific to SF68) in puppies, samples were cross-reactive to commensal organisms in controls. IgA is of interest, particularly in the feces, because it is the major antibody component of the GIT mucosa, and therefore a surrogate marker for the local immune system. An increase in IgA could be interpreted as a marker of enhanced protection against pathogens. However, increased IgA might simply represent a response to increased antigenic stimulation without enhanced immunity or the body’s protective mechanism against a harmful stimulus. Only a challenge with a pathogen can prove that increased IgA represents enhanced protection. The authors of this study suggested that these changes represent no harm to GIT commensals, based on lack of clinical difference in treated compared to controls (liver digest vehicle). Lack of clinical signs does not rule out significant impact on the baseline GIT microbiome or long-term implications of an upregulated immune response. Treated kittens had higher serum and salivary Feline herpes virus-1 (FHV-1) IgA but no significant difference in FHV-1 IgG, FCV IgG, total IgA or total IgG. In contrast to puppies, supplemented kittens did have increased peripheral CD4+ lymphocytes in comparison to controls. Similarly, these results in kittens suggest upregulation of the immune system but without clinical context the risks or benefits of this response is unknown. Healthy seven to eight-month-old research puppies treated with Bacillus subtilis (strain C-3102; added to commercial diet at 0.01% diet) had improved fecal scores and higher dry matter content, as well as lower ammonia levels, compared to untreated puppies. No difference in fecal output was noted however, and fecal scores were ideal in both groups.
In one to six-month-old puppies treated for parvovirus enteritis (diagnosed by fecal ELISA) with standard supportive care, adjunctive treatment with the probiotic combination of *Lactobacillus, Bifidobacterium*, and *Streptococcus* was associated with reduced clinical signs, increased lymphocyte counts, and improved survival when compared to controls; no fecal microbiome evaluations were performed. Although dogs were randomly assigned to treatment groups, method of randomization was not described; three dogs in the unsupplemented group died within the first three days compared to one dog in the supplemented group, which could suggest unequal disease severity at baseline. It is possible that the difference in outcome was the result of selection bias or “absence of optimal care by the owner” and not treatment.

During an acute diarrhea outbreak in kittens, a smaller percentage of kittens treated with *E. faecium* SF68 required other medical interventions in comparison to untreated kittens (9.5% vs 60%, respectively); however, the need for treatment was subjective. Kittens receiving the probiotic experienced faster resolution of clinical signs (18 vs 45 days), and had increased fecal *Bifidobacteria*, decreased fecal *C. perfringens*, and increased serum IgA.

Studies in puppies and kittens mostly have similar limitations as studies in adult dogs and cats, including small numbers, limited evaluations in states of disease, and limited controlled populations for direct comparison. One important note, which is highlighted in studies evaluating puppies and kittens concurrently, is that important species differences in response to probiotics do exist, and therefore, evidence from one species cannot be automatically be extrapolated to the other. Probiotics are also likely to have different effects in immature animals versus adults as the GIT microorganism population transitions to the adult microorganism population during development in immature animals.
Evidence for use in dogs and cats with non-gastrointestinal illness

Table 6 summarizes studies on probiotics in dogs and cats with non-gastrointestinal illness.

(Appendix A. Table 6)

Probiotics have been evaluated in several non-gastrointestinal illnesses because of their potential effects on the immune system and systemic inflammation, with extrapolation from human medicine. 81-91

Atopic Dermatitis

Several studies evaluated research dogs sensitized to *Dermatophagoides farinae*. One breeding pair was bred for two litters. The first litter served as control puppies, with the breeding pair administered *Lactobacillus rhamnosus GG* prior to the second litter. Puppies in the second litter were administered the probiotic from 3 weeks to 6 months of age. All puppies were sensitized to the *D. farinae* and subsequently underwent intradermal allergen testing. While probiotic-treated puppies had reduced reaction to intradermal skin testing and lower IgE titers, clinical signs following allergen exposure were not different in comparison to the first litter; skin biopsy showed no difference in filaggrin expression (a protein decreased in atopic dermatitis). At 3-4 years of age, probiotic-treated dogs had reduced clinical signs following allergen exposure in comparison to the first litter. This study had multiple limitations, including a small population of non-atopic beagles (N= 10) used for generation of cytokine reference ranges, lack of placebo group, and lack of fecal microbiome analysis. Comparisons among puppies were also performed on different litters, which could have different maternal immunity and environmental antigenic exposure. 92-94
In women with recurrent urinary tract infections, oral administration of a probiotic helps to restore normal vaginal microbiota by increasing vaginal lactic acid-producing bacteria (LAPB). Based on this, a study was performed to determine whether oral administration would increase vaginal LABP in dogs. This study found that LAPB are uncommonly isolated from the vaginal vault of spayed healthy female dogs (about 20% of dogs) and that oral administration of a commercial synbiotic \(^1\) \((Lactobacillus, Bifidobacterium, Bacillus, yeast, enzymes, prebiotics)\) did not increase vaginal populations of LAPB in these privately owned dogs.\(^6\) However, in dogs with historical recurrent urinary tract infections LAPB were even less common (<10% of dogs).\(^7\) The effect of LAPB supplementation or other probiotic treatment was not evaluated in dogs with chronic urinary tract infections. It is possible that while there is no effect in healthy dogs, female dogs with reduced LAPB and historical recurrent urinary tract infections might benefit from probiotic treatment.

In contrast to the manufacturer’s claim, administration of a synbiotic product \(^m\) \((Streptococcus thermophiles, L. acidophilus, Bifidobacterium longum, psyllium husk)\) formulated in an enteric capsule had no effect on azotemia (i.e. no significant percent change in BUN or creatinine by study definition) in cats with stable chronic kidney disease (CKD), in a double-blinded, controlled (prebiotic only), randomized clinical trial. Albumin was assessed in this study as a surrogate for hydration status and it too remained within reference range and was unaffected by treatment.\(^8\) However, the probiotic in this study was not administered as an enteric-coated capsule as labeled. Instead, the capsule contents were sprinkled on the food. In an earlier study, an improvement in creatinine was reported in a group of 7 cats with CKD treated with this
However, that study had multiple methodological flaws; it was an uncontrolled, non-blinded study in which the diagnosis of CKD was based on palpation of small kidneys in cats with persistently elevated BUN and creatinine, with no documentation of urine specific gravity and no control for hydration status. Also, cats recruited to that study were treated with a variety of diets and other concurrent medications. Taken together, there is currently no indication for use of probiotics in cats with chronic kidney disease. Importantly, these studies evaluated the effect of the probiotic on “azotemia” in its traditional definition of elevated BUN and creatinine, implying that reduction in azotemia is a marker for improved GFR and renal function. However, probiotics might affect urea processing in the GIT such that serum BUN is decreased via reduced amino acid fermentation in the gut or via ion-trapping of ammonium from luminal acidification without any effect on renal function and GFR. In addition, because creatinine can be affected by muscle mass, the effect of probiotics on renal function in these studies is questionable. Further analysis of the effect of the probiotics on BUN and creatinine separately in a controlled study would be more informative.

Respiratory disease

Treatment of FHV-1 infected research cats with E. faecium SF68 following a two-week acclimation period had no significant impact on viral DNA expression or viral shedding. To account for the potential impact of stress, cats were group housed for 28 days, individually housed for 28 days, and then group housed for an additional 84 days. It should be noted that none of the cats were positive for FHV-1 DNA by fluorogenic PCR assay, and so the effect of SF68 supplementation on the level of FHV-1 shedding could not be determined. All cats had elevated FHV-1 antibody concentrations (measured by ELISA), and those did not change over time. While, cats experienced fewer episodes of conjunctivitis when treated with the probiotic
compared to placebo, some cats had received topical antivirals, introducing a confounding factor.

A potential benefit to treatment with the probiotic was observed in that decreased fecal microbiota biodiversity (via temporal temperature gradient electrophoresis and PCR) was observed in placebo treated cats but biodiversity was maintained in the probiotic-treated cats.\textsuperscript{54}

No attempt was made in this study to examine the mechanism of decreased biodiversity in placebo-treated cats.

Overall, evidence for probiotic use in non-gastrointestinal illness is limited to few studies with limited numbers of animals. Populations often have loosely defined diseases or are not naturally affected with the proposed disease target. These studies have limited microbiome assessment, preventing full understanding of the interaction between the GIT and affected body system.

Clinical Summary

A clear role for treatment of dogs and cats with probiotics is undetermined based on current literature. Evidence in healthy dogs, as well as dogs with gastrointestinal and non-gastrointestinal illness, suggests some impact of probiotics on the GIT microbial population, metabolic status (e.g. fecal SCFA concentrations), and immune system, as well as systemic effects (e.g. changes in serum biochemistry), but no clear clinical benefits. Similar but weaker evidence is available in cats, with fewer controlled studies.

While general conclusions can be drawn to a specific study population, results among studies were variable, and some studies showed no effect of probiotics. Undetected differences may have resulted from few individual animals and low statistical power; however, differences in study design may have resulted in real discrepancies or lack of efficacy. Unfortunately, it is difficult, if
not impossible, to make direct comparisons between studies. There are no standards for probiotic formulation and dose, treatment duration, or timing of clinical evaluation and study end-points. Many studies attempted to document persistence, colonization, and duration of impact, but methods varied among studies, including bacterial counts, PCR, and fecal qualities; alterations in the variables are not fully understood in healthy individuals.

Many studies consider changes in presumably pathogenic or ‘beneficial’ bacteria as important outcomes but do not fully speciate microorganisms (e.g. pathogenic vs. commensal Clostridia) and do not consider interactions between bacteria. GI metabolomics are rarely studied in veterinary medicine, limiting conclusions about the impact of treatment on ‘beneficial’ vs pathogenic species.

Current evidence suggests that probiotic supplementation may play a role in animals with acute gastrointestinal disease, especially in stress-induced diarrhea and mainly in shortening the time period to resolution of clinical signs when compared to standard therapies. Studies in dogs suffering from chronic enteropathies are more difficult to interpret because they are typically confounded by concurrent therapies. No significant side-effects were noted following probiotic administration in any of these studies, suggesting relative safety over a short time period within the microbial populations studied.

Based on current data, no specific product can be recommended. An effective probiotic species is likely disease and individual-specific. Due to questions regarding accuracy of product labeling, products evaluated by independent companies could be considered. Longer term outcomes and administration periods still require evaluation for both safety and efficacy.
Future studies should focus on a clear definition of disease, standardize study populations, and include both healthy and placebo, or at least standard therapy controls, to directly compare probiotic impact within a population. Consistent microbiome and metabolome assessment will help more clearly define mechanisms of response and GIT influences in GI and non-GI diseases. Over time, the most appropriate approach to specific disease processes can be developed.

Footnotes
a. Fortiflora, Nestle Purina PetCare, St. Louis, MO
b. Gore AM. Reynolds A. Effects of Enterococcus faecium SF68 on stress diarrhea, in 2012
d. Iams Prostora, Procter & Gamble Pet Care, USA
e. ZooLac Propaste, Chem Vet A/S, Denmark
f. Synbiotic D-C, Protexin Ltd, Somerset, UK
g. VSL#3, VSL Pharmaceuticals, Inc, Gaithersburg, MD
h. Paciflor, Prodeta, Vannes, France
j. Fermactiv, C. Richter Gesmbh Co, KG, Austria
k. Culturelle HS, Amerifit Brands/Culturelle, Cromwell, CT
l. Y+ Powder, Rayne Clinical Nutrition, Kansas City, MO
m. Azodyl, Vetoquinol, USA
Chapter 3: *Akkermansia muciniphila* and Antibiotic GIT Impact

3.1 GIT Mucin Layer

The mucin layer of the GIT provides a barrier between GIT luminal contents (e.g. microbiota) and the GIT epithelium and is produced by mucous-secreting GIT epithelial cells. Mucous is composed of glycoproteins, as well as components of the innate and adaptive GIT immune system (e.g. lysozyme, immunoglobulins).\textsuperscript{102,103} Though the mucin layer is separated from the GIT epithelium, the outer portion of the mucin layer is susceptible to degradation by pathogenic microorganisms, as well as specialized commensal organisms.\textsuperscript{102} Mucin-degrading commensals express specialized mucolytic enzymes (glycosidases), allowing utilization of mucin glycoproteins as their primary energy source and produce by-products, which are consumed as substrates by other GIT microorganisms.\textsuperscript{102,104} Furthermore, bacterial structural components and degradation products provide additional stimulation for mucous production, resulting in a continuous breakdown-production cycle.\textsuperscript{105}

3.2 *Akkermansia muciniphila*

Microbiota associated with the intestinal mucin layer exert greater proportional impact on the host due to proximity to the epithelium.\textsuperscript{102,103,105} *A. muciniphila*, an anaerobic gram-negative, mucin-degrading bacterium, has the ability to feed on mucin, convert glycans into short chain fatty acids (acetate, propionate), and prevent colonization of pathogens.\textsuperscript{105,106} In healthy humans, *A. muciniphila* (CAGCACGTGAAGGTGGGGAC; CCTTGCGGTTGGCTTCAGAT) is the predominant mucin-degrading microorganism.\textsuperscript{104} While not evaluated in models of antibiotic-
associated diarrhea (AAD), decreased *A. muciniphila* numbers have been observed in humans with ulcerative colitis and Crohn’s disease, and an increase in this bacterial population has been shown in mice given prebiotics.\textsuperscript{104-107} It is known that up to eight different *Akkermansia* species colonize the human GIT, and 16S rRNA sequences with 80-100\% similarity to *Akkermansia muciniphila* have been documented throughout the GIT of multiple mammalian species.\textsuperscript{105,107,108}

Direct treatment with *Akkermansia muciniphila* in rodent models of metabolic syndrome (i.e. high fat diet; diabetic inflammation), improved GIT and systemic markers of GIT barrier function.\textsuperscript{106} Specifically, following administration, portal serum LPS concentrations decreased as cecal *A. muciniphila* counts increased. Additionally, markers of Paneth cell and epithelial cell expression, along with intestinal concentrations of acylglycerols [e.g. 2-OG (2-oleoylglycerol), 2-arachidonoylglycerol (2-AG)], increased following treatment.\textsuperscript{106} As 2-AG serves an anti-inflammatory role in the GIT and 2-OG stimulates GLP-2 release, changes suggested improved mucosal barrier function.\textsuperscript{106} Furthermore, indirect increases in *A. muciniphila* following prebiotic administration in similar rodent models also resulted in decreased serum lipopolysaccharide (LPS) and increased GIT L-cell numbers.\textsuperscript{109}

### 3.3 Antibiotic Associated Diarrhea

Antibiotic-associated diarrhea (AAD) is a common complication of antibiotic therapy in human and veterinary medicine. The pathophysiology of AAD is multifaceted: Antibiotics can enhance selection of pathogenic enteric microorganisms, have direct effects on the GIT epithelium and motility (e.g. amoxicillin-clavulanate), and have immune and metabolic implications secondary to an altered GIT microbiome.\textsuperscript{110} Amoxicillin-clavulanic acid is the most commonly implicated antibiotic in AAD in human medicine, affecting up to 25\% of patients.\textsuperscript{110,111} Other implicated
antibiotics include cefixime (15-20%), ampicillin (5-10%), and others affecting 2-5% of patients (e.g. other cephalosporins, fluoroquinolones, tetracyclines, macrolides).\textsuperscript{110,111} Current recommendations in human medicine include cessation of the implicated antibiotic. However, refractory cases may result in life-threatening complications, such as bacterial translocation, and require additional antibiotic (e.g. metronidazole) therapy directed against the suspected pathogenic bacteria. Therapy with traditional antibiotics is often unrewarding, and relapses following cessation of therapeutic antibiotic therapy are common (20-25\%).\textsuperscript{110} Probiotic administration has been evaluated in human medicine with mixed results\textsuperscript{112-114} but may help stabilize the composition of the natural microbiome and reduce systemic effects. Meta-analyses have suggested an overall benefit in pediatric patients;\textsuperscript{112} however, studies demonstrate variability in protocols, and no specific probiotic formulations or dose recommendations can be extrapolated.

3.4 Antibiotics and the GIT Microbiome

Antibiotics are commonly used in veterinary medicine as therapy for chronic inflammatory enteropathies (e.g. metronidazole), as well as GIT and systemic infections. It is known that both amoxicillin-clavulanate and metronidazole alter the gastrointestinal microbiome composition in dogs,\textsuperscript{55,115,116} but the implications on acute or chronic enteropathies or intestinal permeability are unknown. Experimental models in healthy dogs have described decreased bacterial microbiome diversity and shifts in bacterial populations (e.g. decreased Clostridia spp. and Fusobacteria and increased Bifidobacteria and Enterobacteria) following metronidazole administration.\textsuperscript{55} Microorganism population shifts were not observed in dogs with IBD treated with a combination of metronidazole and prednisone,\textsuperscript{71} suggesting that antibiotic effect might depend on the disease processes or concurrent therapies. Altered microorganism responsiveness to other antimicrobials
(e.g. *E. coli* susceptibility to β-lactams) have been observed following treatment with amoxicillin; these susceptibility shifts differ among antibiotics and likely among microorganisms.\textsuperscript{116}
Chapter 4: *Akkermansia muciniphila* Impact on GIT Permeability in Two Models of Antibiotic Treatment in Healthy Dogs

4.1 Hypothesis
Antibiotic administration to healthy dogs results decreased GIT mucosal barrier function. *A. muciniphila* administration stimulates GLP-2 secretion. GLP-2 decreases GIT permeability and GIT epithelial damage, resulting in decreased plasma LPS and CK-18 concentrations.

4.2 Materials and Methods

*Animals*
Eight healthy, adult research dogs (six spayed, mixed breed females, two intact beagle males) were studied. Dogs were determined to be healthy based on their history, physical examination and fecal examination. At study initiation, median age was 25 months (range, 15-32 months), median weight was 24.7 kg (range, 11.2-31.9 kg), and median body condition score was 6/9 (range, 5-8). Dogs were weighed and body condition score (BCS) assessed prior to study initiation and every two weeks, to correspond with changes in study treatments.

Dogs were housed in individual runs, with visual interaction with other dogs, at The Ohio State University in Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited facilities for study duration. All eight dogs were housed in a single room for the first half of the experiment and then randomly separated into two rooms (four
dogs each) for the second half of the experiment, with two weeks remaining in the washout period. Housing changes were made due to facility considerations, unrelated to the study.

Throughout the study, dogs were fed once daily (Laboratory Canine Diet 5006; LabDiet; St. Louis, MS, USA) in the morning. Dogs had received this diet for three months prior to study initiation and no oral supplements, medications (aside from study treatments), or treats were administered from one week prior to study initiation through study completion; no changes in caloric allocation were made, regardless of BCS at the beginning of the study. This study was approved by OSU-IACUC (Protocol 2014A00000129).

**Study design**

This was designed as a double-crossover, placebo-controlled, prospective study in which the effect of oral *Akkermansia muciniphila* administration was assessed in two models of antibiotic dysbiosis. After a 7-day acclimation period (i.e. study food only), baseline fecal samples were collected daily for 7 days (days 1-7), and then all dogs were administered metronidazole (MT) orally (12.5 mg/kg q12 hours) for 7 days (days 8-14). Following MT administration, all dogs received omeprazole orally (1 mg/kg q24 hours) and either probiotic (PB) *Akkermansia muciniphila* [1-5 x 10^10 CFU/ml diluted 25% in sterile phosphate-buffered saline (PBS) solution; 10^9 CFU/kg; group 1, N= 4] or vehicle (VH) (equal volume PBS solution; group 2, N= 4) after a minimum 12-hour fast for a total of 6 days (days 15-21). PB was administered one hour after omeprazole administration. After a 20-day washout period, this process was repeated but with group 1 receiving VH and group 2 receiving PB. The study was repeated after another 20-day washout period with Amoxicillin-clavulanate (13.5 mg/kg PO q12h). Blood and fecal samples
were collected at the end of each baseline (T0), antibiotic period (T1), and probiotic (or vehicle) period (T2).

A single PB culture batch was used for all dogs per antibiotic. PB and VH were stored in individual cryogenic vials (16-23 vials per culture batch) at -80° C until administration and individual vial allowed to thaw for 30 minutes prior to administration. A new dose aliquot was used daily to avoid freeze-thaw changes. Figure 1 outlines the study timeline.

Figure 1. Study Timeline
Probiotic Design

Akkermansia muciniphila was obtained from the American Type Culture Collection. Product was cultured under anaerobic conditions in hog gastric mucin medium as previously described.\textsuperscript{117} Modifications to environmental conditions (85% nitrogen, 10% carbon dioxide, 5% hydrogen atmosphere) were made to increase microorganism growth rate. The 16S rRNA was amplified, sequenced (Retrogen Inc), and submitted to NCBI for strain confirmation. Product was determined to be \textit{A. muciniphila} strain GP28 16S rRNA by 99\% sequence match. Bacteria were concentrated in PBS to a cell density of $3.5-6 \times 10^{10}$ cells/ml based on hemocytometer counting and frozen at -80°C.

Blood collection

Blood samples (6 ml) were collected after a 24 hour fast, prior to the dog’s morning meal. Dogs were then fed $\frac{1}{2}$ their daily meal and 3-hour post-prandial blood samples (2 ml) were collected. Blood was collected into glass EDTA tubes and centrifuged (20 min, 3000 rpm, 4° C) within 60 minutes. Plasma was stored at -80°C. Samples with gross hemolysis or lipemia were run through a second centrifugation cycle. All samples were analyzed in one batch at the end of the study. All blood samples were collected via jugular vascular access ports placed previously for another study (N=6) or via lateral saphenous venipuncture (N=2).

Fecal collection

Following the 7-day acclimation period, naturally voided fecal samples were collected on days 1-7 for baseline microbiome analysis. Large volume fecal samples were collected into fecal storage containers on 3 consecutive days prior to first antibiotic administration and immediately frozen at -80°C. Following baseline, fecal samples were collected daily from first morning feces for the
duration of the study. Feces were collected into Eppendorf tubes and immediately frozen at -80°C until processing. Fecal scores were performed daily by a single observer on a 1-5 scale, as previously described.118

Sample Analysis

Probiotic analysis

Probiotic product was analyzed via PCR as previously described.119 Briefly, primers were chosen based on 16S rRNA sequences based on GenBank data [S-St-Muc-1129-a-a-20 (sequence 5’ CAG-CAC-GTG-AAG-GTG-GGG-AC); S-St-Muc-1437-a-A-20 (sequence 5’ CCT-TGC-GGT-TGG-CTT-CAG-AT)]. PCR (Applied Biosystems StepOne instrument) was performed using SYBR green master mix. Calculation of copy number (Molar copies/µL) was performed using nanodrop spectrometry based on optical density of purified product (OD260). Melting curve analysis was performed to generate a standard curve (y = -3.3x + 39.7; r² = 0.99). Figure 2 outlines probiotic standard curve.
Fecal analysis

Fecal bacterial DNA extraction was performed on samples collected at the end of each baseline (T0), antibiotic period (T1), and probiotic (or vehicle) period (T2) per study period. DNA was extracted from approximately 200-220 mg thawed fecal material using a commercially available QIAamp DNA stool kit (QIAGEN), per manufacturer’s directions. This kit has previously been used for canine fecal DNA extraction.115

PCR was performed to test for *A. muciniphila* on the above days using the sample PCR analysis and dilution concentrations based on standard curve as described above. To verify that initial negative PCR results from day 55 were not spurious, fecal DNA extraction was repeated on samples from that day on all dogs and second PCR analysis was performed.
Biomarker analysis

To assess intra- and inter-assay variation, 2-3 samples from the first two plates and additional duplicate samples from the third plate were simultaneously performed on the third plate. All samples were run in duplicate. All assays for a single dog per antibiotic were run on one plate.

**CK18:** CK-18 concentrations were measured with a canine ELISA [Canine Cytokeratin 18 (CK-18) ELISA; MyBioSource Inc; San Diego, CA, USA]. The manufacturer reports 4.4-5.6% intra-assay variation, 6.6-7.9% inter-assay variation, and a sensitivity of 1 uIU/mL. The assay standard curve was linear (slope: 0.99 (0.99 – 1.00); intercept: 0.07 (-7.96x10^-5 – 0.21); r^2=0.99). Inter-assay variation was 12.6% and intra-assay variation was 18.6%.

**LPS:** LPS concentrations were measured with a canine ELISA [Canine Lipopolysaccharides (LPS) ELISA; MyBioSource Inc; San Diego, CA, USA]. The manufacturer reports 4.4-5.6% intra-assay variation, 6.6-7.9% inter-assay variation, and a sensitivity of 1 ng/mL. The assay standard curve was linear (slope: 0.99 (0.99 – 1.00); intercept: 1.62 (-2.29x10^-5 – 1.62); r^2=0.99). Inter-assay variation was 14.5% and intra-assay variation was 2.6%.

**GLP-1:** Pre- and post-prandial total GLP-1 concentrations were measured with an ELISA (GLP-1 total ELISA; EMD Millipore; St. Charles, Missouri, USA), which was previously validated in humans, rats, and mice. GLP-1 has 100% homology among mammalian species. Manufacturer reports <5% intra-assay variation, <12% inter-assay variation, working range of 4.1 – 1000 pM, and sensitivity of 1.5 pM. The assay standard curve was sigmoidal (5 parameter logistic; r^2=0.99). Inter-assay variation was 27% and intra-assay variation was 12.2%.
GLP-2: Pre- and post-prandial total GLP-2 concentrations were measured with an ELISA (GLP-2 total ELISA; EMD Millipore; St. Charles, Missouri, USA), which was previously validated in humans, rats, and mice. Canine GLP-2 has 88% homology to human GLP-2; there is 100% homology to the N-terminal sequence in humans and rats, which is responsible for metabolic effects. Manufacturer reports <10% intra-assay variation, <12% inter-assay variation, a working range of 1 ng/mL – 64 ng/mL, and sensitivity of 0.1 µg/mL. The assay standard curve was sigmoidal (4 parameter logistic; $r^2=0.99$). There were not enough samples with concentration above background to calculate inter- and intra-assay variation.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 24.0 (SPSS Inc 2016, NY, USA) and GraphPad Prism (GraphPad Software Inc, CA, USA). Data were assessed for normality using Shapiro–Wilk. Normally distributed data are presented as mean ± standard deviation; non-normal data are presented as median [range]. Significance was set at $p < 0.05$.

For each antibiotic period, absolute biomarker concentrations for BL (i.e. prior to AB) and AB (i.e. end of antibiotic treatment) were compared using paired samples $t$ test (normally distributed data) or Wilcoxon signed-rank test (non-normally distributed data). Because there was no difference, values were combined for statistical analysis as T0 (BL) and T1 (AB). The biomarker concentration following antibiotic administration (T1) was used to calculate change to VH or PB (T2-T1).

Change scores were calculated from T0 to T1 and T1 to T2 and compared between PB and VH groups using paired samples $t$ test or Wilcoxon signed-rank test. Absolute biomarker
concentrations were compared between T0 and T1 and T1 and T2 using paired samples t test or Wilcoxon signed-rank test. Pre- and post-prandial concentrations of GLP-1 were compared at the same time-points using paired samples t test or Wilcoxon signed-rank test. Descriptive statistics were calculated for *A. muciniphila* fecal PCR analysis and GLP-2 concentrations.

4.3 Results

**Fecal DNA PCR**

*Akkermansia muciniphila* was detected in 7/8 (87.5%) dogs. It was detected at three time-points post-PB supplementation (T2) in 4/4 (concentration: $10^1$-$10^2$ molar copies/µL), 4/4 (concentration: $1$-$10^2$ molar copies/µL), and 3/4 dogs (concentration: $10^1$-$10^2$ molar copies/µL), respectively. No quantifiable *A. muciniphila* were identified in any dog at one PB/VH sampling time-point, including the 4 dogs that were given PB in the preceding time-period. This PB/VH period followed a period of MT administration. *A. muciniphila* was detected in two dogs (concentration: 10 molar copies/µL) following VH supplementation, prior to any direct PB supplementation in these dogs. No *A. muciniphila* was detected in any fecal sample at baseline (BL; T0) or after AB (T1) sampling periods.

**Fecal Score**

Baseline fecal scores were higher prior to CL than MT administration (p=0.04). Average fecal score was higher following MT than CL (p<0.05) administration. Average fecal score increased from BL to MT (T1-T0) administration (0.3±0.14) and decreased from BL to CL administration (-0.11±0.13; p<0.01).
**Metronidazole**: Fecal score increased from BL (2.66 ± 0.12) to MT (T1-T0: 2.96 ± 0.31; p<0.01). Average fecal score (T2) was not different between PB (2.81±0.31) and VH administration (2.69 ± 0.29; p=0.32) but there was a trend towards a more significant improvement in fecal score from MT to PB (T2-T1: -0.4±0.30) vs MT to VH (-0.17 ± 0.23) (p=0.09).

**Amoxicillin Clavulanate**: Fecal score decreased from BL (T1-T0: BL 2.65[2.55-3.4]) to CL (2.58[2.45-3]; p=0.03). Average fecal score (T2) was not different between PB (2.69 ± 0.21) and VH administration (2.65±0.20; p=0.46) and the change in fecal score from CL (T2-T1) did not differ between VH (-0.03 ±0.15) and PB (0.03±0.21; p=0.51).

**Cytokeratin-18 (CK-18)**

**Metronidazole**: There was no difference in CK-18 concentration between BL and MT (20.3 ± 9.42 vs 19.86 ± 9.18 ng/ml; p = 0.36). Change in CK-18 concentration (T2-T1) from MT to treatment was significantly different from VH (-0.27 ng/ml) versus PB (2.4 ng/ml; p = 0.03). Overall CK-18 concentration (T2) was not significantly different between VH (20.7 ± 8.1 ng/ml) and PB (20.2 ± 10.3 ng/ml; p = 0.64) administration.

**Amoxicillin Clavulanate**: There was no difference in CK-18 concentration at BL (20.18 ± 8.39 ng/ml) to CL (18.03 ± 9.46 ng/ml; p = 0.05) treatment. Change in CK-18 concentration (T2-T1) was not different from CL to VH (0.76 ± 5.26 ng/ml) versus PB (0.17 ± 1.049) ng/ml. (p = 0.75). Overall CK-18 concentration (T2) was not different between VH (19.04 ± 9.78 ng/ml) and PB (17.96 ± 10.83 ng/ml; p = 0.42) administration.
Lipopolysaccharide (LPS)

Metronidazole: LPS concentration did not differ from BL (17.8 [0-123.5] ng/ml) to MT (18.9[0-121.2] ng/ml; p = 0.41) treatment. There was no significant difference in change (T2-T1) of LPS concentration from MT to treatment with either VH (2.60 ± 4.49 ng/ml) or PB (0.71 ± 3.81 ng/ml; p=0.41). Overall LPS concentration (T2) was not different between VH (21.658[0,121.31] ng/ml) and PB (18.30[0,135.94]) ng/ml; p = 1.00) administration.

Amoxicillin Clavulanate: LPS concentration was unchanged from BL to CL treatment (T1-T0: 0[-17.7,6.53] ng/ml). Change score (T2-T1) was not significantly different from CL to VH (0[-3.1,1.9] versus PB (0[0,2.87]; p= 0.41). Overall LPS concentration (T2) was not significantly different between VH (5.36[0,153.5] ng/ml) and PB (7.10[0-122.1] ng/ml; p = 0.41) administration.

Glucagon-like peptide-1 (GLP-1)

Metronidazole: Post-prandial GLP-1 concentrations (38.17 ± 10.01 pM) were significantly higher than pre-prandial concentrations (15.54 ± 9.59 pM; p < 0.05). Neither pre- or post-prandial GLP-1 concentrations were different from BL to MT (T1-T0) treatment (Pre BL: 8.14[6.23,22.63] pM; Pre MT 9.20[1.74,30.17] pM; p = 0.16; Post BL: 33.66 ± 11.08 pM; Post MT 37.24 ± 9.80 pM; p = 0.31).

Change in pre- or post-prandial GLP-1 concentrations (T2-T1) was not different from MT to VH (Pre: 10.01 ± 8.68 pM; Post: 4.48 ± 10.31 pM) versus PB (Pre: 5.63 ± 16.28 pM; p=0.51; Post: -0.76 ± 14.17 pM; p= 0.29).Overall GLP-1 concentration (T2) was not significantly different between VH (Pre: 19.67 ± 13.96 pM; Post: 39.81 ± 13.27 pM) and PB (Pre: 18.21 ± 13.80 pM; Post: 38.39 ± 10.54 pM; Pre: p=0.84; Post: p = 0.59 ) administration.
**Amoxicillin Clavulanate:** Post-prandial GLP-1 concentrations (33.59 ± 12.44 pM) were significantly higher than pre-prandial concentrations (18.48 ± 7.54 pM; p < 0.05). Neither pre- or post-prandial GLP-1 concentration was significantly different from BL to CL (T1-T0; Pre BL: 13.21 ± 6.69 pM; Pre CL 19.81 ± 8.48 pM; p = 0.11; Post BL: 35.9 ± 13.28 pM; Post CL 37.02 ± 12.89 pM; p = 0.75).

Change in pre- or post-prandial GLP-1 concentration from CL to VH (T2-T1; Pre: -2.09 ± 9.93 pM; Post: -12.57 ± 13.90 pM) was not different than change from CL to PB (Pre: -3.23 ± 7.71 pM; p = 0.84; Post: -1.10 ± 16.24 pM; p = 0.19). Overall GLP-1 concentration (T2) was not significantly different between VH (Pre: 15.91 ± 8.75 pM; Post: 25.53 ± 6.77 pM) and PB (Pre: 18.39 ± 7.69 pM; Post: 34.79 ± 18.31 pM; Pre: p = 0.48; Post: p = 0.11) administration.

**Glucagon-like peptide-2 (GLP-2)**

**Metronidazole:** Minimal GLP-2 concentrations were only detected in three dogs. Pre-prandial concentrations were detected at BL (T0; 0.76 ng/ml) and following VH (T2; 0.35 ng/ml) administration in one dog each, respectively. Post-prandial concentrations were detected in one dog (1.05 ng/ml) following VH administration (T2).

**Amoxicillin Clavulanate:** Minimal GLP-2 concentrations were detected in three dogs. Pre-prandial concentrations were detected in one dog following both periods of CL administration (T1; 1.11, 1.64 ng/ml), PB administration (T2; 1.29 ng/ml), and VH administration (T2; 2.67 ng/ml). Pre-prandial concentrations were detected in an additional dog following CL administration (T1; 2.27 ng/ml). Post-prandial GLP-2 concentrations were detected in one dog at BL (T0; 0.12 ng/ml) and one dog following CL administration (T1; 0.67 ng/ml).
Adverse Events

No adverse effects directly related to PB administration were noted. There were three episodes of vomiting noted during the study period (N= 2 VH; N= 1 MT). One dog ate only 1/4 its daily meal for 7 days during the washout period following VH administration. One dog received an oral non-steroidal anti-inflammatory drug (meloxicam 0.2 mg/kg once; 0.1 mg/kg) for 3 days during the washout period following placebo administration for interdigital cysts.

Figures 3 and 4 summarize biomarker concentrations.

Figure 3. Plasma concentrations of CK-18 (ng/ml), LPS (ng/ml), and post-prandial GLP-1 (pM): A. At BL (T0) and after a week of MT administration (T1). B. At T2, following VH or PB administration. C. Delta T2-T1 comparing VH and PB. A2 represents median[range]. Remaining panels represent mean±SD. * = p<0.05.
Figure 4. Plasma concentrations of CK-18 (ng/ml), LPS (ng/ml), and post-prandial GLP-1 (pM): A. At BL (T0) and after a week of CL administration (T1). B. At T2, following VH or PB administration. C. Delta T2-T1 comparing VH and PB. A2 and B2 represent median[range]. Remaining panels represent mean±SD.

4.4 Discussion

The mucin layer of the GIT comprises a protective layer of the epithelium and is colonized by a specialized subset of mucin-degrading bacteria, which have close interaction with host cells. *Akkermansia muciniphila*, specifically, comprises a large portion of the human GIT mucin-layer microbiome in health, with reduced numbers documented in certain disease states (e.g. Crohn’s disease, ulcerative colitis, acute appendicitis). Antibiotic therapy is associated with
significant cost and morbidity in human medicine, with serious implications resulting from GIT hyperpermeability.\textsuperscript{110} Antibiotic therapy is also a mainstay for treatment of antibiotic-associated diarrhea (AAD) in humans and treatment of chronic enteropathies in canine patients.\textsuperscript{110,114} In this study, we examined the effects of oral administration of a probiotic (PB) formulation of \textit{Akkermansia muciniphila} on markers of GIT permeability following two models of antibiotic therapy in healthy dogs.

We were able to detect \textit{A. muciniphila} in fecal samples of healthy dogs following supplementation, suggesting effective GIT transit. Fecal scores were higher (i.e. worse consistency, less formed stool) following metronidazole (MT) treatment than amoxicillin-clavulanate (CL) treatment. We did not find a difference in fecal scores following PB administration vs vehicle (VH) for either antibiotic. However, fecal score tended to improve with PB administration after MT treatment. In terms of biomarker evaluation, we found that cytokeratin-18 (C\textsubscript{K}-18) concentration was higher following PB administration in MT-treated dogs; the same impact was not observed in CL treated dogs. No impact of antibiotic or PB/VH treatment was noted on serum concentrations of LPS or GLP-1.

Lack of fecal \textit{A. muciniphila} detection at baseline, with detection following supplementation, suggests that \textit{A. muciniphila} does not naturally colonize the canine GIT. Post-supplementation detection confirmed successful GIT transit. Low quantification numbers suggest that colonization of the GIT by \textit{A. muciniphila} did not occur. Growth requirements for \textit{Akkermansia muciniphila} have been studied previously. Studies demonstrated strict anaerobic requirements and a narrow pH range for growth in culture.\textsuperscript{117,119} Given the fastidious nature of \textit{A. muciniphila} it is also possible that DNA degradation occurred either in the environment or GIT, precluding higher fecal
quantification. Colonization followed by subsequent degradation within the GIT cannot be completely excluded. While fecal sample collection is traditionally used to assess efficacy of PB supplementation, previous studies have shown that half of cells from samples collected in this manner may be dead (30%) or damaged (19%). This suggests that other methods, such as direct collection from the GIT, may yield significantly different results and could help to clarify any potential GIT colonization. The lack of detection in any dog at day 55 was surprising, given detection following other supplementation periods. Assessment of samples for house-keeping genes would have helped to clarify a discrepancy between a low-cellularity or poor quality (degraded) sample, unsuccessful DNA extraction, or overall PCR inhibition versus lack of A. muciniphila presence. Negative detection could have resulted from A. muciniphila cell counts below the level of detection or environmental/nutritional inhibitors, which can vary over time. In contrast, A. muciniphila was also detected by fecal PCR prior to direct PB administration, which could have resulted from environmental exposure to fecal material of other dogs. Although the dogs were housed in individual runs, indirect interaction or exposure during cleaning times cannot be excluded. This suggests some degree of environmental viability, which could correlate with recent work demonstrating low-level A. muciniphila growth in ambient air and microaerophilic environments, rather than strictly anaerobic environments, for up to 48 hours.

The finding of higher (worse) fecal scores following MT treatment compared to CL treatment was unexpected. MT is regarded as the treatment of choice in humans with Clostridium-associated AAD and a common therapy for chronic enteropathies in dogs. Although the baseline fecal scores were not equal between antibiotics, scores were higher prior to CL versus MT and improved with CL treatment but worsened with MT. This finding is in contrast to previous studies which showed improvement following MT treatment. That study, however,
used MT therapy in combination with prednisone and controlled studies on MT use for chronic enteropathies are lacking, with one study noting questionable improvement with MT treatment. Additionally, CL is the antibiotic most commonly associated with AAD in human medicine. This may be population-dependent as previous veterinary studies have documented no adverse clinical effects in healthy dogs administered amoxicillin, even with shifts in GIT microbiome diversity and fecal microorganism antibiotic-susceptibility patterns. It should be noted that extrapolation to a potentiated penicillin may not be appropriate, as the side-effect profile differs in human medicine.

The trend toward improved fecal scores in MT-treated dogs following PB therapy may suggest a positive impact of PB supplementation. As no additional signs of illness were noted, the disease model induced by MT may have been insufficient to demonstrate significant improvement following PB administration. Greater severity of AAD may have shown greater PB effect. Furthermore, no change in fecal score was noted in CL-treated dogs following PB therapy. Potentially, CL administration did not result in antibiotic-induced GIT disease in this population, so no additional beneficial effect was noted with PB supplementation.

As CK-18 comprises a filament component of epithelial cells, increased levels in systemic circulation are concluded to be markers of cellular damage. In models of chemotherapy toxicity, CK-18 concentrations increase in people experiencing dose-limiting toxicity to treatment. CK-18 concentrations and fragment levels have also been incorporated into scoring systems for survival prediction in acute liver failure and correlate with disease severity in forms of chronic hepatitis. Rodent models have also demonstrated increased cellular CK-18 expression with early hepatic regeneration and GIT epithelial cell proliferation; however, blood
concentrations have not been evaluated in these models. CK-18 has not been evaluated in the direct context of GIT disease. The increase in CK-18 following PB therapy in MT-treated dogs versus CL-treated dogs is interesting, as fecal scores improved in this population of dogs. The net effect of increased plasma CK-18 in our model could represent not only cell damage but reparative GIT cell turnover following epithelial insult. Alternatively, this finding could be due to direct impact of the PB on GIT epithelium, which would suggest a potential for negative systemic implications of *A. muciniphila* supplementation in dogs. As this increase was not seen following CL administration, results may represent cumulative damage of MT and PB administration, which was not able to be detected following MT alone.

The role of GIT GLP-1 and GLP-2 in gastrointestinal disease has not been explored previously in dogs. In other species, GLP-2 promotes GIT barrier function, increasing mucosal thickness, crypt depth, and villus height.\(^22\),\(^{136}\) GLP-1 and GLP-2 are co-secreted from enteroendocrine L-cells that are dispersed along the GIT epithelium, primarily within the ileum and colon. Secretion from L-cells is largely controlled by intraluminal nutrients but it is also affected by luminal signals, including GIT microorganism metabolic intermediates and by-products.\(^{17}\),\(^{18}\) Importantly, both GLP-1 and GLP-2 increase in mice following treatment with prebiotics, *A. muciniphila*, and mucin-degradation products of *A. muciniphila*.\(^{106,109,137}\) Therefore, we expected an increase in GLP-1 and GLP-2 in response to PB treatment in the dogs of this experiment. The lack of effect of PB administration on GLP-1/GLP-2 could have resulted from a of lack of viability of the bacteria or inability of live bacteria to colonize. It is also possible that *A. muciniphila* (or similar mucin-degrading bacterium) was not decreased in number following AB treatment and therefore already maximally stimulating GLP-1/GLP-2 release by L-cells, with lack of effect from additional PB numbers. Our hypothesis was that AB administration would reduce the numbers of
A. muciniphila and that PB administration would accelerate recovery. However, there is some recent evidence that Akkermansia spp. are susceptible to imipenem and resistant to MT,\textsuperscript{138} which could which could have implications on PB impact and subsequent biomarker effects, particularly if antibiotics are used concurrently or still affecting the GIT.

Treatment with GLP-2 in rodents with experimentally-induced IBD decreased histopathologic markers of mucosal damage;\textsuperscript{139} treated mice showed decreased GIT epithelial permeability\textsuperscript{20} and decreased chemotherapy-induced GIT toxicity.\textsuperscript{140} These findings make GLP-2 an attractive marker and potential therapeutic agent in dogs with GIT disease. GLP-2 concentrations were not successfully detected in our canine plasma samples. The GLP-2 ELISA used was designed to measure human and rat GLP-2. Although GLP-2 is 88\% conserved among humans and dogs,\textsuperscript{17} it is possible that canine GLP-2 was not detected by this ELISA or natural inhibitors existed in our population, preventing ELISA detection. Furthermore, sample timing was extrapolated from peak concentrations in pigs,\textsuperscript{141} and it is possible peak post-prandial release does not occur at the same time in dogs.

Systemically circulating endotoxin has been documented in a large percentage of humans with IBD, with levels correlating with clinical disease.\textsuperscript{30} Rodent models of metabolically-induced (i.e. obesity-driven) GIT hyperpermeability have also documented increased plasma LPS concentrations, with subsequent prebiotic administration and changes in GIT microbiota impacting systemic LPS concentrations.\textsuperscript{142} While we did not find an association with any oral interventions in this study, it is possible that our model did not induce significant changes to impact GIT permeability. Further evaluation in canine models of IBD, particularly protein-losing enteropathies, should be considered.
No adverse effects were observed directly related to PB administration, suggesting that short-term administration of *Akkermansia muciniphila* to healthy dogs is relatively safe. This requires further observation over a longer time duration and in a population of dogs with GIT illness.

There were several limitations to this study. First, this was a small population of dogs, so small effects in biomarker concentrations may not have been detected. The strain of *Akkermansia muciniphila* used has been naturally-detected in humans and mice and noted to have positive effects following administration to mice. However, genomic analysis has documented eight species of naturally-occurring *Akkermansia* in the human GIT, and differences exist among mammalian species. While *Akkermansia*-like species have been detected in the order Carnivora, the exact species in dogs has, to our knowledge, not been documented. This is important as the genus *Akkermansia* can be divided into 5 clades, with clade 2 containing *A. muciniphila*, as isolated from primates and mice. While clades 3-5 contain *Akkermansia* species isolated from both humans and other mammals, no species from clade 1 have been isolated from humans, and *Akkermansia* species in this clade have low structural and functional similarities to human isolates. While the lack of fecal detection at baseline could be an effect of collection method and environmental exposure, we suggest that *A. muciniphila* is not the naturally-occurring *Akkermansia* in dogs. Additionally, although we attempt to preserve viability by administering the PB product immediately after thawing and avoiding freeze-thaw cycles, we did not re-culture the samples from frozen vials to assess viability. While effects of non-viable PB formulations have been documented, previous studies suggest that *Akkermansia* must be viable to exert positive effects. Detection of *A. muciniphila* in some dogs prior to supplementation suggests viability, but studies demonstrating viability at atmospheric and microaerophilic conditions show
significantly reduced growth (25-1% at 24-48 hours, respectively) even when anaerobic conditions show >90% viability. Lowered viability could cause effects below limits of detection. Fecal sample timing and exposure to the environment may have also affected the ability to detect *A. muciniphila* via fecal PCR. Although it was attempted to collect fecal samples shortly after defecation, study design prevented immediate collection. Furthermore, samples collected in this manner do not necessarily represent the microorganism population in the upper GIT. We also cannot completely rule out lasting antibiotic effects on the microbiome at the baseline periods. Although a previous study of MT demonstrated a transient effect, with return to baseline microorganism populations in 30 days, a subsequent study demonstrated longer effects. GIT microorganism diversity was noted to approach baseline characteristics two weeks after cessation of oral treatment with amoxicillin in dogs. This is important if effects of our PB product were impacted by persistent AB effects, as noted above. Furthermore, this suggests consideration of PB-timing and prior treatments when using PB therapy for GIT disease. No fecal microbiome analysis was performed in this study. It is not possible to exclude effects on the local GIT environment that were not detected by systemic assessments. Microbiome effects may have different implications in various disease processes and conditions of naturally-altered GIT permeability, rather than the microbiome in healthy dogs. Our samples had significant inter- and intra-assay variability. Although double-centrifugation was performed on grossly hemolyzed or lipemic samples, some degree did exist, which may have affected evaluation of assay performance.

In conclusion, *Akkermansia muciniphila* was detected in fecal samples of healthy dogs following short-term oral supplementation, with no adverse side-effects. Oral administration resulted in increased plasma CK-18 in metronidazole-treated dogs, suggesting epithelial impact. Oral
administration of amoxicillin-clavulanate led to improved fecal scores in healthy dogs in comparison to metronidazole. Further study is needed to determine the optimal species/strain and dosing of *Akkermansia* for positive probiotic effects and for utility in canine patients with clinical GIT disease.
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randomized-controlled trial. *Journal of Veterinary Internal Medicine / American College of Veterinary Internal Medicine* 2010;24:269-277.


Appendix A: Probiotic Literature Reference Tables
<table>
<thead>
<tr>
<th>Study reference</th>
<th>Bacteria spp.</th>
<th>Study population</th>
<th>Sample type</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kainulainen (2015)</td>
<td><em>Lactobacillus acidophilus</em> LAB20</td>
<td>Research dogs</td>
<td>Canine mucous</td>
<td>Adhesion to mucous Decreased IL-8 production Increased transepithelial electric resistance</td>
</tr>
<tr>
<td>Schmitz (2013)</td>
<td><em>Enterococcus faecium</em> NCIMB 10418</td>
<td>Research dogs</td>
<td>Blood</td>
<td>Increased TNF-α No difference from flagellin stimulation</td>
</tr>
<tr>
<td>Ogue-Bon (2010)</td>
<td><em>Bifidobacterium bifidum</em> O245013</td>
<td>N= 3</td>
<td>Fecal</td>
<td>Decreased <em>Clostridia</em> Altered metabolomics: Increased SCFA Increased lactic acid</td>
</tr>
</tbody>
</table>

Table 1. Probiotic bacteria, sample type, study population, and effect in studies of healthy dogs (in-vitro).
Table 1 Continued

<table>
<thead>
<tr>
<th>Authors</th>
<th>Bacteria Culture</th>
<th>Species</th>
<th>Model</th>
<th>Model</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perelmuter</td>
<td>L. murinus</td>
<td>N= 1</td>
<td>Bacteria culture</td>
<td>Adhesion to mucous</td>
<td>Adhesion to glass</td>
</tr>
<tr>
<td>(2008)</td>
<td></td>
<td>LbP2</td>
<td>Canine mucous</td>
<td>Decreased growth with bile salts</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>LbP6</td>
<td></td>
<td>Viable at pH 2.5</td>
<td>Inhibition of Clostridia growth</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LbP10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lb02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biagi</td>
<td>L. acidophilus LA4</td>
<td>Research dogs</td>
<td>Fecal</td>
<td>Increased LAPB</td>
<td>Decreased: Enterococcus, Clostridium perfringens</td>
</tr>
<tr>
<td>(2007)</td>
<td></td>
<td>N= 2</td>
<td></td>
<td></td>
<td>Altered metabolomics: Decreased ammonia Increased lactic acid</td>
</tr>
<tr>
<td>Laukova</td>
<td>E. spp</td>
<td>Research dogs</td>
<td>Canine chyme</td>
<td>Adhesion probiotic-</td>
<td>Dependently No host effect</td>
</tr>
<tr>
<td>(2004)</td>
<td></td>
<td></td>
<td>Human mucous</td>
<td>dependent</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Porcine mucous</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rinkinen</td>
<td>B. lactis Bb12 L. pentosus SK2A L. rhamnosus GG Enterococcus faecium M47 E. faecium SF273</td>
<td>Research dogs</td>
<td>Mucous</td>
<td>Inhibition of Clostridium perfringens</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Coaggregation of L. rhamnosus, B. lactis, C. jejuni</td>
</tr>
</tbody>
</table>

Continued
Table 1 continued

| Rinkinen (2003) | Bifidobacterium lactis Bb12  
|                | Enterococcus faecium M47  
|                | Lactobacillus bulgaricus AT  
|                | CC11842  
|                | L. casei Shirota  
|                | L. johnsonii La1  
|                | L. rhamnosus GG  
|                | L. pentosus UK1A  
|                | L. pentosus SK2A  
|                | Research dogs  
|                | N= 6  
|                | Chyme  
|                | Adhesion superior with L. rhamnosus  
|                | No host effect  

| Rinkinen (2000) | Bifidobacterium lactis Bb12  
|                | Enterococcus faecium M47  
|                | Lactobacillus casei Shirota  
|                | L. johnsonii La1  
|                | L. pentosus UK1A  
|                | L. pentosus SK2A  
|                | L. rhamnosus GG  
|                | Research dogs  
|                | N= 6  
|                | Chyme  
|                | Adhesion superior with L. rhamnosus  
|                | Adhesion reduced in chyme  

<table>
<thead>
<tr>
<th>Study reference</th>
<th>Bacteria spp.</th>
<th>Dose (CFU per day)</th>
<th>Study population</th>
<th>Duration fed</th>
<th>Diet</th>
<th>Sample type</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nakamura (2015)</td>
<td><em>Bifidobacterium animalis</em></td>
<td>$10^{10}$</td>
<td>Privately owned N=5</td>
<td>14 days</td>
<td>Varied Commercial</td>
<td>Fecal</td>
<td>Effective transit No persistence Pathogen impact</td>
</tr>
<tr>
<td>Strompfova (2014)</td>
<td><em>Bifidobacterium animalis</em></td>
<td>$1.04x10^9$</td>
<td>Research N=10 probiotic N=10 control</td>
<td>14 days</td>
<td>Standardized Commercial</td>
<td>Blood Fecal</td>
<td>Effective transit Pathogen impact Metabolomic effects Serum biochemistry effects</td>
</tr>
<tr>
<td>Tang (2014)</td>
<td><em>Lactobacillus acidophilus</em></td>
<td>$1.7x10^8$</td>
<td>Privately owned N=5</td>
<td>3 days</td>
<td>Varied Commercial</td>
<td>Fecal</td>
<td>Effective transit</td>
</tr>
</tbody>
</table>

Table 2. Probiotic bacteria, dose and duration, study population, and effect in studies of healthy dogs.
Table 2 continued

<table>
<thead>
<tr>
<th>Study</th>
<th>Probiotic Species</th>
<th>Initial Count</th>
<th>Study Design</th>
<th>Course Duration</th>
<th>Delivery Route</th>
<th>Outcome Measures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tang (2014)</td>
<td><em>Lactobacillus acidophilus</em></td>
<td>1.7x10⁸</td>
<td>Privately owned</td>
<td>3 days</td>
<td>Varied</td>
<td>Fecal Effective transit</td>
</tr>
<tr>
<td>Delucchi (2014)</td>
<td><em>Lactobacillus murinus</em></td>
<td>5x10⁹ q48h</td>
<td>Privately owned N=7 probiotic N=6 control</td>
<td>14 days (8 doses)</td>
<td>Standardized</td>
<td>Fecal Immune response</td>
</tr>
<tr>
<td>Gonzalez-Ortiz (2013)</td>
<td><em>Bacillus amyloliquefaciens Enterococcus faecium</em></td>
<td>1x10⁸</td>
<td>Research N=8 probiotic N=8 control</td>
<td>39 days</td>
<td>Standardized</td>
<td>Fecal Effective transit Enterococcus persistence Pathogen decrease</td>
</tr>
<tr>
<td>Strompfova (2013)</td>
<td><em>Lactobacillus fermentum</em> synbiotic</td>
<td>0.1x10⁹/kg</td>
<td>Research N=12 probiotic N=12 synbiotic N=12 control</td>
<td>14 days</td>
<td>Standardized</td>
<td>Fecal Effective transit Prebiotic augmentation</td>
</tr>
<tr>
<td>Tang (2013)</td>
<td><em>Lactobacillus acidophilus</em></td>
<td>5.6-6.7x10⁶</td>
<td>Privately owned N=1</td>
<td>5 days</td>
<td>Commercial</td>
<td>Fecal Effective transit Persistence</td>
</tr>
<tr>
<td>Strompfova (2012)</td>
<td><em>Lactobacillus fermentum</em></td>
<td>2x10⁸ 1x10⁷</td>
<td>Research N=12</td>
<td>14 days 7 days</td>
<td>Standardized</td>
<td>Fecal Pathogen impact</td>
</tr>
</tbody>
</table>

Continued
<table>
<thead>
<tr>
<th>Study</th>
<th>Species</th>
<th>Dose</th>
<th>Source</th>
<th>Period</th>
<th>Mode</th>
<th>Site</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Garcia-Mazcorro</td>
<td><em>Bifidobacterium longum</em> <em>Enterococcus faecium</em> <em>Lactobacillus acidophilus</em> <em>L. bulgaricus</em> <em>L. plantarum</em> <em>L. rhamnosus</em> synbiotic</td>
<td>5x10⁹</td>
<td>Privately owned N= 12 synbiotic</td>
<td>21 days</td>
<td>Varied Commercial Fecal</td>
<td>Effective transit Microbiome impact No immune response No serum biochemistry effect</td>
<td></td>
</tr>
<tr>
<td>O-Mahony (2009)</td>
<td><em>Bifidobacterium animalis</em></td>
<td>1.5x10⁹</td>
<td>Research N= 11</td>
<td>42 days</td>
<td>Standardized Commercial Fecal</td>
<td>Variable pathogen impact</td>
<td></td>
</tr>
<tr>
<td>Biagi (2007)</td>
<td><em>Lactobacillus animalis</em></td>
<td>5x10⁸</td>
<td>Privately owned N= 9</td>
<td>10 days</td>
<td>Varied Commercial Fecal</td>
<td>Effective transit Pathogen effect</td>
<td></td>
</tr>
<tr>
<td>Manninenen (2006)</td>
<td><em>Lactobacillus fermentum</em> <em>L. mucosae</em> <em>L. rhamnosus</em> <em>L. salivarius</em> <em>Weisella confusa</em></td>
<td>2.8-11.8x10⁷</td>
<td>Research N= 5</td>
<td>7 days</td>
<td>Standardized Commercial Chyme</td>
<td>Survival in chyme No persistence (<em>L. fermentum</em> &amp; <em>L. mucosae</em>) Microbiome impact</td>
<td></td>
</tr>
<tr>
<td>Marcinakova (2006)</td>
<td><em>Enterococcus faecium</em></td>
<td>1x10⁹</td>
<td>Research N= 11</td>
<td>7 days</td>
<td>Standardized Commercial Blood Fecal</td>
<td>Commensal impact Pathogen impact Metabolomic fecal effects Serum biochemistry effects</td>
<td></td>
</tr>
<tr>
<td>Strompfova (2006)</td>
<td><em>Lactobacillus fermentum</em></td>
<td>3x10⁹</td>
<td>Research N= 15</td>
<td>7 days</td>
<td>Standardized Commercial Blood Fecal</td>
<td>Effective transit Persistence Serum biochemistry effects</td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>Strain/Species</td>
<td>Dose (Log)</td>
<td>Animal Type</td>
<td>N</td>
<td>Duration (Days)</td>
<td>Diet</td>
<td>Sample Type</td>
</tr>
<tr>
<td>---------------</td>
<td>---------------------------------</td>
<td>------------</td>
<td>-------------</td>
<td>----</td>
<td>----------------</td>
<td>------</td>
<td>-------------</td>
</tr>
<tr>
<td>Swanson (2002)</td>
<td><em>Lactobacillus acidophilus</em></td>
<td>2x10⁹</td>
<td>Research</td>
<td>5</td>
<td>28</td>
<td>Standardized</td>
<td>Fecal</td>
</tr>
<tr>
<td></td>
<td>synbiotic</td>
<td></td>
<td></td>
<td>5</td>
<td>28</td>
<td>Commercial</td>
<td>Fecal</td>
</tr>
<tr>
<td>Weese (2002)</td>
<td><em>Lactobacillus rhamnosus</em></td>
<td>1x10⁹, 1x10¹⁰, 5x10¹⁰, 5x10¹¹</td>
<td>Research</td>
<td>8</td>
<td>5</td>
<td>Standardized</td>
<td>Fecal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8</td>
<td>5</td>
<td>Commercial</td>
<td>Fecal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>5</td>
<td>Fecal</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>5</td>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>Biourge (1998)</td>
<td><em>Bacillus spp</em></td>
<td>7.4x10¹⁰</td>
<td>Research</td>
<td>5</td>
<td>7</td>
<td>Standardized</td>
<td>Fecal</td>
</tr>
<tr>
<td>Kanasugi (1997)</td>
<td><em>Enterococcus faecium</em></td>
<td>100 mg/kg</td>
<td>Research</td>
<td>5</td>
<td>1</td>
<td>Standardized</td>
<td>Blood</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>1</td>
<td>Commercial</td>
<td></td>
</tr>
<tr>
<td>Rochat</td>
<td><em>Enterococcus faecium</em></td>
<td>5x10⁸</td>
<td>Research</td>
<td>21</td>
<td>14</td>
<td>Standardized</td>
<td>Fecal</td>
</tr>
<tr>
<td>Study reference</td>
<td>Bacteria spp.</td>
<td>Dose (CFU per day)</td>
<td>Study population</td>
<td>Duration fed</td>
<td>Diet</td>
<td>Sample type</td>
<td>Effect</td>
</tr>
<tr>
<td>-----------------</td>
<td>---------------------------------------------------</td>
<td>--------------------</td>
<td>------------------</td>
<td>--------------</td>
<td>-----------------------</td>
<td>-------------</td>
<td>-----------------------------------------------------</td>
</tr>
<tr>
<td>Biagi (2013)</td>
<td><em>Bifidobacterium pseudocatenulatum</em> synbiotic</td>
<td>$10^{10}$</td>
<td>Privately owned N= 10</td>
<td>15 days</td>
<td>Standardized Commercial (N= 2)</td>
<td>Fecal</td>
<td>Effective transit No pathogen impact Metabolomic impact</td>
</tr>
<tr>
<td>Garcia-Mazcorro (2011)</td>
<td><em>Bifidobacterium longum</em> &lt;br&gt; <em>Enterococcus faecium</em> &lt;br&gt; <em>Lactobacillus acidophilus</em> &lt;br&gt; <em>L. bulgaricus</em> &lt;br&gt; <em>L. plantarum</em> &lt;br&gt; <em>L. rhamnosus</em> synbiotic</td>
<td>$5 \times 10^9$</td>
<td>Privately owned N= 12</td>
<td>21 days</td>
<td>Blood Fecal</td>
<td></td>
<td>Effective transit Microbiome impact No immune response No serum biochemistry effect</td>
</tr>
</tbody>
</table>

Table 3. Probiotic bacteria, dose and duration, study population, and effect in studies of healthy cats.
<table>
<thead>
<tr>
<th>Study reference</th>
<th>Bacteria spp.</th>
<th>Dose (CFU per day)</th>
<th>Study population</th>
<th>Diagnosis</th>
<th>Duration fed</th>
<th>Diet</th>
<th>Sample type</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schmitz (2015)</td>
<td>Enterococcus faecium synbiotic</td>
<td>2x10⁹</td>
<td>Privately owned dogs N= 7 synbiotic N= 5 control</td>
<td>Food responsive enteropathy</td>
<td>42 days</td>
<td>Standardized Hydrolyzed</td>
<td>Biopsy (intestinal)</td>
<td>Decreased severity No immune response</td>
</tr>
<tr>
<td>Gagne (2013)</td>
<td>Bacillus coagulans Enterococcus faecium Lactobacillus acidophilus synbiotic</td>
<td>10⁷ 10⁸ 10⁹</td>
<td>Privately owned dogs N= 9 synbiotic N= 8 control</td>
<td>Exercise induced diarrhea</td>
<td>42 days</td>
<td>Varied Commercial</td>
<td>Fecal</td>
<td>Effective <em>Lactobacillus</em> transit Pathogen impact Microbiome impact No metabolomics impact Decreased duration clinical signs</td>
</tr>
<tr>
<td>Rossi (2014)</td>
<td>Bifidobacteria, Lactobacillus, Streptococcus</td>
<td>112-225x10⁶/kg</td>
<td>Privately owned dogs N= 10 probiotic N= 10 drug N= 10 control</td>
<td>Inflammatory bowel disease</td>
<td>60 days</td>
<td>Varied</td>
<td>Biopsy (intestinal)</td>
<td>Decreased severity No decrease duration Immune response</td>
</tr>
<tr>
<td>Fenimore (2012)</td>
<td>Enterococcus faecium</td>
<td>10⁸</td>
<td>Shelter dogs N= 16 probiotic N=17 control</td>
<td>Undefined diarrhea</td>
<td>7 days</td>
<td>Standardized Commercial</td>
<td>Fecal</td>
<td>Decreased duration</td>
</tr>
</tbody>
</table>

Table 4. Probiotic bacteria, sample type, study population, diagnosis, and effect in studies of cats and dogs with gastrointestinal illness.
<table>
<thead>
<tr>
<th>Study (Year)</th>
<th>Probiotic Bacteria</th>
<th>Log Concentration</th>
<th>Strain Type</th>
<th>Treatment</th>
<th>Duration</th>
<th>Study Type</th>
<th>Outcome A</th>
<th>Outcome B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gore (2012)</td>
<td>Enterococcus faecium</td>
<td>$10^8$</td>
<td>Privately owned N= 13 probiotic N= 13 control</td>
<td>Exercise-induced diarrhea</td>
<td>7 days</td>
<td>Varied Commercial</td>
<td>Fecal</td>
<td>Decreased severity Decreased duration</td>
</tr>
<tr>
<td>Hart (2012)</td>
<td>Enterococcus faecium, Lactobacillus acidophilus, L. bulgaricus, L. casei, L. plantarum, Streptococcus salivarius</td>
<td>$5 	imes 10^9$</td>
<td>Privately owned cats N= 53</td>
<td>Chronic diarrhea</td>
<td>21 days</td>
<td>Varied Commercial</td>
<td>Fecal</td>
<td>Decreased severity</td>
</tr>
<tr>
<td>Kelley (2012)</td>
<td>Bifidobacterium animalis</td>
<td>$&lt;10^3$, $1.5 	imes 10^7$, $1.5 	imes 10^8$, $1.5 	imes 10^9$</td>
<td>Privately owned dogs N= 121</td>
<td>Stress-induced diarrhea</td>
<td>77 days</td>
<td>Standardized Commercial</td>
<td>Fecal</td>
<td>Effective transit No pathogen impact Decreased disease incidence</td>
</tr>
<tr>
<td>Bybee (2011)</td>
<td>Enterococcus faecium</td>
<td>$2.1 	imes 10^9$</td>
<td>Shelter animals N= 182 cats N= 217 dogs</td>
<td>Undefined diarrhea</td>
<td>Variable</td>
<td></td>
<td>Fecal</td>
<td>Decreased duration (cats) No duration impact (dogs)</td>
</tr>
<tr>
<td>Herstad (2010)</td>
<td>Bacillus subtilis, B. licheniformis, Lactobacillus acidophilus, Lactobacillus farcininis, Pediococcus acidilactici</td>
<td>$10^7$</td>
<td>Privately owned dogs N= 15 probiotic N= 21 control</td>
<td>Acute diarrhea and vomiting</td>
<td>Variable</td>
<td>Variable</td>
<td>Clinical Signs</td>
<td>Variable clinical response</td>
</tr>
</tbody>
</table>

Continued
Table 4 Continued

<table>
<thead>
<tr>
<th>Study</th>
<th>Organism</th>
<th>Dosage</th>
<th>Study Design</th>
<th>Disease Duration</th>
<th>Treatment Duration</th>
<th>Clinical Signs</th>
<th>Benefits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Czarnecki-Maulden (2009)</td>
<td><em>E. faecium</em></td>
<td>5x10^8</td>
<td>Research N= 8</td>
<td>Chronic diarrhea</td>
<td>25 days</td>
<td>Standardized Commercial</td>
<td>Clinical signs Decreased frequency</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>probiotic N= 7</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kelley (2009)</td>
<td><em>Bifidobacterium animalis</em></td>
<td>2x10^10</td>
<td>Privately owned dogs N= 13 probiotic N= 18 control</td>
<td>Acute diarrhea</td>
<td>Variable; 7 days maximum</td>
<td>Standardized Commercial (N= 2)</td>
<td>Clinical Signs Decreased duration</td>
</tr>
<tr>
<td>Simpson (2009)</td>
<td><em>Enterococcus faecium</em></td>
<td>10x10^8</td>
<td>Research N= 20</td>
<td>Chronic Giardia</td>
<td>7 weeks</td>
<td>Standardized Commercial</td>
<td>Fecal No disease impact No immune response</td>
</tr>
<tr>
<td>Pascher (2008)</td>
<td><em>Lactobacillus acidophilus</em></td>
<td>6x10^9/g food</td>
<td>Research N= 6</td>
<td>Chronic diarrhea</td>
<td>12 weeks</td>
<td>Standardized Fecal</td>
<td>Decreased frequency Fecal biochemical impact</td>
</tr>
<tr>
<td>Aktas (2007)</td>
<td><em>Saccharomyces boulardii</em></td>
<td>1000 mg/d</td>
<td>Research N= 25</td>
<td>Antibiotic induced diarrhea</td>
<td>10 days</td>
<td>Standardized Fecal</td>
<td>Prevention Decreased duration Metabolomic effects</td>
</tr>
<tr>
<td>Sauter (2006)</td>
<td><em>Lactobacillus acidophilus L. johnsonii</em> synbiotic</td>
<td>10^10</td>
<td>Privately owned dogs N= 11 probiotic N=10 control</td>
<td>Food responsive enteropathy</td>
<td>4 weeks</td>
<td>Standardized Elimination Biopsy</td>
<td>Fecal Decreased severity</td>
</tr>
<tr>
<td>Study reference</td>
<td>Bacteria spp.</td>
<td>Dose (CFU per day)</td>
<td>Study population</td>
<td>Diagnosis</td>
<td>Duration fed</td>
<td>Diet</td>
<td>Sample type</td>
</tr>
<tr>
<td>----------------</td>
<td>--------------</td>
<td>---------------------</td>
<td>------------------</td>
<td>-----------</td>
<td>--------------</td>
<td>------</td>
<td>-------------</td>
</tr>
<tr>
<td>Gabinaitis (2013)</td>
<td><em>Enterococcus faecium</em></td>
<td>$5 \times 10^5$</td>
<td>Research dogs N=12 probiotic N=12 control</td>
<td>Healthy puppies</td>
<td>3 days</td>
<td>Standardized Commercial</td>
<td>Blood Fecal</td>
</tr>
<tr>
<td>Arslan (2012)</td>
<td><em>Bifidobacterium breve</em> <em>B. longum</em> <em>B.infantis</em> <em>Lactobacillus acidophilus</em> <em>L. bulgaricus</em> <em>L. casei</em> <em>L. plantarum, Streptoccocus thermophilus</em></td>
<td>$4 \times 10^9$</td>
<td>Privately owned N=10 probiotic N=10 control</td>
<td>Parvovirus enteritis</td>
<td>1-3 weeks</td>
<td>Variable</td>
<td>Blood</td>
</tr>
<tr>
<td>Felix (2010)</td>
<td><em>Bacillus subtilis</em></td>
<td>$1 \times 10^{10}$/g food</td>
<td>Research dogs N=6 probiotic N=6 control</td>
<td>Healthy puppies</td>
<td>25 days</td>
<td>Standardized Commercial</td>
<td>Fecal</td>
</tr>
</tbody>
</table>

Table 5. Probiotic bacteria, sample type, study population, diagnosis, and effect in studies of puppies and kittens.
Table 5 Continued

<table>
<thead>
<tr>
<th>Study reference</th>
<th>Bacteria spp.</th>
<th>Dose (CFU per day)</th>
<th>Study population</th>
<th>Diagnosis</th>
<th>Duration fed</th>
<th>Diet</th>
<th>Sample type</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Czarnecki-Maulden (2007)</td>
<td><em>Enterococcus faecium</em></td>
<td>5x10^8</td>
<td>Research cats N= 31</td>
<td>Acute diarrhea (kittens)</td>
<td>1 year</td>
<td>Standardized Commercial Kitten</td>
<td>Blood Fecal</td>
<td>Decreased severity Decreased duration Commensal impact Pathogen impact Systemic immune response</td>
</tr>
<tr>
<td>Veir (2007)</td>
<td><em>Enterococcus faecium</em></td>
<td>5x10^8</td>
<td>Research cats N= 9 probiotic N= 9 control</td>
<td>Healthy kittens</td>
<td>20 weeks</td>
<td>Standardized Commercial Kitten</td>
<td>Blood Fecal</td>
<td>No clinical impact Immune response No pathogen impact</td>
</tr>
<tr>
<td>Benyacoub (2003)</td>
<td><em>Enterococcus faecium</em></td>
<td>5x10^8</td>
<td>Research dogs N= 7 treated N= 7 control</td>
<td>Healthy puppies</td>
<td>44 weeks</td>
<td>Standardized Commercial</td>
<td>Blood Fecal</td>
<td>Peripheral immune response</td>
</tr>
<tr>
<td>Czarnecki-Maulden (unpublished)</td>
<td><em>Enterococcus faecium</em></td>
<td>5x10^8</td>
<td>Research dogs N= 41</td>
<td>Healthy puppies</td>
<td>365 days</td>
<td>Standardized Commercial</td>
<td>Fecal</td>
<td>Commensal impact No pathogen impact</td>
</tr>
</tbody>
</table>

Table 6. Probiotic bacteria, sample type, study population, diagnosis, and effect in studies of non-gastrointestinal illness.
<table>
<thead>
<tr>
<th>Study (Year)</th>
<th>Probiotic Strains</th>
<th>Dosing</th>
<th>Study Subjects</th>
<th>Study Disease</th>
<th>Study Duration</th>
<th>Study Design</th>
<th>Sample</th>
<th>Outcome Measures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marsella (2012)</td>
<td><em>Lactobacillus rhamnosus</em></td>
<td>200x10⁹ 100x10⁹</td>
<td>Research dogs N= 2 adults N= 16 puppies</td>
<td>Atopic dermatitis</td>
<td>5 months; study 3 years after discontinuation</td>
<td>Standardized Commercial</td>
<td>Blood Skin</td>
<td>Decreased severity Variable immune impact</td>
</tr>
<tr>
<td>Rishniw (2011)</td>
<td><em>Lactobacillus acidophilus</em> <em>Bifidobacterium longum</em> <em>Streptococcus thermophiles</em> synbiotic</td>
<td>-----</td>
<td>Privately owned cats N= 10</td>
<td>Chronic kidney disease</td>
<td>2 months</td>
<td>Variable</td>
<td>Blood</td>
<td>No serum chemistry impact</td>
</tr>
<tr>
<td>Lappin (2009)</td>
<td><em>Enterococcus faecium</em></td>
<td>5x10⁸</td>
<td>Research cats N= 6 probiotic N= 6 control</td>
<td>Feline herpes virus-1</td>
<td>4.5 months</td>
<td>Standardized Commercial</td>
<td>Blood Fecal Oral cavity</td>
<td>Decreased frequency No microbiome impact No difference viral expression</td>
</tr>
<tr>
<td>Marsella (2009)</td>
<td><em>Lactobacillus rhamnosus</em></td>
<td>200x10⁹ 100x10⁹</td>
<td>Research dogs N= 2 adults N= 16 puppies</td>
<td>Atopic dermatitis</td>
<td>5 months</td>
<td>Standardized Commercial Adult Puppy</td>
<td>Blood Skin</td>
<td>No clinical impact Decreased skin response Immune impact</td>
</tr>
<tr>
<td>Palmquist (2009)</td>
<td><em>Bifidobacterium longum</em> <em>Lactobacillus acidophilus</em> <em>Streptococcus thermophiles</em> synbiotic</td>
<td>-----</td>
<td>Privately owned cats N= 7</td>
<td>Chronic kidney disease</td>
<td>3 months</td>
<td>Variable</td>
<td>Blood</td>
<td>Serum biochemistry impact</td>
</tr>
</tbody>
</table>