Lactobacillus salivarius L28 in dog kibble results in shifts in microbial indicators in pet fecal samples after feeding

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ABSTRACT

Certain probiotic lactic acid bacteria (LAB), also called Direct fed microbials (DFM), have beneficial effects in the gastrointestinal tract (GI) of pets through competitive exclusion of enteric-pathogen or by modulating the population of GI microflora, which can be monitored through the change in the load or shedding of microbial indicators such as: Generic *E. coli*, fecal coliforms, Entereobacteriaceae and *Salmonella* in fecal samples. Additionally, changes in the microbial populations can improve the overall gut health of the animal. The purpose of this study was to evaluate the impact of various combinations of probiotics including *Lactobacillus salivarius* L28 in the indicators during feeding.

A total of 47 apparently healthy and domesticated dogs from various households within Lubbock County of Texas were randomly assigned to each of the 4 feeding groups; i) Control (without probiotics); ii) L28 iii) L28 and commercial probiotics and iv) commercial probiotics. These dogs differed in breed, gender, and age. During the adjustment phase, the kibble without probiotics was fed to every dogs for 7 days irrespective of the groups assigned. On the 8^{th} day (Day 0 of the treatment phase) the three probiotic groups were given their respective treatment food, whereas the control group was continuously fed with the non-probiotic kibble. The group fed with L28 alone or the combined probiotics (L28 + commercial probiotics) received the L28 at a dose of 10^6 CFU/dog/day, based on average consumption. Fecal samples from each dog were aseptically collected and subjected to microbial enumeration on days 3, 2 and 1 before feeding probiotics (to generate a baseline of the microbial indicators), and on day 5, 10, 20 and 40 of the treatment phase (to assess the impact of probiotics). Simultaneously, the fecal samples were screened for *Salmonella* with Real- Time BAX kit followed by selective enrichment, selective plating, and agglutination test for confirmation, before and during treatment.

The group fed with combination of L28 and commercial probiotics showed a higher reduction in the fecal population of Generic *E. coli*, fecal coliforms, total-coliforms and, Entereobacteriaceae compared to the groups fed with the non-probiotic kibble, L28 or the commercial probiotics. Similarly, the group fed with combined probiotics had the highest

proportion of dogs with greater than 1-log cycle reduction in the four microbial indicators. Like the control group, the group fed with *Lactobacillus salivarius* L28 alone or the commercial probiotics did not exhibit a significant reduction ($p<0.05$) in the microbial indicators over-time. Whereas, the group treated with combination of L28 and commercial probiotics at the given dose of L28 showed a significant reduction ($P<0.05$) in the fecal population of the indicators compared to baseline. However, the efficacy of L28 + commercial probiotics to reduce the indicators in the fecal samples depended on the duration of the feeding. Similarly, the effect of the supplement and its duration varied between the subjects in the group. A significant reduction ($P<0.05$) in all the indicators in the group was observed after 20 days of feeding. There was a rare presence of *Salmonella* in the dogs under study during baseline.

The reduction in microbial indicators suggests the potential of L28 + commercial probiotics to reduce/control the growth of enteric-pathogens and modulate the balance of GI microflora towards healthier ones. This microbial shift could boost the gut health of the animal. Thus, L28 can be a promising component of the commercial probiotics formulation to add better functionality to kibbles for maintaining or improving GI health of pets and lowering incidence of zoonotic disease in humans.

Keywords: Direct-Fed Microbials, Probiotics, Indicators, *Lactobacillus salivarius* L28.

CHAPTER I
INTRODUCTION

Probiotics are viable micro-organisms that promote health of the host" (Food and Agriculture Organization of the United Nations, 2006). Researches have proved that probiotics provide both preventive and supportive care for pets. They are used in the prophylactic approach to diarrhea associated with antibiotics, and to treat diarrhea induced by antibiotic treatment. Probiotics have shown a positive effect on the immune system of pets (Hosbjerg et al., 2016). *Lactobacillus* spp., *Bifidobacterium* spp., or certain species of *enterococci* are commonly used as probiotics in commercially available products sold for use in animals (Kanwar et al., 2016). Currently, different brands of probiotic-supplemented pet-kibble have been commercialized. Each probiotic product may contain one or many species of bacteria. These supplemented kibbles are claimed to have positive effects on Gastro-intestinal health of canines and felines by competitively excluding the pathogen through decolonization or inhibiting their growth by producing inhibitory substances such as organic acids, hydrogen peroxide and bacteriocins and improving intestinal microbial balance (Hosbjerg et al., 2016). However, testing, detecting, and identifying a pathogen in GI tract or fecal samples is very expensive and impractical, so to monitor the efficacy of probiotic treatment on "indicators" are used.

If we consider the traditional definition, indicator micro-organisms are the organisms that suggest the possible presence of microbial hazard (Thomas et al., 2012) or pathogens (Berg, 1978). Both enteric pathogens and indicator organisms are excreted by warm-blooded animals, therefore detection of indicators, may suggests that pathogens could potentially be present (N.H. Department of Environmental Services (DES) 2003)). Indicator microbes are generally selected for their abundance in the matrix to be assayed, presence of relatively rapid, cost effective and accurate analytical method for enumerating the indicator (DES 2003). Usually these organisms are not pathogenic by themselves (EPA 2006) and are easily and rapidly detectable (Thomas et al. 2012). Ideal characteristics of indicator is that it should be more resistant to a treatment process, so that the reduction of the indicators would verify the reduction of the pathogen (Mary 2003).

Fecal coliforms, *E. coli*, Entereobacteriaceae and *Salmonella* are commonly used indicators and are normally present in the intestines and feces of warm-blooded animals. Entereobacteriaceae is a large family of bacteria which includes intestinal pathogens such as *Salmonella*, *E. coli*, *Yersinia pestis*, *Klebsiella* and *Shigella, Proteus, Enterobacter, Serratia* and *Citrobacter* and found in soil and water, as well as in plants and in animals, both vertebrates and invertebrates. The family is responsible for causing mostly intestinal upset or gastroenteritis (Clifford et al., 2012). Coliforms are members of genera or species within the family Entereobacteriaceae. They are facultative anaerobic organism which can readily grow at 37°C. Coliforms are gram-negative, non-spore forming, rod-shaped bacteria, can ferment lactose to produce gas and acid and possess β-galactosidase (HMSO, 1989, 1994). The fecal category of coliforms consists of both pathogen and nonpathogenic bacteria. Normally fecal coliforms dwell in the intestinal tract of warm-blooded animals and present in their feces (DES, 2003). They can grow at 44 °C and are not inhibited by the presence of bile salts (Clifford et al., 2012). *Escherichia coli* is one group of fecal coliform bacteria commonly found in the lower intestines of warm-blooded animals. Although, most of the strains are harmless but some serotypes can cause illness. The non-pathogenic strains are part of normal gut microflora and constitute about 0.1 % of the flora. *E. coli* provide beneficial effects on the host by producing Vitamin K, as well as reducing colonization of intestine by pathogens (Odonkor and Ampofo 2013). *E. coli* are excreted into the environment through feces of warm-blooded animals. They can survive outside the body making them suitable as indicator organism (Feng et al., 2002). *Salmonella* belong to the family Entereobacteriaceae. They are Gram-negative, nonspore-forming, motile and facultative anaerobic bacilli. *Salmonella* have been isolated from a variety of birds, reptiles, insects, and mammals. The genus *Salmonella* consists of only 2 species, *Salmonella* enterica and *Salmonella* bongori. S. enterica is further divided into 6 subspecies: *S. enterica ssp. enterica, S. enterica ssp. salamae, S. enterica ssp. arizonae, S. enterica ssp. diarizonae, S. enterica ssp. houtenae,* and *S. enterica ssp. Indica* (Marks et al. 2011).

Apart from the commercially used strains of probiotics, there may be many other strains or species of LAB that could hold a potential to be used as probiotics for dogs. Among them *Lactobacillus Salivarius* could be the one with such potential. The strains of L salivarius are mostly used in poultry and swine (Chaves et al. 2017) and are considered

safe to be used in the animals and environment (EFSA, 2012). L28 is a recently identified strain of *L. salivarius* with antagonistic effect towards *Salmonella, Escherichia coli and Listeria monocytogenes* (Campos 2016). Therefore, a study was conducted in apparently healthy dogs to evaluate the efficacy of various combination of probiotics including L28 to reduce the generic *E. coli*, fecal coliforms, total coliforms, Entereobacteriaceae, and shedding of *Salmonella* in the fecal of dogs. We hypothesized that feeding dogs with kibble supplemented with L28 alone or in combination with commercially available probiotics will significantly decrease generic *E. coli*, fecal coliforms, total coliforms, Entereobacteriaceae, and shedding of *Salmonella* in fecal samples of dogs.

The specific objectives of this study were to:

1. Define the baseline population of Generic *E. coli*, fecal coliforms and Entereobacteriaceae, and fecal shedding of *Salmonella* in the dog population included in this research.
2. Determine the fecal shedding of *Salmonella* in the dog population included in this research.
3. Determine the impact of various combinations of probiotics including L28 in the Generic *E. coli*, fecal coliforms, total coliforms, and Entereobacteriaceae, during feeding.
4. Evaluate the impact of various combinations of probiotics including L28 in fecal shedding of *Salmonella* during feeding.

CHAPTER II
LITERATURE REVIEW

The GI bacterial microbiome of dogs

The term 'microbiota' is described as "the ecological community of commensal, symbiotic and pathogenic microorganisms found in our body space," and the term 'microbiome' refers to the complete genetic mass ('genome') of corresponding microorganisms or microbiota. These microorganisms include archaea, eucaryotes, bacteria, viruses, and fungi (Silke and Jan 2016). Studies in human population are the source of most of the currently available information on the composition and activity of the GI microbiota and corresponding microbiome. However, there are several investigations on intestinal microbes in dogs (Suchodolski, 2011).

The GI microbiota of dog is composed of Eukaryotes Archaea, and Bacteria. Among these three domains, bacteria are present abundantly and are metabolically active (Garcia-Mazcorroa and Minamoto, 2013). The composition and bacterial populations differ between sites of the intestine (Momozawa et al., 2011). Along the GI tract, populations of bacteria and their diversity increase gradually (Suchodolski et al., 2005). A healthy dog has diverse microbiota in its stomach and 454-pyrosequencing has identified at least 4 phyla (Garcia-Mazcorro et al., 2012a). Total bacterial population present in the stomach of a healthy dog is approximately 10^5 $\log_{10}$ 16S rRNA copy numbers. 99.6 % of the bacterial population belongs to Proteobacteria and 0.3 % is occupied by Firmicutes (Garcia-Mazcorro et al., 2012). Molecular techniques have revealed the presence of bacterial phyla; Firmicutes (47.7%), Proteobacteria (23.3%), Fusobacteria (16.6%), and Bacteroidetes (16.6%) in the intestinal tract of dogs. The results from the molecular studies shows that the proportion of the bacterial phyla is different in each of the intestinal compartment. Duodenum and jejunum contains more than 50% Firmicutes, while the 30% of this phylum is present in ileum and colon. Fusobacteria, Bacteroidetes and Firmicutes (around 30% each) are co-dominant phyla in the colon of healthy dogs followed by Clostridiales (18%). Presence of Proteobacteria are in low proportions (1.4%), whereas the presence of Lactobacillales are like the level in the jejunum (10%) (Suchodolski et al.,

2008). At least ten bacterial phyla have been reported in the jejunum of dogs (Suchodolski et al., 2009). Similarly, the canine fecal microbiome has predominant presence of the phyla Fusobacteria (24–40%), Bacteroidetes (32–34%), Firmicutes (15–28%), Proteobacteria (5–6%) and Actinobacteria (0.8– 1.4%) (Suchodolski et al., 2009; Swanson et al. 2011; Garcia-Mazcorro et al., 2012).

The composition of GI microbiome of dogs plays an important role in determining the health status of dogs. When certain pathogens invade and colonize the GI tract of these animals, the integrity of the epithelial barrier of the intestine is disturbed (Viswanathan et al., 2009). These potential pathogens commonly include *Salmonella* spp., *Clostridium perfringens*, and *E. coli* (Marks et al., 2002). Since these pathogens are also commensals of GI tract, and their isolation at a similar rate from healthy and diseased dogs (Marks and Kather 2003; Unterer et al., 2014; Busch et al., 2015) it becomes necessary to evaluate the cause and effect relationship between GI disease and the organisms.

Acute or chronic GI disease in dogs may result from the non-specific alterations of the microbial community. There are several attempts made by researchers to analyze the characteristics of the fecal microbial composition in diarrheic dogs. Bell et al., (2008), Minamoto et al., (2014b) and Guard et al., (2015), observed a large-scale change in acute diarrhea, while using both culture and sequencing techniques. Researchers reported an increase of *E. coli*, *Clostridium* spp., *Lactobacillus* and *Enterococcus* spp., and at the same time, reductions of normal colonic microbiota consisting of *Faecali bacterium*, *Ruminococcaceae* and *Blautia* spp. Whereas in chronic diarrhea, there was an increase of Bacteroides sp. (Jia et al., 2010) and decreased abundances of Fusobacteria, Ruminococcaceae, Blautia spp. and *Faecalibacterium* spp. Similarly, Minamoto et al., (2014b) observed higher *Bifidobacterium* spp., *Lactobacillus* spp. and *E. coli* counts in comparison to healthy dogs. The change in microbial composition has been extensively studied in dogs with Inflammatory Bowel Syndrome (IBD). Significant reduction in species richness and increase in Entereobacteriaceae has been observed in duodenal brush samples than healthy dogs (Xenoulis et al., 2008). Duodenal biopsies of dogs with IBD has revealed an abundance of Proteobacteria and lower number of *Clostridia* (Suchodolski et al., 2010). Similarly, when the fecal samples of dogs with IBD were analyzed, dysbiosis

was seen. Bacterial diversity was significantly lower in the feces, accompanied by an increase in *E. coli* and reduction in *Clostridia* and *Bacteroidia*. It was hypothesized that these bacterial changes could be due to alternation of metabolic functions of the microbiota such as modified amino acid metabolism, changed in redox equilibrium, change in bile acid metabolism and reduction in Short-chain fatty acids (SCFA) concentrations. These changes could potentially worsen the inflammatory state of the host (Minamoto et al., 2014a).

Enteropathogenic Bacteria in Dogs and their Zoonosis

The primary enteropathogenic bacteria in dogs include *Clostridium perfringens, Clostridium difficile, Salmonella* spp., *Campylobacter* spp., and *Escherichia coli* and are associated with self-limiting diarrhea. Among these enteropathogens, *Salmonella* and *Campylobacter* are well-documented zoonoses that may be present in the GI tract or shed in the feces of the animal and spread to humans and the environment (Marks et al., 2011).

Clostridium difficile

Clostridium difficile is a Gram-positive, fastidious, anaerobic, and spore-forming bacillus. It is responsible for 10–21% of cases of diarrhea in dogs in the general population. In some cases, *C. difficile* are also found to be involved in acute hemorrhagic diarrheal syndrome in dogs (Cave et al., 2002), but the causation has not been proven. Prevalence of *C. difficile* is 0–58% of healthy, non-diarrheic young dogs that visit human hospitals (Cave et al., 2002). Fecal shedding of *C. difficile* is variable and transient. The shedding is due to frequent exposure from food or the environment or short-term colonization. Currently, the risk of zoonotic transmission *C. difficile* is unclear and it is wise to consider this pathogen as potentially zoonotic (Weese et al., 2010). (Marks et al., 2011). (Cave et al., 2002),

Clostridium perfringens

Clostridium perfringens inhabits the GI tract of humans and animals and is among the most widespread pathogenic bacteria. *C. perfringens* is found normally in canine intestinal microflora. It has been cultured readily from greater than 80% of diarrheic and non-diarrheic dogs (Mark et al., 2002). Diarrhea associated to Canine *C. perfringens* is usually caused by *C. perfringens* enterotoxin (CPE). The enterotoxin is found in up to 34%

of diarrheic dogs, and in 5–14% of non-diarrheic dogs. This is more likely due to disruption of the intestinal microenvironment (Mark et al., 2002).

Enteric *Escherichia coli*

Escherichia coli are part of the normal intestinal microflora (Marks et al., 2011). They are Gram-negative, non-spore-forming rods that belongs to the family Entereobacteriaceae. *Escherichia coli* can be associated with gastroenteritis when bacterial factors of virulence are present and systemic or local immunity is impaired (Marks et al., 2011). There are 7 pathotypes currently being identified which include enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), necrotoxigenic *E. coli* (NTEC), enteroinvasive *E. coli* (EIEC), enteroaggregrative *E. coli* (EAEC), and adherent-invasive *E. coli* (AIEC) strains (Sancak et al., 2004). Dogs with and without diarrhea are important reservoirs of many strains of *E. coli* but the involvement of many of these strains in disease causation in dogs is poorly known. In susceptible dog breeds, evidence has been found supporting the role of AIEC strains (Sancak et al., 2004).

Salmonella

Salmonella belongs to the family Entereobacteriaceae. They are Gram-negative, non-spore-forming, motile and facultative anaerobic bacilli. *Salmonella* have been isolated from a variety of birds, reptiles, insects, and mammals. The genus *Salmonella* consists of only 2 species, *Salmonella enterica* and *Salmonella bongori*. *S. enterica* is further divided into 6 subspecies: *S. enterica* ssp. *enterica*, *S. enterica* ssp. *salamae*, *S. enterica* ssp. *arizonae, S. enterica* ssp. *diarizonae, S. enterica* ssp. *houtenae*, and *S. enterica* ssp. Indica (Marks et al., 2011). *Salmonella* is a pathogen for dogs and the infectious dose determines the ability of different *Salmonella* strains to cause disease. *Salmonella* has been involved in various foodborne outbreaks affecting both humans and animals (Marks et al., 2011). Feeding dogs with raw meat have tremendously increased the risk of infection in humans by *Salmonella* (Lefebvre et al., 2008). Similarly, contact with contaminated dry dog food also has been a common cause of *Salmonella* infections in people (Behravesh et al., 2010).

Over the years, the prevalence of *Salmonella* has been widely studied, and these studies have reported the prevalence to range from 0 to 3.6%. in healthy dogs (Fukata et al., 2002), 0-3.5 % in diarrheic dogs (Weber et al., 1995) and 0-51 % in stray or shelter

dogs (Kocabiyik et al., 2006). Lowden et al., (2015) investigated 436 apparently healthy dogs without diarrhea within the Midlands region of the United Kingdom. They reported the feces from one dog (0.23 %; 95 % confidence limit 0.006 %, 1.27 %) as positive for *Salmonella*. Similarly, studies have estimated the prevalence of subclinical carriage of *Salmonella* in clinically healthy dogs from the UK to range from 0 to 44 % (Carter and Quinn, 2000). A study carried on 1,391 dogs across Trinidad reported a prevalence of 3.6 % (Seepersadsingh et al., 2004.) In addition, investigations conducted in Tehran, Iran, and Florida, USA reported a prevalence of 4.4 % (21 of 474 dogs) and 15 % (n = 1,626), respectively (Galton et al., 1952). The geographical variation in the prevalence of *Salmonella* in dogs can be explained by taking into consideration the factors such as sample size, year of sampling, sampling strategies, isolation methods performed, and favorable climate conditions for bacterial growth and survival (Lowden et al., 2015).

Studies have shown higher prevalence of *Salmonella* in dogs that are fed raw food diets. In a study on greyhounds that were fed a raw chicken diet, researchers isolated *Salmonella* from 80% of the diet samples and 30% of the stool samples (Joffe and Schlesinger, 2002). The prevalence rate of *Salmonella* was evaluated in dogs in Aba city, Nigeria. Nwiyi (2014) sampled the rectal swabs of 125 dogs from five different veterinary clinics located in Aba. The researcher reported a significant prevalence of *Salmonella* ($P > 0.05$) ranging from 4% to 16% which was unacceptable and suggested the meat fed to the dogs to be cooked adequately and to discourage scavenging.

Various methods are being currently used to detect *Salmonella* in feces or food samples, although it is difficult to determine the best method. The two mostly used methods include; Culture method and molecular method. For culture method, commercially available media such as, Xylose Lysine Deoxcycholate (XLD) agar, Xylose Lysine Tergotol 4 (XLT4) and Brilliant Green agar are used. *Salmonella* detection and confirmation steps involve pre-enrichment, selective enrichment (Rappaport Vasiliadis broth or Tetrathionate broth) and selective agar plating. The presumptive *Salmonella* colonies are further identified through biochemical techniques and discriminated by serotyping (Marks et al., 2011). Molecular techniques or PCR assays detect *Salmonella* in a variety of different matrices, from water to human stool samples and is a rapid and non-

laborious process. This technique requires overnight enrichment in a nonselective broth, followed by selective enrichment for isolation and identification. This method requires, 3 consecutive cultures to be negative, to achieve 90% confident that the fecal sample is truly negative and for 99 % confident 6 cultures is required (Marks et al., 2011).

Direct-fed Microbials for animals

Direct-fed microbials (DMF) are defined as supplements that can inhibit gastrointestinal infection and provide a controlled or regulated environment for microbials in the digestive tract (Ja Kyeom Seo et al. 2010). Microorganisms such as lactic acid producing bacteria (LAB), lactic acid utilizing bacteria (LUB), and yeast products containing *Saccharomyces* and *Aspergillus* have been used as DMF (Ja Kyeom Seo et al., 2010). The mode of action for DMF is yet to be clarified. The way in which DMF may function includes: maintaining the presence of beneficial commensal microorganisms in the gut and balancing the population of diverse microfloral species (Reid and Friendship 2002), competitive exclusion of pathogenic bacteria, and stimulate immune function (Ng et al., 2009). Yeast DFM are found to reduce harmful oxygen, prevent excess lactate production, increase feed digestibility, and improve fermentation in the rumen. LAB could reduce incidences of diarrhea and improve weight gain and feed efficiency. Similarly, LUB may improve weight gain in young animals (Ja Kyeom Seo et al., 2010). Researchers have reported the improvement of dry matter intake, milk yield, and milk fat content in mature animals from the use of DMF. Spore forming bacteria, due to their convenience of preparation and effectiveness of delivering DMF to target organs, are being used as DFM strains (Ja Kyeom Seo et al., 2010).

Recent studies have supported the positive effects of DFM on animal performance. Abu-Tarboushetal (1996), fed *L. acidophilus* 27SC to calves and established the beneficial effect of lactobacilli to reduce the incidence of diarrhea. The researcher hypothesized that the decreased incidence of diarrhea could be due to consistent increased shedding of *Lactobacillus* and decreased coliform's shedding (Bruce et al., 1979) in feces caused by the *Lactobacillus* treatment. When new beef calves enter a feed and undergo different types of stress such as recent transport, vaccination, weaning, dehorning and, castration, such stress could decrease the calf's performance and increase morbidity (Williams and

Mahoney 1984) as these stresses might change the gut microbial population. Several research trials conducted in the early to mid-1980s to evaluate the efficacy of combining bacterial DFM containing live cultures (*L. acidophilus, L. plantarum, L. casei,* and *S. faecium*) on the performance of beef calves reported 13.2% increase in daily gain, 2.5% increase in feed consumption, and a 6.3% improvement in feed: gain (Krehbie et al., 2003). Similarly, Gill et al., (1987) reported a 9.3% increase in daily gain, 9.5% improvement in feed efficiency, and a 10.9% reduction in morbidity when a bacterial DFM was fed during a 28-d receiving period.

Prevalence of *E. coli* O157:H7 has been found to be high in feedlot cattle and their feces. Ohya et al., (2000) conducted a study on the effect of DFM supplemented with lactic-acid producing *Streptococcus bovis* LCB6 and *L. gallinarum* LCB 12 and found reduced fecal shedding of *E. coli* O157:H7 from cattle. There have been numerous studies with DMF at Texas Tech University as pre-harvest interventions to control-food borne pathogens at the feedlot. A feeding trial was conducted by Brashears et al., (2003a) to evaluate the efficacy of *Lactobacillus*-based DFM fed on feedlot cattle. For the study 180 steers of British breed were bought from a single source. The steers were separated into one of three treatment groups and fed a 90 % concentrate diet for an average of 70 days. Two different strains of *Lactobacillus* were used as DFM and their efficacy was investigated. The fecal samples were collected from the steers, prior to shipment and the presence of *E. coli* O157 was analyzed, which helped to generate baseline *E. coli* O157 carriage for each treatment group. The results showed that the *L. acidophilus* NP51 treatment was able to lower the fecal shedding of *E. coli* O157 in treated animals by 75%, when compared to the controls and the treatment had no detrimental effects on the performance of animals.

Pond et al., (2013) conducted an evaluation of the impact of NP51 on the reduction of *E. coli* O157:H7 and six non-O157 O serogroups in cattle feces. The study was sub-divided into commercial feedlot, and research feedlot. The commercial feedlot consisted of approximately 1,800 cattle blocked by weight and randomized into treatment and control pens. The treatment pens were fed with NP51. Similarly, research feedlot consisted of 112 heads of cattle randomly assigned to treatment and control pens at the Texas Tech

University Research feedlot. Fecal were taken from each pen before the animals were transported to slaughter. The samples were analyzed for *E. coli* O157, and the "Big 6" non-O157 serogroup (*E. coli* O26, O45, O103, O111, O121 and O145). When the results were analyzed, a significant (44 %) reduction of *E. coli* O157 in fecal samples was observed when comparing control groups in the commercial feedlot. The results of the "Big Six" Non-O157 serogroups, indicated a reduction in O serogroups O26, O45, O103, and O121 with a reduction of 52.7% ($P = 0.02$), 36.0% ($P = 0.001$), 33.0% ($P = 0.03$), and 42.0% ($P = 0.02$), respectively. Similarly, a significant (60%) reduction of *E. coli* O157 among the treatment cohort compared to the controls and a numerical reduction in O serogroups O26, O45, O103, O111 and O145 was observed in the research feedlot. The results allowed the researchers to conclude that NP51 could serve a potential pre-harvest food safety intervention.

Wolfenden et al., (2011) evaluated isolates PHL-MM65 (a *Bacillus laterosporus*) and PHL-NP122 (a *Bacillus subtilis*) and were evaluated using poultry raised under commercial conditions. The study reported that Bacillus isolates PHL-NP122 and PHL-MM65 resulted in a significant reduction ($P \leq 0.05$) in the frequency of *Salmonella* by more than 25% in the treated poultry compared with the controls. A study was conducted by Lascano et al., (2014) to determine the dose effect of live yeast culture (YC) on rumen fermentation of heifers and total cell concentrations of microbes in precision-fed dairy heifers. In the experiment, the heifers were exposed to different rapidly fermented carbohydrates diets. The results indicated diet dependency between readily available carbohydrates source and YC addition in dairy heifers. There was an increase of viable, non-viable, and total fluid-associated bacteria, particle-associated bacteria, and total bacteria. When YC dose was increased linearly beyond 10 g $d^{-}1$, there was a decrease in viable and total fluid-associated bacteria.

Mathew et al., (1998) conducted a phase feeding program involving weaning pigs to evaluate the effects of a *Saccharomyces cerevisiae* culture on performance, ileum microflora, and short-chain fatty acids in the pigs. A total of 36 pigs were cannulated at 12 d of age and weaned at 17 d of age. The pigs were then randomly assigned to one of 3 treatments: 1) a pelleted phase feeding program, 2) a similar program with the inclusion of

a live *S. cerevisiae* culture (1 g/ kg), and 3) a non-pelleted feeding program. The pigs fed the yeast diet had higher total intake and greater overall gains than the pigs fed the control diet. Researchers concluded that supplementing the diets of weaning pigs with live yeast affects performance and intake but doesn't alter net concentrations of fermentation products and intestinal microflora.

Probiotics as Direct-fed microbials in pet food

Probiotics are defined as "Viable micro-organisms when administered in adequate amounts, promote, or confer a health benefit on the host" (Food and Agriculture Organization of the United Nations, 2006). Researches have proved that probiotics provide both preventive and supportive care for pets. They are used in the prophylactic approach to diarrhea associated with antibiotics, and to treat diarrhea induced by antibiotic treatment. Probiotics have shown a positive effect on the immune system of pets (Hosbjerg et al., 2016). *Lactobacillus* spp., *Bifidobacterium* spp., or certain species of *enterococci* are commonly used as probiotics in commercially available products sold for use in animals (Kanwar et al., 2016). Lactic acid bacteria (LAB) belongs to the order *Lactobacillales* and includes environmental organisms, commensals of humans and animals, members of plant microbiota, and opportunistic/ obligate pathogenic organisms (G€anzle et al., 2015).

Probiotics are already present in the intestinal system of all animals and are required for the digestion of nutrients and the balance of the intestinal microflora, as it is important for optimal health, since 70% of the immune system originates in the digestive tract. There are several possible modes of action of probiotics depending on the strain of bacteria. Competition for nutrients and adhesion sites, alteration of microbial metabolism, stimulation of the immune system, and direct antimicrobial effect are some of the actions pertinent to probiotics (Hosbjerg et al., 2016).

Based on the increased awareness of probiotics and their health benefits, several pet foods have emerged in the market. Each probiotic product may contain one or many species of bacteria. It is important to consider the difference in potential of each bacterium when selecting probiotics for potential use (Lefebvre et al., 2011). The in-vivo effects of bacteria genus may be different, even though they show similar behavior in laboratory conditions. Whenever probiotic supplements are used in animal feed or human food, one

must ensure that they are non-pathogenic, non-toxic, and unable to transfer antibiotic resistance and instead maintain genetic stability.

According to FAO/WHO definition of probiotics, viability is an essential element of probiotics (Lefebvre et al., 2011). In the United States, probiotic products are commercially available in many forms such as granules, liquids, powders, pastes, liquids, tablets, and capsules. The organisms should be able to survive formula preparation. The challenge for survival is greater when probiotics are incorporated into pet food due to their manufacturing processes. Most commercial pet food that contains probiotics are a dry and kibble variety. Organisms are added to the food after the heating process (Lefebvre et al., 2011). A study was conducted to assess the possibility of including probiotic *Bacillus CIP* 5832 in a commercial dry dog food (Biourge, 1998). The researchers incorporated the bacterium into dog food prior the production steps that involves heat. During the manufacturing process of the kibble, > 99 percent of viable bacterial spores were lost, but when the organism was coated onto the kibble, only 60 percent or less of the spores' viable spores remained afterward. *L. acidophilus* was later added by a post extrusion coating process during production (Baillon et al., 2004).

The viability of probiotics is affected when they pass through the GI tract. The probiotics must reach the intestinal tract in adequate number, so they should be able to survive the transit time through the acidic environment of the stomach and survive in the presence of bile (Hosbjerg et al., 2016). For a probiotic to be successful, it should colonize the GI tract when the pre-existing microflora are present (Tuomola (2001), Bezkorovainy (2001) and Strompfova (2004)). There is evidence that suggests that certain probiotics can survive GI transit in dogs. Weese (2002) conducted an experiment where varying doses of *Lactobacillus rhamnosus* strain GG (LGG) was given to groups of healthy and adult Beagles. A commercially available freeze-dried form of LGG was mixed in with canned dog food. The food was given to the dogs for 5 days (once every day). Fecal samples were collected up to 11 days after treatment for isolation of LGG to evaluate the survival and colonization of LGG in the GI tract. According to the obtained results, LGG was able to survive passage through the GI tract. The researcher suggested the use of strains of canine origin, as the strains are adapted to GI environment of canines and thus can colonize the

gut better than human strains of bacteria (Lefebvre et al., 2011). A similar study was conducted on healthy dogs with *Bacillus* CIP 5832 (Biourge et al., 1998) and *L. acidophilus*. The outcome of the research suggested that colonization achieved was temporary and depends on regular probiotic consumption. So, to maintain colonization, probiotics should be consumed continuously. However, no studies to evaluate the effects of consuming probiotics for the long-term in dogs are available. Studies have reported changes in microfloral diversity and/or transient colonization in healthy dogs for *L. animalis*, *L. fermentum*, *L. salivarius*, *Weissella confuse*, *L. rhamnosus*, *L. mucosae*, *Bifidobacterium animalis* AHC728 and *E. faecium* SF68 (Lefebvre et al., 2011).

If we refer to available literature on the benefits of probiotics for improving the health in dogs, most of the studies are conducted in controlled experimental conditions. With such controlled conditions, one can take control over factors such as differences in ages, breeds, and management practices that may affect the efficacy of a probiotic under study. Any observed effects can be accredited to the probiotic with confidence than performing trials in less controllable environments or real-life conditions. In controlled conditions, researchers can examine and evaluate a probiotic in the animals for its intended purpose (Lefebvre et al., 2011). Such controlled studies have reported the positive effect of feeding probiotics alone and in combination on the behavior of canines and immunomodulation, as well as the efficacy of the probiotic to treat various forms of GI abnormalities caused by pathogens.

In a study conducted over 10 months, 8-weeks old puppies of various breeds received one of two kibble diets. In this study, only one kibble had a commercially available strain of *E. faecium* SF68. The puppies under study were given vaccinations against canine distemper at 9 and 12 weeks. Results showed a higher plasma concentration of CDV-specific IgA and IgG in puppies that received the probiotic compared to puppies that did not receive the probiotic supplement, which suggested that canine distemper could be prevented effectively in puppies treated with probiotics (Benyacoub et al., 2003). Similarly, a study was conducted to evaluate the efficacy of three strains mixture of *Lactobacillus* (*L. acidophilus* NCC2628 and NCC2766 and *L. johnsonii* NCC 2767 in influencing cytokine production in dogs suffering from chronic enteropathies. The

evaluation was performed ex vivo, and specimens from duodenum were cultured from both affected and controlled dogs. In affected dogs, the expression of pro-inflammatory cytokine (TNF-α, IFN-γ and IL-12p40) was low compared to regulatory cytokine expression (IL-10), which suggested that this combination of bacteria may be promising to treat dogs with chronic enteropathies. The researchers were unable to show a similar immunomodulation effect when they conducted other trials involving the same probiotic cocktail, as they did not perform any in vitro studies following in vivo trails before they forwarded their conclusions (Saunter et al., 2005).

In a study that involved the feeding of kibble coated with *L. acidophilus* to healthy dogs, there was a decreased volume of clostridia and an increased volume of lactobacilli in the feces of dogs. Similarly, there was an increase in various hematologic parameters that includes monocytes, neutrophil, red blood cell (RBC) counts, and a decrease in the fragility of RBC in the dogs studied. However, these findings were not evaluated for their clinical importance (Baillon et al., 2004). A study involving *B. animalis,* reduction in production of abnormal fecal within a short time was observed in dogs suffering from acute idiopathic diarrhea and fed with probiotics. Diarrhea in the dogs fed with the probiotics resolved in 3.9 days compared to 6.6 days for dogs fed a placebo. Similarly, only 5 treated dogs versus 9 control dogs required metronidazole treatment, suggesting the usefulness of *B. animalis* to treat acute idiopathic diarrhea (Kelly et al. 2009).

A small laboratory-based study was conducted on six German Shorthair Pointers with non-specific dietary sensitivity. These dogs were fed with a *L. acidophilus* DSM13241 coated diet. The feeding enhanced quality and frequency of defecation, however the effect on the concentration of fecal microflora, including *Clostridium perfringens*, *Escherichia* spp., *Lactobacillus* spp. and *Bifidobacterium* spp was not significant (Pascher et al., 2008).

Attempts have been made to manipulate GI microbiota of dogs by using probiotics to enhance their health. Unfortunately, few studies have fully evaluated the properties and effects of probiotics on the intestinal microbial ecosystem, and most of the studies are carried out only in selected intestinal bacterial groups (Strompfová et al., 2004, Cutrignelli et al., 2009) and in vivo (Vanhoutte et al., 2005, Biagi et al., 2007). A study to monitor the in vitro and in vivo effects of *Lactobacillus animalis* LA4 (isolated from a healthy adult dog)

on the composition of dog intestinal flora was conducted by Biagi et al., (2007). The researchers reported the reduction of *enterococci* as well as *C. perfringens* and increment in lactobacilli counts throughout the study when a freeze-dried preparation of LA4 was given to the dogs for 10 days. The results suggested that LA4 was able to survive gastrointestinal passage, colonized the intestine of dogs, and positively influenced the composition of intestinal microflora.

The effect of a commercial preparation of probiotics on the fecal microbial composition of healthy dogs and cats was recently investigated using molecular techniques, such as a high-throughput sequencing technique (Garcia-Mazcorro et al., 2011). The authors observed increases in fecal abundance of the ingested microorganisms, and a quantitative change in specific bacterial groups. Although probiotics are reported to have the potential to modify intestinal microbiota of humans (still controversial) when evaluated molecular techniques (Larsen et al., 2011), the overall phylogenetic composition of the fecal microbiota did not change in the animals. Worthy et al., (2009) reported that consumption of probiotics together with prebiotics can potentially affect bacterial populations, but no significant differences in fecal chemistry. Pagnini et al., (2010) have attributed this difference in the results to the amount and types of probiotics used in food/feed administered, and the combination of microorganisms used and their possible synergistic effects. While most of the available data are based on the use of single strains, there is few data favoring a more beneficial effect of multi-strains/species preparation, although encouraged by some researchers (Timmerman et al., 2004).

Lactobacillus salivarius and its application to improve animal health

Lactobacillus salivarius is a producer of bacteriocin. It is often isolated from gastrointestinal tracts of humans, porcine and avian, as well as from human milk, and other sources. Currently, several strains have been identified with a potential to be used as probiotics, as they can produce antimicrobial substance, change gut microbiota, inhibit fecal enzymatic activity, stimulate protective immune response, and produce short chain fatty acids (Messaoudi et al., 2013). There are several experimental studies in animal models to evaluate the probiotic activity of *L. salivarius* and their applications in animal health. *L. salivarius* has been mostly applied in animals to improve immune status and

reduce colonization of pathogenic bacteria in swine and poultry with an aim to improve animal production (Chaves et al., 2017). In an experiment conducted by Mare et al., (2006) to determine the adhesion site of *L. salivarius* 241 in pre- and postweaned piglets using in situ fluorescent hybridization technique, researchers found that the strain colonized to the duodenum in the preweaned piglets. In postweaned piglets, 241 were present at high level in the duodenum and posterior colon. *L. salivarius* 241 lowered 25% of cells of *Enterococcus* in pre-weaned piglets potentially because of competitive exclusion effects on *E. faecalis*. In a similar experiment by Zang et al., (2011a), neonatal piglets were administered with 10^9 CFU per ml of *L. salivarius*. In early lactation, no significant changes in the composition of the intestinal microflora was observed except for the increase in the count of *Bifidobacterium*.

Deng et al., (2013) conducted an experiment to evaluate the potential of co-administration of *L. salivarius* B and *Bacillus subtilis* RJGP16, to stimulate local immune response. In the experiment 32 newborn piglets were divided into 4 groups and varying combination of probiotics (none; RJGP16; B1:RJGP16 and B1) was orally administered to the piglets. After weaning, the parameters of the mucosal immunity of the piglets was analyzed. The results showed that when the two strains are co-administered, the gene expression of interleukin (IL)-6 in the duodenum and ileum significantly increases. Similarly, there is an increase in porcine beta-defensins (pBD)-2 in the duodenum and an increase in the expression and release of TLR-2 and the number of immunoglobulin (Ig) A-producing cells. The results suggested that when the two bacterial strains are given in combination, intense mucosal immunity can be stimulated compared to single bacterium. Similarly, Rondon et al., (2013) evaluated the probiotic effect of a biopreparation with *L. salivarius* C65 on the productive and health indicators in lactating piglets and were able to confirm the effect. In the experiment piglets were given two treatments: basic diet (control) and basic diet + C65 biopreparation. The probiotic treatment improved the live weight of the animals at five weeks, and reduced diarrhea incidence compared to the control group. The researchers confirmed the biopreparation of the probiotics has a potential to create positive effects on lactating piglet yield.

L. salivarius has been used in poultry as a probiotic for over 15 years (Chaves et al., 2017). There are several studies currently available that report this bacterium can reduce colonization by *Salmonella* Enteriditis (SE) through its effects of competitive exclusion (Chaves et al., 2017). Strain CTC2197 along with SE directly was orally administered into the proventriculus in 1-day old chickens. This administration eliminated the pathogen after 21 days. Similar results were reported when the probiotic was administered in the feed and drinking water (Pascual et al., 1999). Similarly, a study was conducted by Zang et al., (2007) to develop a defined competitive exclusion bacteria (CE) culture that will prevent or substantially reduce *Salmonella* colonization of poultry. The potential CE was fed to day-of-hatch chicks at 10^6–10^8 CFU per chick, and *Salmonella* were administered by gavage 2 days later at 5.5 x 10(3) to 5.0 x 10(4) CFU per chick. The outcome of the study showed that when chickens were fed with an overnight CE culture of *L. salivarius* strains Salm-9, List40-18, or List40-41, there was a reduction of *Salmonella* carriage in caecal content. Similarly, a mixture of Streptococcus cristatus List 40-13 and *L. salivarius* List 40-41 was also able to reduce *Salmonella* carriage in chickens. The results allowed the authors to conclude that CE isolates of *Salivarius* Salm-9, List40-18 and List40-41 and *S. cristatus* List40-13, when used individually or in combination, can effectively prevent *Salmonella* colonization of chickens. In a recent study by Sornaplang et al., (2015), 150 newborn broiler chicks were divided into five groups. Group 1(control) was given feed and water only, group 2(positive control) was given feed, water, and SE infection, group 3(L61 treated) was given feed water, SE infection followed by *L. salivarius* L61 treatment, group 4(L55 treated) was given feed water, SE infection followed by *L. salivarius* L55 treatment, and group 5 was given feed, water, SE infection followed by L61+L55 combination treatment. The treatment with L61 and L55 resulted an increase in survival rate of the chicks after SE infection and reduced the recovery rate of SE from caecal tonsils. It was thus suggested that SE infection in young chicks can be prevented by *Lactobacillus* due to their stimulatory effect on immune cells.

Every year, thousands of cases of gastroenteritis caused by *Salmonella* spp. are reported in the European Union. Probiotics are being proposed to have potential to improve this situation. A study was conducted to assess the effect of a LAB mixture consisting of two strains of *L. murinus* and one strain each of *Lactobacillus salivarius* subsp. salivarius,

L. pentosus, and *Pediococcus pentosaceous*, on both clinical and microbiological signs of *Salmonella* enterica serovar Typhimurium infection. In the study, 15 weaned pigs were administered control milk or a mixture of five probiotic strains as either a milk fermentate or milk suspension for a total of 30 days. These animals were orally infected with serovar Typhimurium after 6 days of probiotic administration. Their feces were monitored for 23 days post infection. Improvement in the clinical and microbiological outcome of *Salmonella* infection was found in the animal when probiotic was administered and incidence, severity as well as duration of diarrhea reduced. Fecal of the treated animals showed significant reduction in mean fecal number of *Salmonella* at 15 days post infection ($P = 0.01$) (Casey et al., 2007).

Various animal models and in-vitro studies of *L. salivarius* have demonstrated the potential of this probiotic to modulate the immune system, exert anti-inflammatory effects, and prevent acute liver disease. Li et al., (2010) conducted a study to investigate the effect of *Lactobacillus salivarius* PM-A0006 on ovalbumin (OVA)-sensitized asthma model in BALB/c mice that was challenged with an antigen. Serum OVA-specific antibody levels, airway responsiveness to methacholine, influx of inflammatory cells to the lung, and cytokine levels in bronchoalveolar lavage (BAL) fluid and splenocytes were evaluated in the mice after challenging with the antigen. The treatment reduced the influx of eosinophils to the airway lumen, serum OVA-specific IgE and eotaxin level in BAL fluid, and allergen-induced airway hyperresponsiveness. The observed results enabled the researchers to conclude that the strain of *L. salivarius* may have therapeutic potential in the treatment of allergic airway disease.

Drago et al., (2015) found beneficial immunomodulatory activity and probiotic properties of *L. salivarius* LS01 and Bifidobacterium breve BR03 when they evaluated these two strains in asthmatic human subjects. Peran et al., (2005) investigated Salivarius CECT5713 in the TNBS model of rat colitis. The probiotic *L. salivarius* (5×10^8 CFU suspended in 0.5 mL of skimmed milk) was orally administered to female Wistar rats for 3 weeks. After 2 weeks of starting, 10 mg of TNBS dissolved in 0.25 mL of 500 mL/L ethanol was administered to the rats induce colitis. Results obtained after biochemical tests and microbiological analysis showed that when colitis rats were treated with *L. salivarius*

ssp. salivarius there was a reduction of colonic TNF-α levels, reduction in necrosis and inflammation, and higher counts of Lactobacilli species in colonic contents when compared control colitic rats. The researchers concluded that *L. salivarius* ssp. salivarius CECT5713 could have a potential to beneficially alter the immune response that occurs during colitis.

Lv et al., (2014) have indicated the potential of *L. salivarius* LI01 to prevent acute liver failure. The researchers gave intragastric supplements of *L. salivarius* LI01, *L. salivarius*LI02, *L. paracasei* LI03, *L. plantarum* LI04, or *Pediococcus pentosaceus* LI05 for 8 days to Sprague–Dawley rats. On the eighth day, there was an induction of acute liver injury on the rats. After 24 h, samples from rats were analyzed for levels of liver enzymes, liver function, histology of the terminal ileum and liver, serum levels of inflammatory cytokines, bacterial translocation, and composition of the gut microbiome. Pretreatment with *L. salivarius* LI01 or *P. pentosaceus* LI05 reduced the elevated liver enzymes, and the level of alanine aminotransferase and aspartate aminotransferase. The pretreatment also prevented the increase in total bilirubin, reduced the histological abnormalities of both the liver and the terminal ileum. Based on these results, researchers suggested that *L. salivarius* LI01 and *P. pentosaceus* LI05 can be used as potential probiotics in the treatment or prevention of acute liver failure.

Potential of *L. salivarius* to exert effect in cancer has been studied in various animals. Zhang et al., (2011b) investigated the effect of a carcinogen, 4-nitroquinoline-1-oxide (4NQO) on colonic microflora and the ability of *L. salivarius* Ren to prevent its effects. The researchers found that both 4-Nitroquinoline-1-Oxide and *L. salivarius* REN could alter the bacterial communities of rat colons. When the rats were treated with the carcinogen, it increased the two potential pathogens (one *Helicobacter* strain and one *Desulfovibrio* strain) in the intestine, whereas, *L. salivarius* REN treatment effectively lowered the growth of the *Helicobacter* strains detected initially in the microflora of rats treat with the carcinogen. Similarly, two experiments conducted on separate occasions by the researchers have reported that feeding rats with a large dose of Ren (10^{10} CFU per kg body weight per day) can suppress 4NQO induced oral carcinogenesis through suppression of nuclear antigen and induction of apoptosis (Zhang et al., (2013b)). 1,2-

dimethylhydrazine (DMH)-induced colonic carcinogenesis thus prevented colorectal cancer (Zhang et al., 2015).

Rarely, specific *L. salivarius* strains have been assessed for their safety on animals. Literature that points towards the negative effects of *L. salivarius* is also minimal. Lara-Villoslada et al., (2007) conducted an animal model study in mice to evaluate the oral toxicity of a potential probiotic bacteria, *Lactobacillus salivarius* CECT5713. In the study, 50 Balb/C mice were divided into 5 groups. Varying doses of CECT5713 were orally administered to 3 groups for 28 days at a concentration of: 10^8, 10^9 or 10^{10} CFU per mouse per day. Each group was then analyzed for food intake, body weight, bacterial translocation, serum alpha-amyloid protein, and different biochemical parameters. Results did not show any adverse effect of CECT5713 on the body weight and food intake of mouse. Similarly, no bacteremia and bacterial translocation to the liver or spleen was observed. This indicates that the strain of *L. salivarius* is nonpathogenic for mice, even if the consumed dose is 10,000 times higher than the dose normally used by humans.

Lactobacillus salivarius (CNCM I-3238) and *Lactobacillus casei* (ATTC PTA-6135) are considered safe by European Food Safety Authority (EFSA). These strains are considered safe to be used for livestock, in the environment, and in animal feed. European Food Safety Authority (2012) conducted a study to evaluate the efficacy of the two strains to improve the ensiling process when used as technological additives. In the study, silos with differing water soluble-carbohydrates were treated with *L. salivarius* and *L. casei*, at a proposed dose of 1.3×10^7 and 1.3×10^6 CFU/kg fresh material, respectively. The additive that contained L. casei improved the production of silage by reducing the pH and increased the preservation of dry matter, while *L. salivarius* containing additive showed limited potential to reduce dry matter loss. However, the FEEDAP Panel doubts the benefits of the additives in the ensiling process.

Similarly, a preparation consisting of several strains of *Enterococcus faecium*, *Bifidobacterium animalis* and *L. salivarius*, also known as BiominC3, is considered a safe product (FEEDAP, 2015). The European Union has currently authorized this biopreparation for use in feed for fattening chickens. An EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP,2015) conducted a tolerance study,

where a dose which was 100 times (5×10^{12} CFU/L) the dose currently authorized in feed was given to chickens for fattening. No adverse effects in the chickens were observed. FEEDAP concluded that there would be no safety implications of the maximum dose of the additive, if delivered via water for drinking. BiominC3 could improve the performance of chickens for fattening at a minimum dose of 1×10^8 CFU/kg of complete feeding stuffs or the equivalent dose (5×10^7 CFU/L) when water for drinking is used as the delivery method. (FEEDAP, 2015). Although most of the studies have reported positive sides of *L. salivarius*, certain strains of *L. salivarius* could be cariogenic (Chaves et al., 2017**).**

Lactobacillus salivarius (L28)

L28 is a newly discovered strain of *L. salivarius*, isolated from ground beef, while screening environmental cattle fecal samples/ Retail meat samples for lactic acid bacteria capable of rendering antagonistic effect towards pathogenic bacteria: *Salmonella, Escherichia coli and Listeria monocytogenes* (Campos, 2016). The preliminary unpublished experimental data show that when L28 was co-cultivated with *Escherichia coli* O157:H7, *Salmonella* spp., and *Listeria monocytogenes*, it was able to reduce the pathogens by 4.5, 6.5, and 8.5 $\log_{10}$ cfu/ml, respectively, compared to controls cultivated without L28. The strain showed best inhibition of *Listeria monocytogenes* which was below the detection limit by direct agar plating method.

Based on the results of the antagonistic effect of L28 against the pathogens, Ayala et al., (2017) sequenced the genome of the strain to understand the antagonistic mechanisms. In the study, L28 was cultivated in MRS broth, and genomic DNA was isolated to prepare library. The DNA libraries were sequenced, and the genome was annotated using the NCBI Prokaryotic Genome Annotation Pipeline. In the general annotation, 2 pre-peptides or factors that induce bacteriocin synthesis were identified, along with 2 incomplete prophages and one potential plasmid. The results suggested that the strain produces bacteriocin that exerts bactericidal effect on pathogens.

To establish the most effective dose of L28 as an intervention, Castelli et al., (2017) conducted a laboratory experiment, in which fecal samples were collected from cattle fed three different rations: 1. monensin and tylosin with no probiotic (BASE), 2. monensin with a probiotic (L28) (MONPRO), and 3. no probiotics, tylosin or monensin

(CONTROL), and were inoculated with a cocktail mixture of three Shiga-Toxin producing *E. coli* (STEC) strains or *Salmonella* strains to an initial concentration of 3.0-4.0 log10 CFU/g. Three concentrations of *Lactobacillus salivarius* L28, (10^6, 10^7, and 10^8 log10 CFU/ml) were used to determine dose-response. The pathogens in the samples were enumerated 1, 6, 12, and 24 hours after inoculation. Inhibitory effect of L28 on the growth of STEC and *Salmonella* was observed in all three cattle fed diets however there was no significant difference between the dose response by time. Thus, it was hypothesized that the application L28 at 10^7-10^8 CFU/ml would be effective in inhibiting STEC and *Salmonella* in the manure.

Safety and Challenges of probiotics

There is much literature available that suggest the safety of commercially available probiotic organisms for dogs, however in studies that determined the positive effects of probiotics on diseased animal safety was not evaluated or reported. Although we don't have many reports on adverse clinical effects of probiotics on health, there is evidence showing potentially harmful effects of probiotics. In an in vitro study, to evaluate the efficiency of certain lactobacilli and enterococci to prevent the colonization of the mucosa of dog's jejunum by pathogen, investigators found that the two strains of *E. faecium* (M74 and SF273) increased mucosal adhesion of *Campylobacter jejuni* when compared to adhesion in untreated control mucosa. Since *C. jejuni* is zoonotic, it has implications on both human and animal health (Rinikinen et al., 2003). Another factor of importance that needs to be accounted for while evaluating the safety of probiotics is the antimicrobial resistance in the organisms. Pets may acquire resistance at the same time determinants of resistance may be transferred from non-pathogenic bacteria to opportunistic pathogens present in the host (Lefebvre et al., 2011). According to the FAO/WHO recommendation, patterns of antimicrobial resistance should be evaluated for all products (FAO/WHO, 2006). In the case of dogs, studies on antimicrobial resistance are available currently but are not reported.

When we analyze the definition of probiotics, one of its important aspects include administration of probiotics in an adequate amount and exerting beneficial effects in the host. Until now, there is only one study limited to dogs that evaluates the effect of various

dosage of *Lactobacillus rhamnus* strain GG (LGG) (Weese and Anderson 2002). The study showed that a dose of 5 X 10^{11} CFU of LGG/day results in significantly larger fecal colonization with LGG than when smaller doses are used, but the clinical effectiveness of this dose was not evaluated**.** There are studies that have evaluated the effectiveness and efficacy of differing doses of specific organisms in dogs, but the sufficiency of the dosage to exert their intended effect is unknown. One of the most prevalent or common quality control issue includes misstatement or falsification of bacterial species added to probiotic supplements that are commercially available (Weese and Martin 2011). There are cases where products lack information on the organisms contained and no growth or growth of unexpected organisms have resulted when attempts were made to culture the product in laboratory. There is poor control over the microbial content in canine diet claiming to contain probiotics. A study was conducted on 19 unidentified commercial pet foods consisting of 13 canines and 6 felines to confirm their claim of containing probiotics on the label. Unfortunately, no product had all the organisms stated on the labels and five diets could not give relevant growth of the bacteria. The research also questioned the clinical relevancy of the degree of treatment of the diets that yielded growth (Weese and Arroyo 2003). There is need of extensive clinical trials and experiments to identify species of bacteria with probiotic potential, to determine their minimum effective dosages, potential adverse effects, and their impact on the health of pets. There should be more regulation and control at all the stages of manufacturing. Veterinarians should be cautious while approaching new probiotics and should consider their experiences and published evidence. Finally, probiotic pet food needs to be monitored or scrutinized for their label claims (Lefebvre et al., 2011).

CHAPTER III
LACTOBACILLUS SALIVARIUS L28 IN DOG KIBBLE RESULTS IN SHIFTS IN MICROBIAL INDICATORS IN PET FECAL SAMPLES AFTER FEEDING

Introduction

Gastro-intestinal tracts of dogs are a home to number of pathogens that can have direct implications on dog's health as well as direct-indirect implication on health of humans, animals, and the environment (soil and water) encountering the fecal of infected dogs ((Weese et al., (2010), Marks et al., (2011), and Cave et al., (2002)), According to the American Pet Product Association (APPA), $15.4 billion was spent on veterinary care in 2015. The expenses have risen faster than the rate of human health-care (Close 2016). Researches have proved that probiotics provide both preventive and supportive care for pets. However, if we refer to available literatures on the benefits of probiotics for improving the health of dogs, most of the study are conducted in controlled experimental conditions where one can take control over factors such as differences in ages, breeds, management practices which may affect the efficacy of a probiotic under study (Lefebvre et al., 2011). Rarely, there are studies reporting the effect probiotic supplemented kibbles on fecal microbial population of dogs in uncontrolled environment.

Several researchers have reported the beneficial effect of various strains of *Lactobacillus Salivarius* on animal's health by altering the composition and balance of gut bacterial population towards more beneficial one. Apparently healthy dogs have been reported to be the subclinical carrier of *Salmonella* and shed them intermittently posing a risk of Salmonellosis to the owners. Several probiotics are proven to be effective in reducing the fecal shedding of *Salmonella* in many animal studies. *Lactobacillus Salivarius* L28 was recently isolated in Texas-Tech University from ground beef and preliminary invitro laboratory experiments have reported the antagonistic effect of L28 on *Escherichia coli* O157:H7 and *Salmonella* spp (Unpublished data from Texas-Tech University). Since, testing, detecting, and identifying a pathogen, whether in food or fecal is very expensive and impractical, so to monitor pathogens (EPA, 2006) or to monitor the efficacy of process

or food supplementation, "indicators" are used. Direct inoculation studies, to evaluate the potential of supplementation to reduce a specific pathogen would be impractical or unethical. Similarly, monitoring the shift in balance of gut microflora in the GI tract itself would be difficult. Therefore, change in microbial indicators or generic microorganisms in the fecal must be monitored to assess the efficacy of the probiotic supplementation to potentially reduce the enteric pathogens and to shift the microbial balance of gut microflora. Similarly, intended benefits of a probiotic supplement have been achieved in clinical trials, when dose of 10^6–10^{12} CFU/animal/day (FEEDAP 2015, Zang et al. 2007, Zhang et al. 2013b, Zhang et al. 2015, Villoslada et al. 2007, and Shornikova et al. 1997) is used without any negative effects on the subjects and the dose further depends on strain of probiotics. The L28 was developed to be effective at a lower dose thus reducing the cost. A study was conducted in dogs exposed to real-life conditions. These dogs were fed with kibbles supplemented with various combinations of probiotics including L28 (daily dose of 10^6 CFU/animal). The impact of the supplements to reduce the fecal population of Generic *E. coli*, fecal coliforms, Entereobacteriaceae, and to reduce fecal shedding of *Salmonella* in the dogs was evaluated.

The foremost objectives of this study were to i.) Define the baseline population of Generic *E. coli*, fecal coliforms and Entereobacteriaceae, and fecal shedding of *Salmonella* in the dog population included in this research. ii.) Determine the fecal shedding of *Salmonella* in the dog population included in this research. After the dogs were given kibble containing various combinations of probiotics including L28, our objectives were to i.) Determine the impact of the probiotics in the Generic *E. coli*, fecal coliforms, total coliforms, and Entereobacteriaceae, during feeding, and ii.) Evaluate the impact of various combinations of probiotics including L28 in fecal shedding of *Salmonella* during feeding.

Materials and Methods

Formulation of pet Kibble

Upon, receiving approval from Animal Care and the Institutional Animal Care and Use Committee(IACUC) #16111-12, at Texas Tech University, kibble without any probiotics and kibble supplemented with probiotics (*Lactobacillus plantarum, Bacillus subtilis, Lactobacillus acidophilus, Lactobacillus reuteri, Enterococcus faecium,*

Bifidobacterium animalis) were obtained from a commercial pet food company producing product in the United States. The probiotic L28 was added to control Kibble at Department of Animal Science at Texas Tech University following the procedure detailed below.

A portion of uncoated control kibble (without probiotics) was supplemented with the L28. *Lactobacillus salivarius* (L28) was grown in MRS broth for 12 hours. It was estimated that the culture was log 8.00 CFU/ml after the incubation. The cells were super concentrated in a centrifuge at 6000 rpm for 6 minutes. The pelleted cells were re-suspended in 20 ml of the De-Mans Sharpe and Rogosa (MRS) supernatant. It was estimated that the culture was at log 10.0 CFU/ml. This 20 ml of concentrate was added to 20 ml of chicken fat for a total of 40 ml. This 40 ml was added to ½ pound of uncoated pet kibble. The kibble was then allowed 4 hours to dry underneath a hood. A portion of kibble with L28 and was mixed to the kibble with commercial probiotics in 1:1 ratio. Eventually four types of kibble were obtained; Kibble without probiotics, kibble with L28, kibble with commercial probiotics and kibble with L28+ commercial probiotics. The kibbles only differed in probiotic treatment and had similar nutritional composition. These kibbles were then used for the feeding trial on canines. Total populations of the probiotic were determined by plating onto MRS agar.

Similarly, L28 concentration in the kibble were estimated over 4 months, to generate death curve of long term storage in typical ware house condition by opening a new bag of kibble each time. Twenty-five grams of dried kibble with L28 was weighed and homogenized with 225 ml Buffered Peptone Water ((BPW), Merck KGaA). The mixture was homogenized, using a laboratory stomacher, at 230 rpm for 2 minutes, and serially diluted by transferring 1 ml of the aliquot to 9 ml BPW tubes. The similar procedure was followed for obtaining successive dilutions. 0.1 ml of each dilutions was spread plated onto MRS agar in duplicates. The plates were incubated at 37^{o}C for 48 hours, and white colonies typical to Lactic Acid Bacteria on MRS agar were counted using microscope, following the incubation.

Group assignment and Feeding

The feeding experiment was conducted in Lubbock, Texas. Total of 47 different breeds of healthy and domesticated dogs (males=21 and females=26) from different

households of the Lubbock county were included in the study and were given unique numeric ID. Owners voluntarily participated their dogs in the feeding trial. The age of the dogs ranged from 1-13 years. The demographic information of the dogs is presented on Table A.1. These dogs were randomly assigned to one of the four feeding groups: Group 1 or the control group, consisted of 12 dogs (male=5 and, female=7), and were fed kibble without probiotic treatment. Group 2, consisted of 11 dogs (male=4 and, female=7) and were fed kibble supplemented with *Lactobacillus Salivarius* (L28). Similarly, group 3 (n=12, male=7 and, female=5), and group 4 (n=12, male=5 and, female=7) were fed with kibble supplemented with L28 plus commercial probiotics and commercial probiotics, respectively. The kibble given to each group only differed in their probiotic supplements keeping all essential formulations of kibble the same. During the feeding period, fresh kibbles were supplied to the owners of dogs in each groups and instruction was given to follow the normal feeding pattern and the amount the dogs were usually fed. The dogs under study were not allowed to eat food other than the experimental food. All the 47 dogs in this trial were given the control feed for seven days during the adjustment phase to allow adjustment to the food. The three probiotic groups were given their respective treatment food on day 8 of the feeding trial. This 8^{th} day represents the day 0 0f the treatment phase which lasted for a total of 40 days. The dogs in the control group were continued with the control food during the entire trial (Fig A.8). Dogs were monitored by their owners for any signs of weight loss, due to starvation and nutritional deprivation or illness during and after feeding the kibble.

Sampling

The samples were aseptically collected in fecal cups once a day by the owners. Researches collected the samples from the owners, transported them to Department of Animal and Food Sciences in coolers and froze them at -20 ^{o}C within 24 hours of collection. The samples were then transferred to the lab of International Center for Food Industry Excellence located at Experimental Science Building of Texas-Tech University for further microbiological analysis. Fecal samples from every dog were collected on day 5, 6 and 7 of the adjustment phase (day 3, 2, and 1 before starting the treatment food) to generate baseline profile of Generic *Escherichia coli*, fecal coliforms, total coliforms and Entereobacteriaceae in the feces and on day 5,10, 20, and 40 of treatment phase to analyze

the shift in the fecal shedding or excretion of these indicators because of treatment (Fig A.8). The generation or study of baseline is important as it helps to observe and evaluate the efficacy of a treatment to modulate GI microbial population as suggested by the commensals bacterial indicators in feces for this study.

Quantification of Indicator Organisms in Fecal Samples.

Processing of samples

Upon arrival at the laboratory, each fecal sample was weighed, homogenized manually, and approximately half the sample was placed into a 10 ml sterile filter bags; the remaining sample was used to prepare gDNA extractions for metagenomics sequencing and stored at -80°C. BPW was added to each bag at 1:10 ratio and the contents in the bags were homogenized for 2 minutes at 230 rpm. Serial dilutions of the homogenate (1:100 and 1:1000) was carried out in BPW by aseptically transferring 1 ml of the sample to tubes with 9 ml BPW. Each tube was homogenized vigorously using a laboratory vortex before carrying out successive dilution.

Enumeration of Generic *E. coli* and Fecal Coliforms

Following homogenization and serial dilution, 1 ml of each dilution was placed on 3M Petrifilm *E. coli*/ fecal Coliform Count Plate on the center for plating. With flat side down, Petrifilm spreader was placed on top film over inoculum and pressure was applied gently on spreader to distribute inoculum over circular area before gel is formed. The plates were incubated with clear side up in stacks of up to 20. The plates were incubated for 24±2 hours at 35°C and 48±2 hours at 35°C for coliforms and *E. coli* respectively. The *E. coli*/coliform count plates were counted on a 3M standard colony counter by referring to interpretation guide. Blue colonies on the plates associated with gas bubbles were counted as *E. coli* and red colonies associated with entrapped gas bubbles around the colonies were counted as fecal coliforms. Total coliforms were reported by counting both, red and blue colonies associated with entrapped gas.

Enumeration of Entereobacteriaceae

Following homogenization and serial dilution, 1 ml of each dilution was placed on 3M Petrifilm Entereobacteriaceae count plate on the center for plating. With flat side down,

Petrifilm spreader was placed on top film over inoculum and pressure was applied gently on 3M Petrifilm spreader to distribute inoculum over circular area before gel is formed. The plates were incubated with clear side up in stacks of up to 20. The plates were incubated for 24±2 hours at 37°C. The Entereobacteriaceae count plate were counted on a standard colony counter by referring to interpretation guide. Bacteria producing gas and/or acid having one of the following characteristics on the plates; colonies associated with gas bubbles and no acid zones, colonies with yellow acid zones but no gas production, or colonies producing both gas and acid were counted as Entereobacteriaceae.

Screening of fecal samples for *Salmonella*

A. Pre- enrichment

To screen each of the fecal samples for the presence of *Salmonella* homogenized samples were incubated at 37°C for 18h and screened for genetic elements unique to *Salmonella* using *Salmonella* real-time PCR BAX test (Hygiena LLC, USA,) following manufacturers' instructions.

B. *Salmonella* Real-Time PCR BAX

Equipment Preparation

Before preparing the samples for BAX, the heating blocks was turned on to 37ºC and 95ºC. The Q7 instrument was powered on BAX® System application was launched. A rack file was created following User Guide.

Lysis performance

The cluster tubes were broken apart, labelled, and arranged in the rack according to rack file. Lysis reagent was prepared by adding 150 µL protease to one 12 mL bottle of lysis buffer. 200 µL lysis reagent was transferred to each cluster tube and 5 µL enriched sample was added to the corresponding cluster tube. The contents in the tube were heated at 37°C for 20 minutes followed by heating at 95°C for 10 minutes. The tubes were cooled at 2-8°C for at least 5 minutes.

Hydration of PCR Tablets

The instrument was initialized by selecting RUN FULL PROCESS from the OPERATION menu. A PCR tube rack was placed onto a chilled (2-8°C) PCR cooling block. Strips of PCR tubes were arranged according to rack file. Caps were removed from the first strip of tubes with the decapping tool and 30 µL lysate was transferred into PCR tubes, then sealed with flat optical caps. The process was repeated with remaining strips of PCR tubes until all PCR tablets were hydrated. The PCR tubes were let to sit in the cooling block for 10-30 minutes before loading into the BAX® System instrument.

Amplification and Detection

At the "Ready for Rack Load" prompt, the NEXT button was clicked, and the instrument drawer was opened. The rack of PCR tubes was placed over the wells in the drawer. The drawer was closed, and the NEXT button was clicked to begin automated processing.

Results Review

Qualitative results were displayed as a grid of color-cued icons in the top half of the screen.

Confirmation of *Salmonella*

Selective enrichment: BPW enrichments from PCR screened positive were transferred to the secondary selective enrichment broths Rappaport Vassiliadis Broth (RV) (Merck KGaA) and Tetrathionate Brilliant Green broth (TT) (Neogen corporation), following Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM). 0.1 mL aliquot of the pre-enrichment was transferred to 10 mL of RV and 1mL to 10 mL of TT and were incubated at 42ºC and 35ºC for 24h respectively. Before inoculating selective TT broth, 180 µl of iodine was added to each tube and homogenized vigorously.

Selective plating: After incubation 10 µl of TT and RV incubated aliquot was streaked onto Xylose Lactose Tergitol™ 4 (XLT-4) (Difco ™) agar plates, and Chromagar *Salmonella* (CHROMagar) plate. These streaked plates were incubated at 37ºC for 24 hours. Black or black-centered with a yellow periphery on XLT4 and rose-violet (mauve)

to blue-violet colonies on ChromSal agar plates were presumed as *Salmonella*. Up to four presumptive *Salmonella* colonies from both the plates were sub-streaked on to blood agar plates for further antigenic confirmation.

Antigenic confirmation for *Salmonella*

Potential *Salmonella* positive samples were confirmed through both microbiological culture and serological assay. For the antigenic confirmation, Wellcolex Colour *Salmonella* agglutination test (ThermoFisher Scientific) was used. Following manufacturer's instructions, approximately 200 µl of saline was dispensed into a suspension tube. From an overnight culture, one suspected *Salmonella* colony was picked from the Blood Agar plates plate using the flat end of a sampling stick. The bacteria were carefully emulsified in the saline. Latex reagents 1 and 2 was resuspended by shaking vigorously for a few seconds. One free-falling drop of each latex reagent was dispensed into a separate circle on a flat reaction card. Using a disposable sample dispenser held vertically, one free-falling drop (40 µl) of bacterial suspension was dispensed to each of the two circles. Using a sampling stick, the contents of each circle were mixed and spread to cover the whole area of the circle. The card was placed on a suitable flat-bed rotator and run at 150±5 rpm for 2 minutes. The rotator was switched off and observed for agglutination without removing the card from the rotator. WellColex Color *Salmonella* reading guide was used for reading results.

Statistical analysis

The experiment was a randomized complete split-plot design with individual dog in each of the four-feeding group defined as experimental units. Each group was analyzed individually from others. All plating was carried out in duplicates for each dilution and the duplicate counts were averaged. The counts of each indicator obtained at different observation period for all the dogs were converted to log CFU per gram before analysis. For every indicator, negative plates with counts <10 CFU/ml, an arbitrary small value of ''1.0 log CFU/g'' was assigned and too numerous to count (TNTC) plates for dilution 10^{-3}, an arbitrary value of 6.00 log CFU/g was assigned prior to normalize the data distribution. Log counts of each indicators were considered a dependent variable of interest. Data were imported into R-Studio version 3.3.1, for analysis. A two-way ANOVA was performed to

evaluate any variation between the four feeding groups over time due to respective treatment food. The ANOVA test was followed by Post-hoc pairwise t-test and Welch t-test to determine the statistical difference between them. For group comparison, ratio of baseline to each observation during treatment phase was calculated. Similarly, a Two-way repeated measures ANOVA was performed individually for each feeding group (taking counts of the indicators as response variable), to analyze the effect the treatment in the indicators over-time taking into consideration the dog effect followed by Post-hoc pairwise t-test and Welch t-test to determine the statistical significance. Significance level of 0.05 was used for all the analysis. The baseline was used to compare among 5, 10, 20 and 40 days of each treatment.

Results and discussion

Results

When the shelf-life of the kibble was evaluated in terms of L28 over 4 months, the counts of L28 remained consistent for the period of 3 months at 6.12 log CFU/Lb on average (Table A.2). However, on the fourth month the average count was reduced to 5.20 CFU/Lb (Fig A.1).

Upon pair-wise comparison there was no significant difference ($p>0.05$) between the baseline of the three probiotic groups and the control group (Table A.7, A.8, A.9, and A.10) in terms of Generic *E. coli*, fecal coliforms, total coliforms and Entereobacteriaceae. The Two-way ANOVA for comparison between the four treatments (ratio of baseline to treatment) overtime taking into consideration the interaction between treatment and time revealed no significant interaction ($p>0.05$) between the treatments and time for the four microbial indicators. There was a significant variation in the ratio (($p<0.05$) between the treatment groups for Generic *E. coli*, fecal coliforms and Entereobacteriaceae except for total coliforms (Table A.12), irrespective of time. When the three probiotic groups were compared with the control group, only the group fed with L28+ commercial probiotics showed a significantly higher ($p<0.05$) baseline to treatment ratio. This ratio was significantly higher for fecal coliforms ($p=0.03$) and Entereobacteriaceae ($p=0.005$) and numerically higher ratio for Generic *E. coli* and total-coliforms than the control group.

Similarly, when the comparison was made among the three probiotic groups for each of the four indicators no significant differences was observed between the group fed with L28 and the commercial probiotics. However, there was a significant difference ($p<0.05$) between the group fed with the combined probiotics and the L28 or the commercial probiotics. The group fed with the probiotic combination had a significantly higher baseline to treatment phase ratio for Generic *E. coli* ($p=0.0003$) and fecal coliforms ($p=0.002$) than the commercial probiotics fed group. A significantly higher ratio of total coliforms was observed for the group fed with the combined probiotics compared to L28 ($p=0.03$) and as well as the group fed with the commercial probiotics ($p=0.002$). Similarly, a significantly higher ratio of Entereobacteriaceae was observed for the group fed with the combined probiotics compared to L28 ($p=0.02$) and as well as the group fed with the commercial probiotics ($p=0.04$) (Table A.12).

A two-way repeated measures ANOVA was performed on each of the four-feeding group, that examined the variation in Generic *E. coli*, Coliforms, total-coliforms, and Entereobacteriaceae, over time taking (between-subjects factor) dogs as covariate (within-subjects factor). The counts of each indicator for each feeding group were normally distributed, as assessed by Shapiro-wilks test ($p>0.05$). Homogeneity of variance was not violated, as assessed by Levene's test ($p>0.05$). There was no statistically significant effect ($p>0.05$) of supplement duration on Generic *E. coli*, total coliforms, fecal coliforms, and Entereobacteriaceae, with no significant difference ($p> 0.05$) among the baseline and 5, 10, 20, and 40 during treatment for any of the four indicators in the control group (Tables A.7, A.8, A.9, and A.10). The counts varied throughout the treatment period (Figures A.2, A.3, A.4, and A.5). Consistently with the control group, no significant difference ($p>0.05$) was observed among the baseline and days 5, 10, 20, and 40 during treatment phase in the groups fed with L28, or the commercial probiotics, for each of the microbial indicator, however, significant interaction ($p<0.05$) existed between treatment duration and dogs for some indicators. Whereas, unlike the control, L28 + commercial probiotics showed a statistically significant effect of treatment duration ($p<0.05$), statistically significant differences between the dogs in the group ($P<0.05$) as well as significant interaction ($p<0.05$) between the effects of treatment duration and the subjects in the group except Generic *E. coli* (Table 12). Upon post-hoc, pairwise comparison, there was a significantly

lower count of Generic *E. coli*, in the fecal samples of L28 + commercial probiotics fed group on day 20 [p=0.001, 95% CI (0.72, 2.53), t = 3.7348] compared to the baseline with a reduction of 1.63 $\log_{10}$ CFU/g fecal (Table A.7), where 75% of dogs had greater than 1-log cycle of reduction. Similarly, there was a significantly lower count of fecal coliforms in the fecal samples of the group than the baseline, with the reduction of 1.18 $\log_{10}$ CFU/g fecal on both days 10 [p=0.006, 95% CI (0.37, 2.00), t = 3.02], and 20 [p=0.01, 95% CI (0.27, 2.1), t = 2.74], (Table A.8), where highest percentage of dogs (67 %) had greater than 1-log cycle reduction on day 10. The fecal population of total coliforms decreased significantly than its baseline with a reduction of 1.46 $\log_{10}$ CFU/g fecal on day 20 [p=0.005, 95% CI (0.50, 2.42), t = 3.21], with 42 % of dogs having greater than 1-log cycle of reduction (Table A.9). Similarly, the fecal count of the Entereobacteriaceae decreased significantly on day 20 [p=0.004, 95% CI (0.83, 2.36), t = 4.42], and day 40 [p=0.002, 95% CI (0.72, 2.6), t = 3.71] with a reduction of 1.60 and 1.70 $\log_{10}$ CFU/g fecal, respectively (Table 10), where highest percentage of dogs (83 %) had greater than 1-log cycle reduction on day 20. No significant differences ($p>0.05$) in excretion of the indicators existed between each successive days of the treatment period. A linear decrease in average count of Generic *E. coli*, fecal coliforms and total-coliforms was observed on day 5 ,10, and 20 of the treatment period followed by insignificant increase ($p> 0.05$) on day 40 (Figure A.3, A.4, and, A.5), whereas, the reduction remained linear and consistent for Entereobacteriaceae after day 10 (Figure A.6).The linear regression model on the reduction of Generic *E. coli*, total coliforms, fecal coliforms, and Entereobacteriaceae as a function of treatment duration, for the L28 + commercial probiotics fed group was significant ($p<0.05$) with, adjusted R-squared value= 0.65, 0.68, 0.74, and 0.61 and, F-statistic= 3.68, 4.05, 5.18, and 3.29, on 59 and 24 degrees of freedom, respectively.

The overall reduction in the population Generic *E. coli*, fecal coliforms, total-coliforms, and Entereobacteriaceae for the L28 + commercial probiotics fed group during the treatment was significant at 1.12 (p=0.006), 0.92 (p=0.002), 1.02 (p=0.001), and 1.19 ($P<0.05$) $\log_{10}$ CFU/g fecal respectively, when compared to the indicators baseline, individually. The overall change in the indicators in the fecal samples of dogs assigned to each treatment group during the treatment period was compared to the respective baselines. A reduction of greater than 1-log cycle in Generic *E. coli*, Coliforms, total-coliforms, and

Entereobacteriaceae was observed in 58 % (7 of 12), 50 % (6 of 12), 50 % (6 of 12), and 75 % (9 of 12) of the dogs during the treatment period of the L28 + commercial probiotics, respectively. This proportion of the dogs with the reduction was highest for the L28 + commercial group in terms of the four indicators (Fig A.6).

Five fecal samples were identified as potential *Salmonella* positive through PCR during the baseline study period. These samples were collected from Joey on day -2 and Jagger on day -1(Group 1), Daisy on day -2 and -1 (group 4) and Bear on day -1 (group 3). However, after selective enrichment in TT and RV and plating onto XLT-and ChromSal agar, no colonies with typical *Salmonella* morphology were observed, thus these fecal were reported as negative. During the treatment period, only fecal sample collected from Cinnamon fed with commercial probiotics (Group 2) were PCR screen positive. The fecal sample was collected on day-40 of the feeding period. The presumptive *Salmonella* positive colonies from the selective plates gave a positive agglutination reaction, and thus confirmed as *Salmonella*. The presumptive serogroup of *Salmonella* was serogroup C. The prevalence of *Salmonella*, in this study was determined to be 2.12 % (Figure A.7).

No signs of weight loss due to starvation and nutritional deprivation or illness during and after feeding the kibble in the dogs was reported by the owners.

Discussion

Available literature has supported the positive effect of probiotics on the GI health of humans and animals by maintaining the population of beneficial microflora, and through antagonistic effects on enteric-pathogens. The efficacy of a probiotic treatment is affected by various factors such as differences in age, breed, gender, feeding and management practices/environment, and health condition (Lefebvre et al., 2011). The study was conducted in real-life conditions, and subjects were randomly assigned to the feeding groups. It was not possible to control all confounding variable that occur when working with pet owners that could have affected our treatment and fulfill intended purpose of probiotics. Despite of the above-mentioned fact. L28+ commercial probiotics was able to significantly reduce ($p<0.05$) generic *E. coli*, total coliforms, fecal coliforms, and Entereobacteriaceae over time in its feeding group as well as exhibited reduction effect in

larger proportion of dogs assigned to the group. However, no significant effect was observed in the groups fed with control kibble, L28, or the commercial probiotics, respectively. In this study we could attribute with 65, 68, 74, and 61% of the variation in the population of *E. coli*, total coliforms, fecal coliforms, and Entereobacteriaceae respectively, to L28 + commercial probiotics treatment for the group. The control over factors becomes important to observe the intended effect and attribute with greater confidence the effects to probiotics. Similarly, a larger proportion of the dogs assigned to L28 + commercial probiotics group had a reduction of the four indicators greater than 1-log cycle after 20 days of feeding. This suggests at least 20 days of treatment may be required to observe the beneficial effect of L28 + commercial probiotics on dogs. Thus, the results indicate towards prophylactic potential of the supplement. Similarly, the group fed with kibble containing the probiotic combination had a higher baseline to treatment ratio of each microbial indicator on. This suggests a greater reduction effect of the supplement compared to the normal kibble and the kibble with L28 alone or with commercial probiotics.

The available literature report that multi-strain probiotics have greater efficiency than single strains including strains that are components of the mixtures themselves (Chapman et al, 2011). Multispecies probiotics can colonize several niches in the GI tract when compared to a single strain (Kajander 2007). Probiotic effect is limited to strain specific property, therefore in multi-strain probiotic mixture, synergistic effects between strains may increase adhesion to mucus, reduce adhesion of pathogen (Bergogne, 2000), and symbiosis may enhance growth characteristics of certain strains (Timmerman et al., 2004). Apart from affecting adhesion, probiotic have antagonistic effects that include competitive exclusion of pathogenic microorganisms, production of anti-microorganism substances such as organic acids, hydrogen peroxide, and bacteriocin (Miriam et al., 2012). According to Timmerman et al., (2004): "The design and use of multi-strain and multi-species probiotics should be encouraged. Strains used in multi-strain/species probiotics should be compatible or, preferably, synergistic." (Page 219). The significant reduction of indicators by treatment with a combination of L28 and commercial probiotics consisting of *Lactobacillus* spp., *Bacillus subtilis, Enterococcus faecium,* and *Bifidobacterium animalis* observed in our study, can be related to higher probiotic dose, possible strong

synergistic interactions, or symbiosis between L28 and at least one of the strains in the mixture of commercial probiotics, and antagonistic effects resulting from antimicrobials such as organic acid and bacteriocin. *Lactobacillus* and *Bifidobacterium* are known to produce bactericidal proteinaceous molecules or bacteriocins (Eijsink et al., 2002), organic acids (Makras et al., 2006), and hydrogen peroxide (Silva et al., 1987) against gram-negative bacteria. However, in our study despite of being multi-species/strains and having a high dose, commercial probiotics did not show any significant reduction effect on the population of indicators. This situation may be explained possibly by weak synergistic interaction between the strains of commercial probiotics.

Results of our Two-way ANOVA for each group suggest that, not every dog may benefit from the treatment. Similarly, not every dog will be benefitted by the same duration of treatment. Which also supports the fact that the efficacy of a probiotic supplement is affected by various uncontrolled factors; such as differences in age, gender, breed, health-status, and management practices. Relevant to the above-mentioned context, although it was not the objective of this study, a three-way ANOVA was performed to analyze the effect of supplements, supplement duration and gender. There was a significant interaction ($P<0.05$) between treatment and gender for Generic *E. coli*, fecal coliforms, and total coliforms. Upon simple main effect analysis, irrespective of duration of supplement Control and L28 + commercial probiotics had stronger reduction effect ($p<0.05$) on male dogs in terms of Generic *E. coli*, Coliforms, and total-coliforms. The result thereby supports the role of gender in determining the efficacy of probiotics. However, the results are inconclusive due to small sample size and need further studies.

Although, the shifts in indicator population of group 1, 2 and 4 was non-linear and insignificant when compared to the baseline on any day of treatment period, some dogs in the groups showed significant reduction in the indicators during treatment. The results support the fact that the performance of a supplement differs among dogs within a group. Therefore, these supplements could give better output for certain breed, age-group, or an organism associated to the indicators than the factors in general. Similarly, our study shows that L28 treatment resulted in an insignificant but larger drop in *E. coli* count on day 40 and linear but insignificant reduction trend in coliforms and Entereobacteriaceae after day

10 respectively. These results suggest that a longer period of continuous L28 treatment may be required to observe its significant effect over time. Our results for L28 or commercial probiotics are like the results reported by Marcinakova et al., (2006), where no change in *E. coli* was observed in feces of healthy dogs after administering *Enterococcus faecium*, however the results for coliforms are contradictory to reduction observed in healthy dogs that were given *L. fermentum AD1* (Strompfova et al., 2012a indicating microbial shifts are specific to the type of probiotic.

According to Sander (2003), concentration of probiotics needed to obtain clinical effects is reported as 10^6 CFU/g in the small bowel and 10^8 CFU/g in the colon. The viability of probiotics is affected when they pass through the GI tract. The probiotics must reach the intestinal tract in an adequate number, so they should be able to survive the transit time through the acidic environment of the stomach and survive in the presence of bile (Hosbjerg et al., 2016). For a probiotic to be successful, it should colonize the GI tract when the pre-existing microflora are present (Tuomola 2001, Bezkorovainy 2001 and Strompfova 2004). The inability of various strains of *L. salivarius* to colonize the small intestine, despite being highly acid tolerant and passing the ileum at concentration above 10^5 CFU/g, has been reported by Elisa and Anna (2008). Although, the average concentration of L28 in the kibble was 10^6 CFU/lb, and each dog in the group received on average a dose of 10^6 cfu/day, the bacteria possibly might have failed to colonize the colon of significant proportion of the dogs fed with kibble consisting the L28 only due to absence of symbiotic effect as hypothesized between the L28 + commercial probiotics. Therefore, we could not observe any average significant change in microbial indicators in fecal samples of the group during treatment.

Mary (2003) has stated that ideally an indicator should be more resistant to a treatment process, so that the reduction of the indicator would verify the reduction of pathogens. The significant reduction of indicators observed on fecal samples suggest the efficacy of treatment L28 + commercial probiotics to shift their population in the GI tract of dogs, and possibly render an antagonistic effect on existing enteric pathogens in symbiotic relationship with the indicators. Reduced fecal shedding of coliforms has been observed in animals corresponding to decrease in incidence of diarrhea by supplementing

probiotics (Abu-Tarboush et al., 1996). Similarly, a lower population of Entereobacteriaceae (Xenoulis et al., 2008) and *E. coli* (Suchodolski et al., 2010; Minamoto et al., 2014a) has been observed in the GI tract of healthy dogs compared to diseased ones. Although, this study does not involve the effect of probiotic treatment to reduce disease incidence but since the dogs were apparently healthy, the ability of the combined treatment to significantly reduce coliforms, Entereobacteriaceae and *E. coli* may be beneficial to dogs. It can provide prevention against disease incidence caused by enteric pathogens associated to the indicators and promote or maintain gut health. The reduction in disease incidence in the GI tract might have several positive implications such as reduced use of antibiotic, and the healthcare cost. According to the American Pet Product Association (APPA), $15.4 billion was spent on veterinary care in 2015. The expenses have risen faster than the rate of human health-care (Close, 2016). The beneficial effect of the probiotics in dogs could minimize human zoonosis, especially to the owners who are in direct contact with the animals. Similarly, it could lower the environmental contribution to pathogens by the animals.

However, according to DES (2003) change in the population of indicators or generic microorganisms does not reliably indicate the efficacy of a treatment process to reduce or destroy individual species or groups of pathogens and presence of indicators do not correspond to the presence of pathogens as well. Therefore, the significant reduction of indicators achieved in our study may not be reliable to make any conclusion on the reduction of pathogenic microorganisms present in the GI tract of dogs. Since, the occurrences of gastro-enteric diseases indicate the presence of enteric pathogens, disease incidence could be a reliable indicator to monitor the effectiveness of the supplement to reduce enteric pathogens. Similarly, some members associated to the microbial indicators monitored in this study are the part of normal beneficial gut flora, any assumptions regarding gut health thus may not be reliable. Adverse health effects of treatment on dogs were not reported by the owners during the study period, therefore we could be assured of no negative impacts of supplements on gut health.

Salmonella was detected in 2.12 % of the total dogs in this study. The rare presence of *Salmonella* could be attributed to factors such as: i) healthy status of the dogs, where the

organism was completely absent in the animal; ii) intermittent fecal shedding of a carrier dog; iii) specificity of the PCR screening; and iv) viable but not culturable state (VNBC) of *Salmonella,* and v presence of dead cell with intact DNA. Several scientific literatures have reported intermittent shedding of *Salmonella*. Dogs shed the agent irregularly for the subsequent 3–4 weeks and in rare cases this shedding could continue continues for up to 100 days (Tanaka et al., 1976). Therefore, sampling time is very important while assessing a dog for its carrier status, as the agent is shed at intervals (Lowden et al., 2015). The fecal samples collected from the dogs in this study could be negative to *Salmonella* due to intermittent shedding, where it was not excreted in the fecal on the day of sampling, even if the dogs were carrier. Since, the samples were screened using PCR, method and were culture only once, which might have affected the specificity and sensitivity of our test. PCR screening requires, 3 consecutive cultures to be negative, to achieve 90% confident that the fecal sample is truly negative and for 99 % confident 6 cultures is required (Marks et al., 2011). Similarly, study have shown that pathogens such as *Salmonella* can enter VNBC state, due to stress making them unculturable using normal agar. During the baseline study, the PCR screen positive fecal did not yield any typical colonies in the selective plates possibly due to shedding of injured cells that were not culturable or dead cells with intact DNA. Since, PCR involves DNA, positive results do not necessarily suggest the presence of living cells, or viable and culturable cells. When we consider the possibility of intermittent fecal shedding before probiotic treatment, low specificity of PCR screening, as wells as VNBC state of *Salmonella* we might not be able to conclude any effects of probiotic treatment on Fecal shedding of *Salmonella*.

Studies on the effect of probiotic treatment on *Salmonella* in canines are rarely been conducted. Most of such studies are focused on poultry and cattle. Based on the results of various studies evaluating the effect of feeding probiotics on *Salmonella* such as a significant reduction in the frequency of *Salmonella* by more than 25% in the treated poultry compared with the controls on orally administering isolates PHL-MM65 and PHL-NP122 (Wolfenden et al., 2011), reduction in colonization by *Salmonella* Enteriditis (SE) as reported by Pascual et al., (1999) upon orally administering *Lactobacillus salivarius* strain CTC2197 to Chickens and reduction in mean fecal number of *Salmonella* at 15 days post infection observed in weaned pigs when a LAB mixture consisting of two strains of

L. murinus and one strain each of *Lactobacillus salivarius* subsp. *salivarius, L. pentosus, and Pediococcus pentosaceous* was administered (Casey et al., 2007), it is tempting to speculate that some dogs may have been shedding *Salmonella* intermittently before probiotic treatment and that probiotic treatment was able to control *Salmonella* fecal shedding in our dogs. However, future larger scale and more directed studies that include a higher proportion of dogs naturally shedding *Salmonella* are necessary to test the hypothesis that probiotic treatment in dry dog food reduces *Salmonella* fecal shedding in dogs.

CHAPTER IV
SUMMARY AND CONCLUSION

The group fed with L28+ commercial probiotic supplemented Kibbles had a higher reduction in the fecal population per indicator compared to the group fed with the non-probiotic kibble, L28 or the commercial probiotics. Like the control, the kibble with *Lactobacillus salivarius* L28 alone or with the commercial probiotics did not result in any significant overall shifts in microbial indicators in the fecal samples of the respective group during feeding. A significant average reduction in the fecal population of indicators was observed in the group of dogs fed with kibble consisting the combination of L28 and commercial probiotics. The higher combined dose and possible synergistic effect or symbiosis between L28 and commercial probiotics reduced GI population of microbial indicators in dogs assigned to the feeding group. The L28 + commercial probiotics was able to significantly reduce *E. coli* population in the fecal samples of the assigned dogs on day 20 by an average of 1.63 Log_{10} CFU/g fecal. The average reduction was significant on both day 10 and 20 for fecal coliforms at 1.18 Log_{10} CFU/g fecal. Similarly, on day 20, the total-coliforms reduced significantly by 1.46 Log_{10} CFU/g fecal, when compared to the baseline. The average reduction of fecal Entereobacteriaceae population was highest for group 3 on day 40 at 1.70 Log_{10} CFU/g fecal, respectively. However, supplement duration to observe significant effect differed among the dogs and the effect was not significant in all dogs assigned to the feeding group. Similarly, the average overall reduction in the generic *E. coli,* fecal coliforms, total coliforms, and, Entereobacteriaceae in the fecal samples of the L28 + commercial probiotics group during the treatment was the highest at 0.98, 0.92, 1.02, and 1.19 log_{10} CFU/g fecal, respectively. The reduction effect of the treatment on the indicators was stronger for male dogs in the group compared to females, except Entereobacteriaceae.

The reduction of the indicators suggests the potential of the treatment containing the probiotics combination to reduce enteric-pathogens in GI tract, and thus reduce incidence of gastro-intestinal diseases. Therefore, L28 have a potential to be used as a probiotic supplement in specific multi-species probiotic formulation to add better functionality in pet kibble for maintaining or improving gut health of dogs.

CHAPTER V
LIMITATION AND RECOMMENDATIONS

This preliminary feeding trial evaluated the efficacy of various combination of probiotic supplement including L28 to shift microbial indicators in fecal samples in dogs during feeding. The reductions of the microbial indicators were suggestive of potential reduction of enteric pathogens and improvement in dog's GI health. However, the results cannot be used to make any reliable conclusions. Future works should be focused on evaluating the potential of the supplements to control a disease or a pathogen then the organisms in general. Since, the indicators monitored in the study are a part of natural gut microflora and benefit canines as well, any negative health implication of the microbial shift must be assayed.

Similarly, considering the effect size and sample size the statistical power of this study is weak. The results may be inadequate or inconclusive to confirm the reduction effect, and attribute it to a probiotic supplement with confidence. Future studies be accompanied with large sample size in each feeding group to achieve a strong statistical power of the study. Whenever any supplement is designed they may not benefit canines of every breed, gender, or age. Therefore, evaluating the effect of factors such as breed, sex, gender, and management practices on the efficacy of probiotic supplement would be appropriate to target the supplement to a specific demographic group rather than dogs in general.

Although we don't have many reports on adverse clinical effects of probiotics on health, there is evidence showing potentially harmful effects of probiotics. Factor of importance that needs to be accounted for while evaluating the safety of probiotics is the antimicrobial resistance in the organisms. Pets may acquire resistance at the same time determinants of resistance may be transferred from non-pathogenic bacteria to opportunistic pathogens present in the host. Therefore, patterns of antimicrobial resistance should be evaluated for all products, and studies on antimicrobial resistance should be conducted before adding probiotics as dietary supplements in pet-food.

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APPENDIX

Table A.1: Demographic info of the dogs assigned to each of the four-feeding groups.

Dog ID	Dog Name	Treatment	Sex	Age	Breed
104	Jagger	1	M	2	Pit Mix
107	Joey	1	M	6	Corgi/Chihuaha Mix
109	Lowery	1	M	1	Pointer
112	Jager2	1	F	6	Lab Mix
120	Mountie	1	F	4	Cattle dog Mix
122	Zoe	1	F	9	Chow/Golden Mix
129	Diesel	1	M	13	Pit Mix
137	Sasha	1	F	11	Shep Mix
138	Tosca	1	F	8	Lab Mix
139	Sully	1	M	5	Pit Mix
145	Maya	1	F	4.5	Terrier Mix
146	Sadie	1	F	6	Lab Mix
106	Nebbie	2	F	6	Min Pin Mix
111	Lola	2	F	7	Lab Mix
115	Poot	2	M	10	Beagle Mix
118	Lily	2	F	6	Poodle Mix
124	Cinnamon	2	F	13	Sharpei Mix
130	Bree's	2	F	8	Lab Mix
136	Wally	2	M	5	Pit Mix
140	Momma	2	F	7	Shep Mix
141	Misfit	2	M	9	Pit Mix
143	Blue	2	M	11	Husky Mix
103	Lizzie	2	F	5	Pit Lab Mix
101	Bear	3	M	10	Border Collie Mix
108	Trouble	3	F	6	Lab

Table A.1 continued.

Dog ID	Dog Name	Treatment	Sex	Age	Breed
110	Yoshi	3	F	3	Pit Mix
114	Scruff	3	M	1	Terrier Mix
119	Gauge/Reid	3	M	1	Lab Mix
126	June	3	F	12	Pit Mix
131	Cherry	3	F	8	Pit Mix
132	Shorty	3	M	8	Dacuh Mix
133	Rucker	3	M	6	Jack Russel Mix
142	Phoenix	3	M	7	Chihuaha Mix
144	Luna	3	M	11	Husky Mix
147	Lena	3	F	5	Collie Mix
102	Cola	4	F	5	Shepherd Mix
105	Daisy	4	F	6	Pit Boxer Mix
113	Tasia	4	F	5	Min Pin Mix
116	Ralphie	4	M	3.5	Pit Mix
117	Rosie	4	F	3	Pit Mix
121	Liam	4	M	2	Silken Windhound
123	Pickles	4	F	4	Pit Mix
125	Anne	4	F	1	Dacuh Mix
127	Zeus	4	M	11	Lab Mix
128	CJ	4	M	10	Pit Mix
134	Tony	4	M	6	Shep Mix
135	Annie	4	F	4	Basenji Mix

Here, Treatment 1= kibble without probiotics, 2= kibble with L28, 3= kibble with L28+ commercial strain and 4= kibble with commercial strains, F=Female and M=Male.

Table A.2: Average Log-Transformed counts of L28 in the kibbles over a period of 4 months.

Date	Concentration (log CFU/pound)
03/08/2017	6.10
03/24/2017	6.14
04/21/2017	6.17
05/16/2017	6.11
06/30/2017	5.20

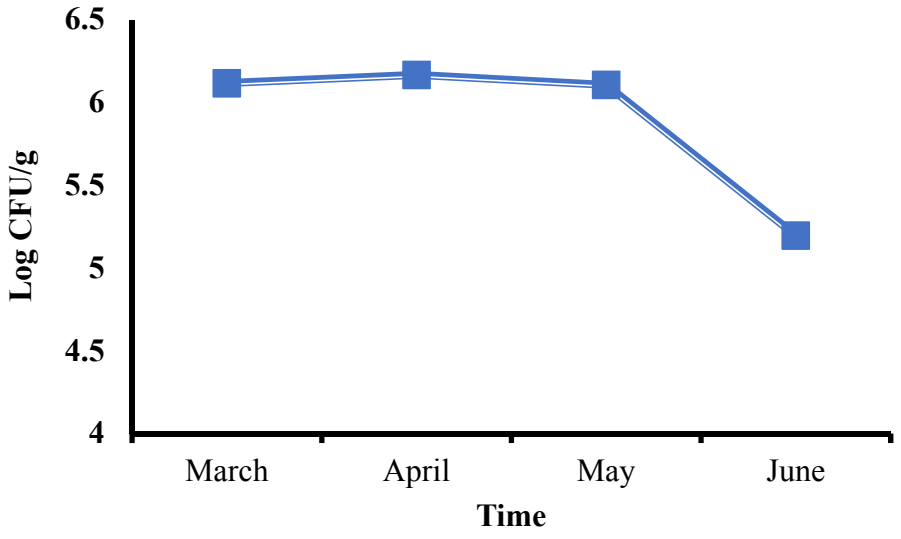

Figure A. 1: Long-term death curve of L28 over the period of 4 months stored in a typical warehouse condition.

Table A. 3: Log- Transformed counts of Generic *E. coli* in fecal samples of dogs before and during treatment.

		DAY							
ANIMAL ID	**GROUP**	**-3**	**-2**	**-1**	**Baseline**	**5**	**10**	**20**	**40**
104	One	4.23	4.29	2.30	**3.61**	2.30	3.00	1.00	1.90
107	One	4.25	4.47	4.07	**4.26**	2.30	1.47	1.30	1.00
109	One	3.30	2.00	2.70	**2.67**	4.62	2.60	4.07	2.04
112	One	5.17	5.53	4.94	**5.21**	4.65	4.14	4.23	5.11
120	One	4.73	5.20	5.17	**5.03**	1.00	4.69	5.20	4.48
122	One	2.00	1.86	5.41	**3.09**	5.63	3.30	4.36	2.61
129	One	3.00	3.47	4.00	**3.49**	1.90	1.00	1.00	4.51
137	One	2.54	2.60	1.78	**2.31**	3.00	1.00	2.00	5.27
138	One	4.56	3.00	4.32	**3.96**	5.43	2.30	2.60	1.00
139	One	2.44	2.00	2.41	**2.28**	5.70	3.00	4.41	3.30
145	One	5.73	5.00	1.00	**3.91**	3.40	3.90	1.00	3.15
146	One	4.40	3.00	1.00	**2.80**	4.56	5.62	4.58	2.60
103	Two	4.27	4.30	3.60	**4.06**	1.00	1.00	1.00	2.30
106	Two	4.81	4.74	5.47	**5.01**	2.84	1.69	4.90	1.00
111	Two	3.70	4.50	5.43	**4.54**	5.25	4.87	3.00	4.32
115	Two	2.30	4.93	4.95	**4.06**	4.88	1.30	4.28	1.60
118	Two	2.00	1.30	1.00	**1.43**	3.47	4.23	3.00	1.00
124	Two	5.53	3.00	4.72	**4.42**	1.30	5.27	2.85	5.63
130	Two	3.30	2.47	1.32	**2.36**	3.11	4.11	4.30	3.48
136	Two	1.84	1.95	1.60	**1.80**	1.56	1.60	4.11	1.00
140	Two	5.27	5.59	5.51	**5.46**	5.11	5.70	5.00	3.00
141	Two	5.74	3.00	4.73	**4.49**	4.68	4.18	3.48	2.90
143	Two	1.48	2.11	2.17	**1.92**	1.17	3.00	5.59	4.80
101	Three	5.50	5.15	5.30	**5.32**	2.00	2.73	1.30	4.54
108	Three	3.81	2.60	2.00	**2.80**	2.60	1.00	1.00	1.00
110	Three	4.79	5.43	5.11	**5.11**	5.20	4.60	5.00	5.53

Table A. 4 continued.

		DAY							
ANIMAL ID	**GROUP**	**-3**	**-2**	**-1**	**Baseline**	**5**	**10**	**20**	**40**
114	Three	2.60	1.30	4.51	**2.80**	1.53	3.00	1.00	1.00
119	Three	2.47	4.68	2.76	**3.30**	1.00	2.17	2.30	2.57
126	Three	4.57	3.30	3.30	**3.72**	1.60	1.00	1.00	2.81
131	Three	5.52	5.47	5.27	**5.42**	2.00	4.04	1.85	1.48
132	Three	2.23	1.83	1.13	**1.73**	1.00	1.00	1.00	1.48
133	Three	2.76	2.57	1.32	**2.22**	1.00	1.00	1.00	4.43
142	Three	3.38	2.70	3.41	**3.16**	4.66	1.60	1.00	1.00
144	Three	2.60	2.00	2.00	**2.20**	3.00	3.00	3.00	3.70
147	Three	4.41	5.84	2.30	**4.18**	4.41	5.07	3.00	2.00
102	Four	4.76	3.61	1.47	**3.28**	4.91	3.47	4.04	4.94
105	Four	3.41	3.77	2.47	**3.22**	1.77	1.00	1.69	3.60
113	Four	4.23	5.39	2.77	**4.13**	3.00	4.61	2.47	1.70
116	Four	5.38	5.49	5.30	**5.39**	5.30	5.49	4.95	4.11
117	Four	2.00	2.35	2.67	**2.34**	1.00	3.79	1.47	2.91
121	Four	2.52	4.20	2.13	**2.95**	4.41	1.30	3.62	2.30
123	Four	1.00	2.00	1.00	**1.33**	1.00	4.32	2.48	3.85
125	Four	6.00	1.74	5.04	**4.26**	5.00	3.47	3.30	5.56
127	Four	4.48	4.57	5.11	**4.72**	5.14	2.00	4.87	3.30
128	Four	5.17	3.14	4.59	**4.30**	3.30	3.48	3.78	4.61
134	Four	2.30	2.00	2.34	**2.21**	2.07	1.48	2.25	1.00
135	Four	4.67	3.00	3.77	**3.81**	1.00	3.84	4.92	5.42

Here, Group One= kibble without probiotics, Two= kibble with L28, Three= kibble with L28+ commercial strain and Four= kibble with commercial strains,

Table A.5: Log- Transformed counts of fecal coliforms in fecal samples of dogs before and during treatment

		DAY							
ANIMAL ID	**GROUP**	**-3**	**-2**	**-1**	**Baseline**	**5**	**10**	**20**	**40**
104	One	4.65	4.58	3.20	**4.14**	3.04	4.22	3.69	3.41
107	One	5.26	5.41	3.48	**4.72**	1.98	1.47	2.89	1.00
109	One	3.37	3.39	2.70	**3.16**	5.54	3.28	3.72	2.96
112	One	5.30	5.82	4.85	**5.32**	4.44	4.16	3.59	4.00
120	One	5.02	4.29	4.48	**4.59**	2.67	4.23	4.29	5.43
122	One	3.76	1.44	4.39	**3.20**	5.76	3.48	4.15	4.02
129	One	3.00	3.01	2.98	**2.33**	3.34	1.00	1.00	3.43
137	One	4.35	3.41	3.90	**3.89**	4.59	3.20	2.97	5.64
138	One	3.80	1.00	4.16	**2.99**	4.41	2.95	3.25	1.00
139	One	3.53	1.58	1.08	**2.06**	5.44	3.59	4.18	3.80
145	One	5.75	5.18	4.04	**4.99**	4.32	5.79	2.11	4.41
146	One	4.32	3.93	2.00	**3.42**	4.51	5.30	4.61	2.11
103	Two	4.40	3.54	4.11	**4.02**	2.60	2.57	2.50	3.43
106	Two	4.35	4.10	5.80	**4.75**	2.49	2.87	3.75	1.00
111	Two	4.03	4.46	5.41	**4.63**	4.99	4.55	2.24	4.04
115	Two	2.30	4.17	4.45	**3.64**	4.12	2.25	1.00	3.35
118	Two	2.77	3.19	2.41	**2.79**	3.68	4.10	1.00	1.00
124	Two	5.62	3.00	4.22	**4.28**	3.19	5.38	2.48	5.36
130	Two	3.69	3.30	2.83	**3.27**	2.69	2.96	3.88	3.98
136	Two	3.18	1.04	2.98	**2.40**	3.64	2.65	3.85	1.00
140	Two	5.87	5.65	5.42	**5.65**	4.47	5.41	4.58	5.37
141	Two	5.10	3.47	4.66	**4.41**	3.53	3.27	3.84	2.95
143	Two	1.84	2.87	2.55	**2.42**	3.10	3.83	5.41	4.09
101	Three	5.18	4.65	4.84	**4.89**	2.29	1.71	3.62	4.48
108	Three	4.16	4.96	3.34	**4.15**	3.61	2.30	3.25	1.00
110	Three	4.56	5.63	5.88	**5.36**	4.44	3.27	4.46	4.48

Table A.6 continued.

		DAY							
ANIMAL ID	**GROUP**	**-3**	**-2**	**-1**	**Baseline**	**5**	**10**	**20**	**40**
114	Three	2.95	2.99	4.19	**3.38**	4.36	3.15	1.00	1.00
119	Three	3.23	4.47	2.41	**3.37**	2.62	3.59	2.59	3.41
126	Three	2.94	1.00	2.98	**2.31**	3.33	1.00	1.00	3.92
131	Three	5.10	5.47	5.33	**5.30**	3.53	3.28	2.59	3.15
132	Three	3.15	2.12	1.68	**2.32**	2.50	1.00	1.00	3.46
133	Three	3.69	1.66	1.90	**2.42**	2.85	1.00	2.86	4.42
142	Three	4.10	3.94	4.39	**4.14**	4.62	3.04	1.00	1.00
144	Three	3.94	2.88	3.36	**3.39**	2.78	3.94	3.78	1.00
147	Three	4.11	4.90	4.36	**4.46**	4.21	4.00	4.15	2.85
102	Four	4.00	4.14	2.66	**3.60**	4.21	2.88	4.25	3.31
105	Four	3.25	3.31	3.17	**3.25**	2.80	3.07	3.16	2.27
113	Four	5.26	4.56	3.54	**4.45**	3.84	4.59	2.90	3.46
116	Four	4.62	4.99	4.47	**4.70**	5.80	5.58	4.04	5.99
117	Four	3.66	2.94	3.00	**3.20**	3.55	4.42	3.13	1.00
121	Four	2.41	4.36	4.14	**3.64**	4.64	2.55	3.88	4.17
123	Four	3.00	2.70	3.69	**2.46**	2.93	4.39	2.91	5.27
125	Four	6.00	2.16	4.21	**4.12**	4.24	3.29	3.43	5.54
127	Four	4.25	4.07	4.35	**4.22**	4.31	2.38	3.54	3.30
128	Four	3.84	3.56	4.36	**3.92**	3.51	3.48	3.28	4.20
134	Four	3.47	2.00	4.03	**3.17**	3.94	2.11	3.18	1.00
135	Four	4.17	3.00	1.00	**2.72**	1.00	4.26	4.09	5.59

Here, Group One= kibble without probiotics, Two= kibble with L28, Three= kibble with L28+ commercial strain and Four= kibble with commercial strains

Table A.7: Log- Transformed counts of total coliforms in fecal samples of dogs before and during treatment.

		DAY							
ANIMAL ID	**GROUP**	**-3**	**-2**	**-1**	**Baseline**	**5**	**10**	**20**	**40**
104	One	4.79	4.76	3.25	**4.27**	3.11	4.25	3.69	3.43
107	One	5.30	5.46	4.17	**4.97**	2.47	1.77	2.90	1.00
109	One	3.64	3.41	3.00	**3.35**	5.59	3.36	4.23	3.01
112	One	5.54	6.00	5.20	**5.58**	4.86	4.45	4.32	5.15
120	One	5.20	5.25	5.25	**5.23**	2.68	4.82	5.25	5.48
122	One	3.77	2.00	5.45	**3.74**	6.00	3.70	4.57	4.04
129	One	3.00	3.60	4.04	**3.55**	3.36	1.00	0.30	4.54
137	One	4.36	3.47	3.90	**3.91**	4.60	3.20	3.01	5.80
138	One	4.63	3.00	4.55	**4.06**	5.47	3.04	3.34	1.00
139	One	3.56	2.14	2.43	**2.71**	5.89	3.69	4.61	3.92
145	One	6.04	5.40	4.04	**5.16**	4.37	5.79	2.12	4.44
146	One	4.66	3.98	2.00	**3.55**	4.83	5.79	4.89	2.72
103	Two	4.64	4.37	4.23	**4.41**	2.60	2.57	2.50	3.46
106	Two	4.94	4.83	5.97	**5.25**	3.00	2.90	4.93	1.00
111	Two	4.20	4.78	5.72	**4.90**	5.44	5.04	3.07	4.51
115	Two	2.60	5.00	5.07	**4.22**	4.95	2.30	4.28	3.36
118	Two	2.84	3.20	2.43	**2.82**	3.89	4.47	3.00	1.00
124	Two	5.88	3.30	4.84	**4.67**	3.20	5.63	3.00	5.82
130	Two	3.84	3.36	2.84	**3.35**	3.25	4.14	4.44	4.10
136	Two	3.20	2.00	3.00	**2.73**	3.64	2.69	4.30	1.00
140	Two	5.97	5.92	5.77	**5.89**	5.20	5.88	5.14	5.37
141	Two	5.83	3.60	5.00	**4.81**	4.71	4.23	4.00	3.23
143	Two	2.00	2.94	2.70	**2.55**	3.11	3.89	5.81	4.88
101	Three	5.67	5.27	5.43	**5.46**	2.47	2.77	3.62	4.81
108	Three	4.32	4.96	3.36	**4.21**	3.65	2.30	3.25	1.00
110	Three	4.99	5.84	5.95	**5.59**	5.27	4.62	5.11	5.57

Table A.8 continued.

		DAY							
ANIMAL ID	**GROUP**	**-3**	**-2**	**-1**	**Baseline**	**5**	**10**	**20**	**40**
114	Three	3.11	3.00	4.68	**3.60**	4.36	3.38	1.00	1.00
119	Three	3.30	4.89	2.92	**3.70**	2.62	3.61	2.77	3.47
126	Three	4.58	3.30	3.47	**3.78**	3.34	1.00	1.00	3.96
131	Three	5.66	5.77	5.60	**5.68**	3.54	4.11	2.66	3.16
132	Three	3.20	2.30	1.79	**2.43**	2.51	1.00	1.00	3.46
133	Three	3.74	2.62	2.00	**2.79**	2.85	1.00	2.86	4.73
142	Three	4.18	3.96	4.43	**4.19**	4.94	3.06	1.00	1.00
144	Three	3.96	2.93	3.38	**3.42**	3.20	3.99	3.85	3.70
147	Three	4.59	5.89	4.36	**4.95**	4.63	5.11	4.18	2.90
102	Four	4.83	4.25	2.69	**3.92**	4.99	3.57	4.46	4.95
105	Four	3.64	3.90	3.25	**3.60**	2.84	3.07	3.17	3.62
113	Four	5.30	5.45	3.61	**4.79**	3.90	4.90	3.04	3.47
116	Four	5.45	5.61	5.36	**5.47**	5.92	5.84	5.00	6.00
117	Four	3.67	3.04	3.17	**3.29**	3.55	4.51	3.14	2.91
121	Four	2.77	4.59	4.14	**3.83**	4.84	2.57	4.07	4.18
123	Four	1.00	2.78	3.69	**2.49**	2.93	4.66	3.05	5.28
125	Four	6.30	2.30	5.10	**4.57**	5.07	3.69	3.67	5.85
127	Four	4.68	4.69	5.18	**4.85**	5.20	2.53	4.89	3.60
128	Four	5.19	3.70	4.79	**4.56**	3.72	3.78	3.90	4.76
134	Four	3.50	2.30	4.04	**3.28**	3.95	2.20	3.23	1.00
135	Four	4.79	3.30	3.77	**3.95**	1.00	4.40	4.98	5.81

Here, Group One= kibble without probiotics, Two= kibble with L28, Three= kibble with L28+ commercial strain and Four= kibble with commercial strains,

Table A.9:Log- Transformed counts of Entereobacteriaceae in fecal samples of dogs before and during treatment.

		DAY							
ANIMAL ID	**GROUP**	**-3**	**-2**	**-1**	**Baseline**	**5**	**10**	**20**	**40**
104	One	6.00	6.00	2.30	**4.77**	4.04	4.07	3.95	2.76
107	One	3.25	3.11	3.81	**3.39**	4.07	4.41	3.41	1.00
109	One	4.27	4.23	4.20	**4.23**	4.32	4.15	3.93	2.81
112	One	4.38	4.11	5.30	**4.60**	4.75	4.11	4.25	4.56
120	One	5.30	4.23	5.11	**4.88**	3.04	4.99	5.36	5.49
122	One	4.25	3.34	5.30	**4.30**	3.07	3.62	4.38	4.22
129	One	3.18	3.90	4.78	**3.95**	3.07	3.34	2.17	4.69
137	One	4.14	3.36	4.32	**3.94**	4.46	2.64	2.68	6.00
138	One	3.80	4.25	4.45	**4.17**	3.93	3.11	2.90	1.00
139	One	4.07	3.04	4.41	**3.84**	2.11	2.36	4.74	4.18
145	One	5.76	5.20	4.22	**5.06**	4.05	5.72	2.00	4.45
146	One	4.61	3.43	1.00	**3.01**	4.72	6.00	4.79	2.15
103	Two	4.28	4.16	3.27	**3.90**	4.78	4.79	3.20	3.84
106	Two	4.78	4.30	5.07	**4.72**	3.00	3.90	4.53	1.84
111	Two	4.25	4.83	5.54	**4.87**	3.47	4.17	2.98	4.42
115	Two	4.25	4.93	5.39	**4.86**	4.23	2.95	3.74	3.08
118	Two	3.49	3.25	3.00	**3.25**	3.72	4.14	2.83	1.00
124	Two	5.69	3.84	4.70	**4.74**	2.20	2.44	2.56	5.66
130	Two	3.54	4.00	3.47	**3.67**	3.27	4.15	4.59	3.60
136	Two	4.23	3.77	4.25	**4.08**	3.78	1.85	4.00	1.00
140	Two	5.07	5.72	5.23	**5.34**	5.04	6.00	5.15	2.26
141	Two	5.79	3.04	4.14	**4.32**	4.71	4.29	3.79	3.00
143	Two	3.69	2.63	2.69	**3.00**	3.28	4.04	2.30	5.17
101	Three	4.83	4.94	5.50	**5.09**	3.47	4.78	2.77	4.68
108	Three	3.30	3.90	3.34	**3.51**	3.30	3.00	2.50	2.23
110	Three	5.19	4.74	5.94	**5.29**	3.60	4.69	5.07	4.45

Table A.10 continued.

		DAY							
ANIMAL ID	**GROUP**	**-3**	**-2**	**-1**	**Baseline**	**5**	**10**	**20**	**40**
114	Three	4.00	3.60	5.27	**4.29**	5.32	4.53	1.70	1.00
119	Three	4.20	4.65	3.07	**3.97**	3.00	2.63	2.95	3.04
126	Three	4.49	4.27	4.30	**4.35**	3.14	2.40	2.30	3.18
131	Three	5.69	4.11	4.49	**4.76**	3.27	3.75	2.60	2.81
132	Three	4.34	4.36	3.17	**3.96**	2.90	2.57	2.60	1.00
133	Three	4.26	4.20	3.14	**3.87**	1.00	1.00	2.62	4.45
142	Three	2.20	3.54	5.04	**3.60**	4.75	3.20	1.00	1.00
144	Three	3.32	2.15	3.64	**3.04**	2.70	3.73	3.95	1.00
147	Three	4.53	5.72	3.30	**4.52**	4.78	5.50	1.00	1.00
102	Four	4.30	4.10	2.00	**3.47**	5.76	4.17	4.07	4.89
105	Four	2.47	2.77	3.34	**2.86**	4.36	4.17	3.30	3.99
113	Four	5.44	5.54	4.90	**5.29**	4.36	4.55	1.00	3.08
116	Four	4.66	3.77	5.41	**4.61**	6.00	6.00	5.07	1.60
117	Four	4.17	4.27	4.07	**4.17**	4.11	3.62	1.00	2.94
121	Four	4.23	4.14	3.32	**3.90**	4.46	1.95	4.14	3.51
123	Four	1.00	2.53	1.00	**1.51**	2.70	2.23	2.57	5.40
125	Four	6.00	3.04	4.99	**4.68**	5.20	3.92	3.60	5.59
127	Four	4.60	4.69	5.20	**4.83**	5.34	3.11	5.11	3.86
128	Four	5.11	4.17	4.45	**4.58**	3.90	3.43	4.01	4.82
134	Four	4.38	3.84	4.23	**4.15**	3.98	1.48	2.11	1.60
135	Four	4.36	4.30	3.90	**4.19**	2.30	1.00	4.63	6.00

Here, Group One= kibble without probiotics, Two= kibble with L28, Three= kibble with L28+ commercial strain and Four= kibble with commercial strains.

Table A.11: Counts of Generic *E. coli* (Log CFU/g fecal) in fecal of each feeding group before and during treatment.

	Day							
	Adjustment Phase			Treatment Phase				
Treatment	**3**	**2**	**1**	**Baseline**	**5**	**10**	**20**	**40**
Control	3.86±1.18	3.53±1.32	3.26±1.59	**3.55±0.97^{ax}**	3.71±01.16^{ax}	3.00±1.44^{ax}	2.98±1.64^{ax}	3.08±1.49^{abx}
L28	3.66±1.58	3.44±1.42	3.68±1.81	**3.59±1.43^{ax}**	3.12±1.68^{ax}	3.36±1.70^{ax}	3.77±1.28^{ax}	2.82±1.62^{abx}
Commercial+L28	3.72±1.21	3.57±1.63	3.20±1.53	**3.50±1.26^{ax}**	2.50±1.50^{axy}	2.52±1.47^{axy}	1.87±1.26^{by}	2.63±1.58^{axy}
Commercial	3.83±1.55	3.44±1.30	3.22±1.49	**3.50±1.16^{ax}**	3.16±1.75^{ax}	3.19±1.42^{ax}	3.32±1.25^{abx}	3.61±1.44^{abx}

^{ab} Means in column without common letters differ ($P \leq 0.05$). ^{xy} Means in row without common letters differ ($P \leq 0.05$). Mean ± Standard Deviation. Comparison made between baseline and treatment phase.

Table A.12: Counts of fecal coliforms (Log CFU/g fecal) in fecal of each feeding group before and during treatment.

	Day							
	Adjustment Phase			Treatment Phase				
Treatment	**3**	**2**	**1**	**Baseline**	**5**	**10**	**20**	**40**
Control	4.18±1.25	3.59±1.60	3.44±1.11	**3.73±1.05^{ax}**	4.17±1.19^{ax}	3.56±1.37^{ax}	3.37±1.02^{ax}	3.44±1.49^{ax}
L28	3.92±1.32	3.53±1.15	4.08±1.22	**3.84±1.04^{ax}**	3.50±0.80^{ax}	3.62±1.12^{ax}	3.14±1.41^{ax}	3.23±1.61^{ax}
Commercial+L28	3.93±0.77	3.72±1.55	3.72±1.33	**3.79±1.10^{ax}**	3.43±0.83^{axy}	2.61±1.16^{ay}	2.61±1.31^{ay}	2.85±1.46^{axy}
Commercial	3.83±1.28	3.48±0.96	3.55±0.99	**3.62±0.69^{ax}**	3.73±1.17^{ax}	3.58±1.06^{ax}	3.48±0.47^{ax}	3.76±1.70^{ax}

^{ab}Means in column without common letters differ ($P \leq 0.05$). ^{xy}Means in row without common letters differ ($P \leq 0.05$). Mean ± Standard Deviation. Comparison made between baseline and treatment phase.

Table A.13: Counts of total Coliforms (Log CFU/g fecal) in fecal of each feeding group before and during treatment.

	Day							
	Adjustment Phase				Treatment Phase			
Treatment	**3**	**2**	**1**	**Baseline**	**5**	**10**	**20**	**40**
Control	4.54±0.91	4.04±1.33	3.94±1.10	**4.17±0.88^{ax}**	4.44±1.25^{ax}	3.74±1.43^{ax}	3.60±1.39^{abx}	3.71±1.57^{ax}
L28	4.18±1.40	3.94±1.14	4.32±1.35	**4.15±1.12^{ax}**	3.91±1.00^{ax}	3.98±1.24^{ax}	4.04±1.04^{ax}	3.43±1.75^{ax}
Commercial+L28	4.27±0.88	4.23±1.36	3.95±1.36	**4.15±1.07^{ax}**	3.62±0.97^{axy}	3.00±1.42^{axy}	2.69±1.41^{by}	3.23±1.54^{axy}
Commercial	4.26±1.42	3.83±1.13	4.07±0.87	**4.05±0.83^{ax}**	3.99±1.33^{ax}	3.81±1.10^{ax}	3.88±0.78^{abx}	4.29±1.47^{ax}

^{ab}Means in column without common letters differ ($P \leq 0.05$). ^{xy}Means in row without common letters differ ($P \leq 0.05$). Mean ± Standard Deviation. Comparison made between baseline and treatment phase.

Table A.14: Counts of Entereobacteriaceae (Log CFU/g fecal) in fecal of each feeding group before and during treatment.

	Day							
	Adjustment Phase				Treatment Phase			
Treatment	**3**	**2**	**1**	**Baseline**	**5**	**10**	**20**	**40**
Control	4.42±0.89	4.02±0.88	4.10±1.26	**4.18±0.60^{ax}**	3.80±0.81^{ax}	4.04±1.13^{ax}	3.71±1.08^{ax}	3.61±1.65^{abx}
L28	4.46±0.79	4.04±0.90	4.25±1.02	**4.25±0.74^{ax}**	3.77±0.86^{ax}	3.88±1.13^{ax}	3.61±0.92^{ax}	3.17±1.56^{abx}
Commercial+L28	4.20±0.93	4.18±0.88	4.18±1.04	**4.19±0.66^{ax}**	3.44±1.14^{axy}	3.48±1.27^{axy}	2.59±1.13^{by}	2.49±1.49^{ay}
Commercial	4.23±1.32	3.93±0.84	3.90±1.32	**4.02±1.02^{ax}**	4.37±1.12^{ax}	3.30±1.43^{ax}	3.38±1.43^{abx}	3.94±1.47^{bx}

^{ab}Means in column without common letters differ ($P \leq 0.05$). ^{xy}Means in row without common letters differ ($P \leq 0.05$). Mean ± Standard Deviation. Comparison made between baseline and treatment phase.

Table A. 15: Baseline to treatment ratio for generic *E. coli*, fecal coliforms, total coliforms, and Enterobacteriaceae for the four feeding groups.

	Organism			
Treatment	***E. coli***	**Fecal coliforms**	**Total coliforms**	**Entereobacteriaceae**
Control	1.56[ab]	1.25[a]	1.52[ab]	1.26[a]
L28	1.49[ab]	1.37[ab]	1.26[b]	1.35[a]
L28+commercial	1.89[a]	1.65[b]	1.69[a]	1.79[b]
Commercial	1.27[b]	1.14[a]	1.14[b]	1.35[a]

[ab] Means in column without common letters differ ($P \leq 0.05$).

Table A. 16:ANOVA for Treatments and Days including their interaction for generic *E. coli*, fecal coliforms, total coliforms, and Enterobacteriaceae.

Organism	**Variables**	**(Df1, Df2)**	**F value**	**P values**
E. coli	Treatment	3,172	3.11	0.03
	Day	3,172	0.10	0.95
	Treatment: Day	9, 172	2.11	0.76
Fecal Coliforms	Treatment	3,172	3.67	0.01
	Day	3,172	3.16	0.03
	Treatment: Day	9, 172	0.46	0.89
Total Coliforms	Treatment	3,172	2.25	0.08
	Day	3,172	1.50	0.21
	Treatment: Day	9, 172	0.72	0.68
Enterobacteriaceae	Treatment	3,173	3.68	0.01
	Day	3,172	3.85	0.01
	Treatment: Day	9, 172	1.02	0.42

Where, Df= Degree of Freedom.

Table A. 17: ANOVA (Day and Subjects as variables) for L28 + commercial probiotics for each of the four indicators.

Organism	**Variables**	**(Df1, Df2)**	**F value**	**P values**
E. coli	Day	4,24	9.23	<0.001
	Subjects	11, 24	9.25	<0.001
	Day: Subjects	44, 24	1.78	0.06
Fecal Coliforms	Day	4,24	12.5776	<0.001
	Subjects	11, 24	8.2302	<0.001
	Day: Subjects	44, 24	2.9038	0.003292
Total Coliforms	Day	4,24	18.3759	<0.001
	Subjects	11, 24	13.375	<0.001
	Day: Subjects	44, 24	3.4769	0.0008515
Entereobacteriaceae	Day	4,24	19.4622	<0.001
	Subjects	11, 24	4.6884	0.0007608
	Day: Subjects	44, 24	3.0037	0.0025777

Where, Df= Degree of Freedom, P-values obtained in scientific notation is denoted as "<0.001"

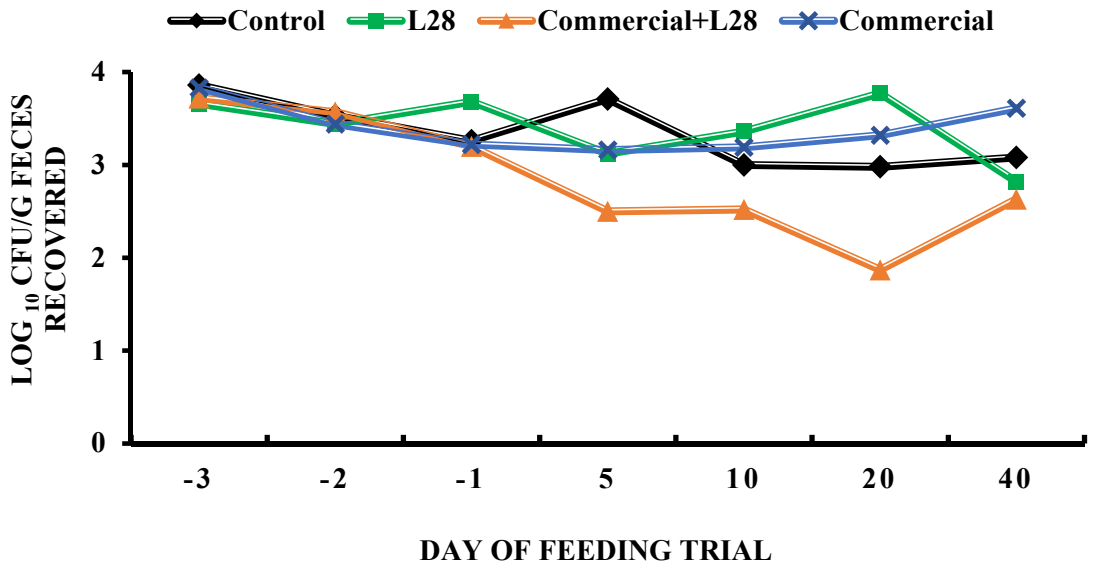

Figure A. 2: Generic *Escherichia coli* in dog fecal samples of each of the feeding group collected before and during probiotic treatment.

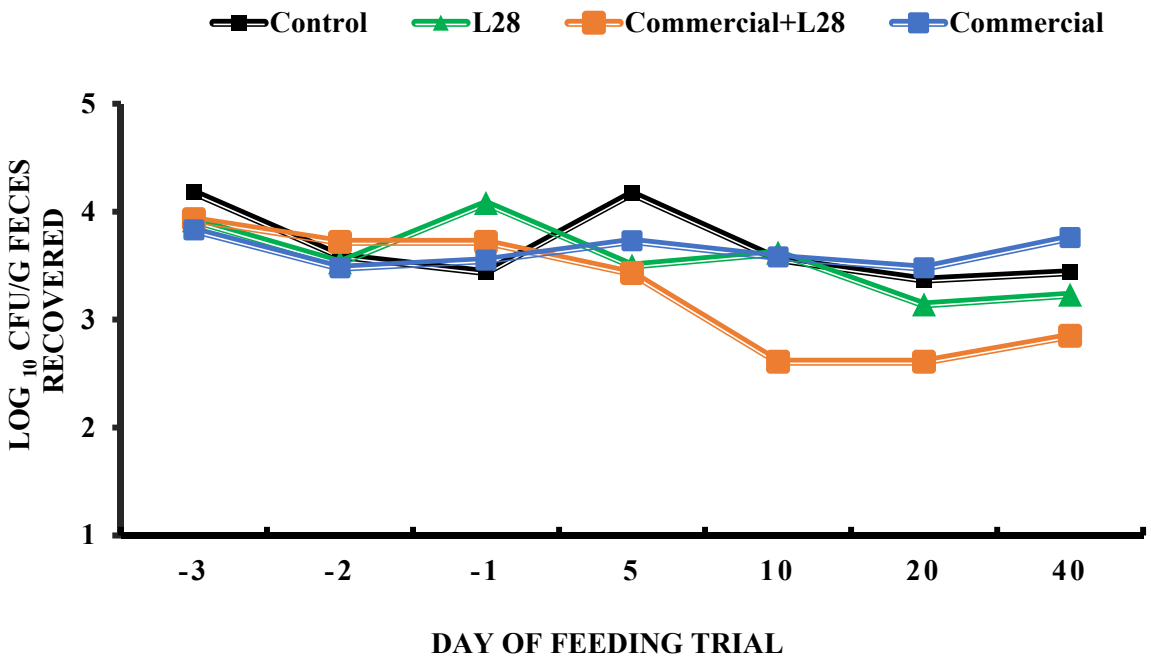

Figure A.3: Fecal coliforms in dog fecal samples of each of the feeding group collected before and during probiotic treatment.

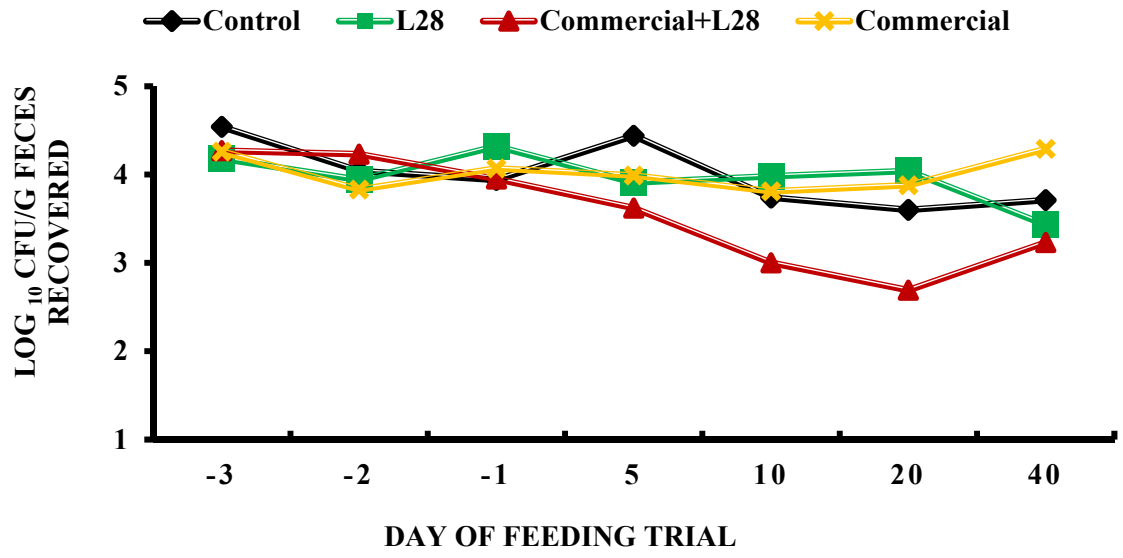

Figure A.4: Total coliforms in dog fecal samples of each of the feeding group collected before and during probiotic treatment.

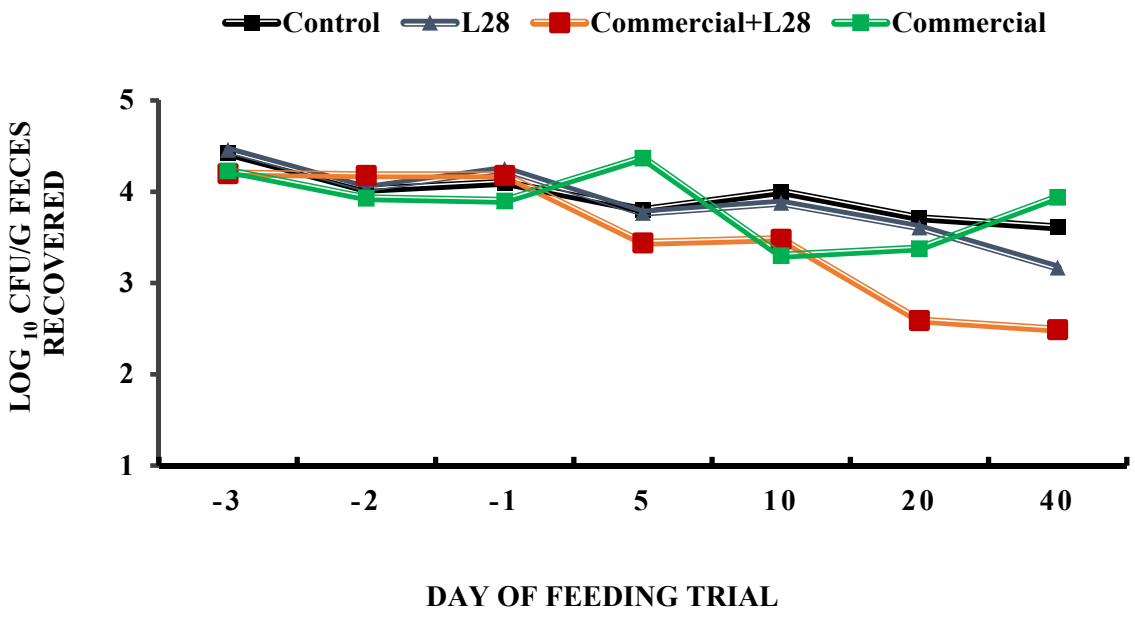

Figure A.5: Entereobacteriaceae in dog fecal samples of each of the feeding group collected before and during probiotic treatment.

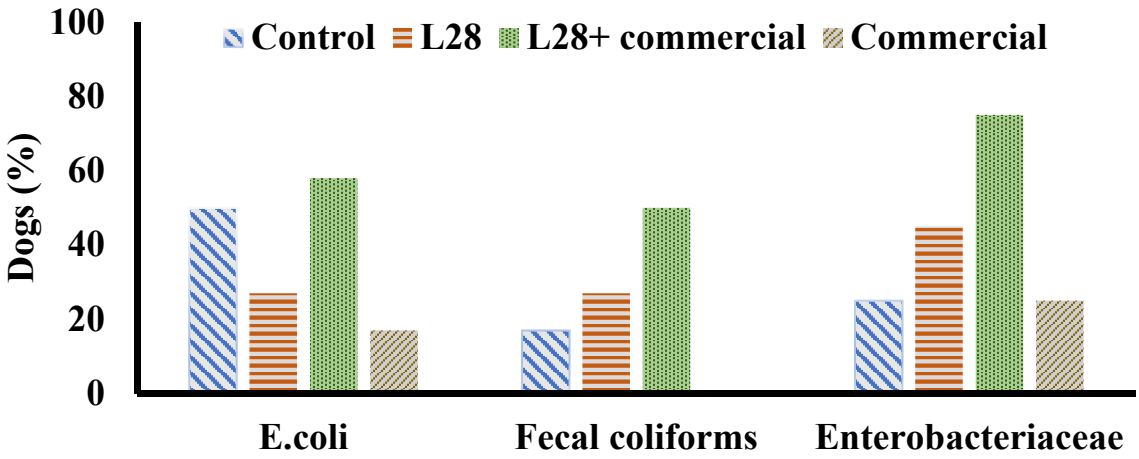

Figure A. 6: Percentage of dogs in each feeding group with reduction of indicators greater than 1- log cycle during treatment.

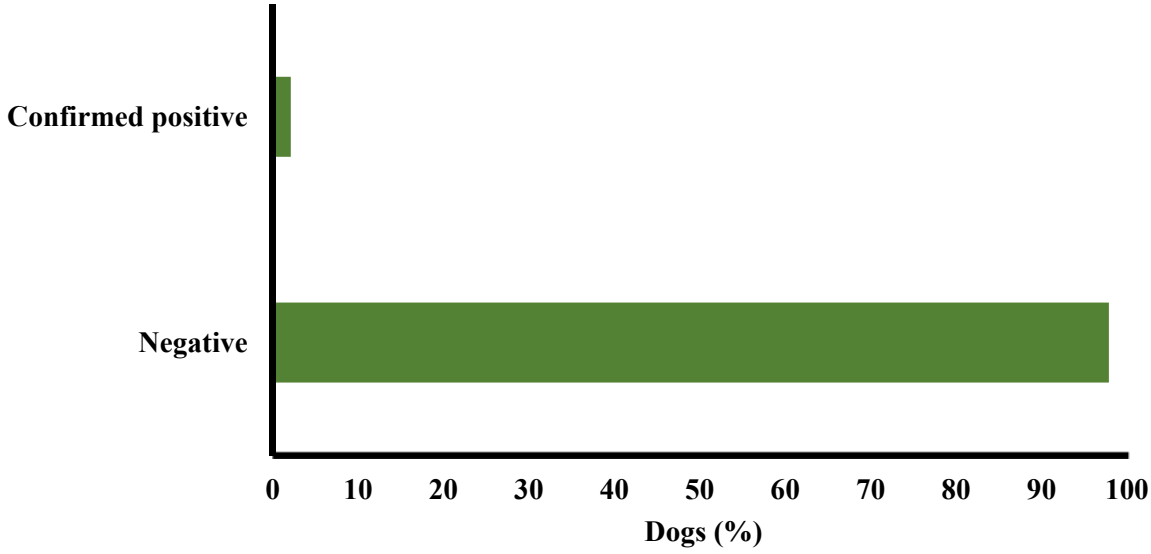

Figure A.7: Detection of *Salmonella* in the dogs under the feeding trial.

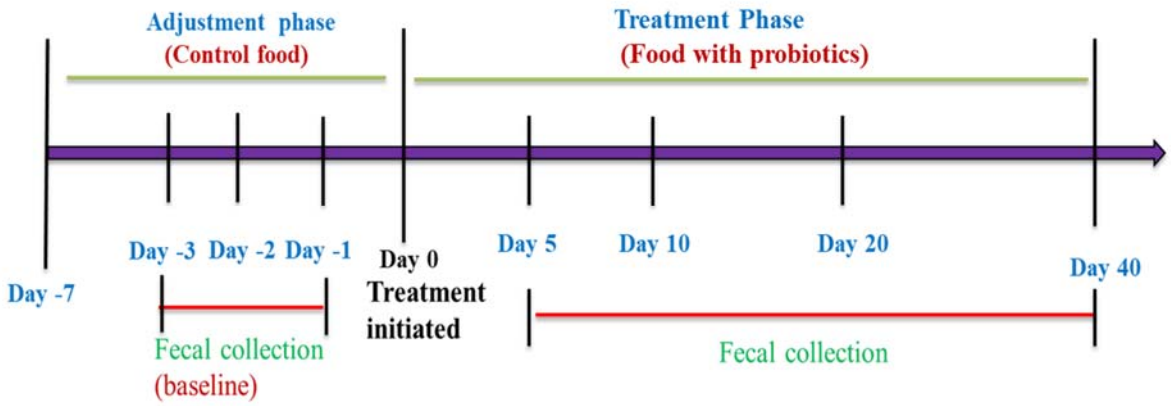

Figure A.8: Timeline of the feeding trial