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Investigating the Potential of Plant-derived Antimicrobials and Probiotic Bacteria for Improving the Microbiological Safety of Dry Pet Food

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**Investigating the Potential of Plant-derived Antimicrobials and Probiotic Bacteria for
Improving the Microbiological Safety of Dry Pet Food**

Chi-Hung Chen

B.A., National Chiayi University, Taiwan, 2010

A Thesis

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Masters of Science

at the

University of Connecticut

2017

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APPROVAL PAGE

Masters of Science Thesis

**Investigating the Potential of Plant-derived antimicrobials and Probiotic Bacteria for
Improving the Microbiological Safety of Dry Pet Food**

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List of Abbreviations

PDAs	Plant-derived antimicrobials
TC	Trans-cinnamaldehyde
CR	Carvacrol
TY	Thymol
EG	Eugenol
CA	Caprylic Acid
NA	Nalidixic acid
CH	Chitosan
TSB	Tryptic Soy Broth
XLD	Xylose Lysine Desoxycholate
KCl	Potassium Chloride
MRS	De Man-Rogosa-Sharpe
LAB	Lactic Acid Bacteria
MALDI-TOF	Matrix assisted laser desorption ionization
PCR	Polymerase chain reaction

**Investigating the Potential of Plant-derived Antimicrobials and Probiotic Bacteria for
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Chi-Hung Chen, M.S.

University of Connecticut, 2017

Salmonella is a major human pathogen in the United States, with contaminated food products as the major source of infections. However, contaminated pet food and contact with infected companion animals can potentially transmit salmonellosis to humans. Recent multistate human outbreaks of salmonellosis linked to commercial contaminated dry dog foods underscore the need for controlling the pathogen in pet food for protecting pet health and human health. In this M.S. thesis project, the efficacy of five Generally Recognized as Safe (GRAS)-status, plant-derived antimicrobials (PDAs), namely trans-cinnamaldehyde (TC), carvacrol (CR), thymol (TY), eugenol (EG), and caprylic acid (CA) applied as a vegetable oil or chitosan-based antimicrobial spray on dry dog food for reducing *Salmonella* Schwarzengrund was investigated. The effect of betaine, a compatible solute on the survival of probiotic bacteria, namely *Lactobacillus plantarum* NRRL B 4496, *Lactobacillus casei* ATCC 334, and *Lactobacillus brevis* NRRL B 3365, on dry pet food during long-term storage was determined. Subsequently, the efficacy of *L. plantarum* in combination with or without thymol, a phytochemical present in thyme oil, for reducing *S. Schwarzengrund* on dry dog food was studied. Furthermore, lactic acid bacteria (LAB) from canine feces with potential antimicrobial activity against *Salmonella* spp. and *L. monocytogenes* were isolated.

Results revealed that TC, CR, TY, EG and CA in the combination with vegetable oil or chitosan significantly reduced *Salmonella* on dry pet food ($P < 0.05$). Betaine with 0.8 mM potassium chloride increased the survival of *L. plantarum*, *L. casei*, and *L. brevis* on dry pet food for up to 6 months ($P < 0.05$). Additionally, TY in combination with *L. plantarum* significantly reduced *Salmonella* populations on dry pet food ($P < 0.05$). A total of 8 LAB cultures isolated from canine feces exerted strong inhibitory effect against both *S. Schwarzengrund* and *L. monocytogenes*, and these isolates were identified as *L. plantarum* and *Pediococcus pentosaceus* by matrix assisted laser desorption ionization (MALDI-TOF) and polymerase chain reaction (PCR), whereas one isolate was unidentified. Collectively, the above results underscore the potential use of aforementioned PDAs and probiotics for controlling *Salmonella* on dry dog food.

Chapter I
Introduction

Salmonella is a Gram negative bacterium that causes gastrointestinal illness in both humans and animals (CDC, 2012). It is estimated that *Salmonella enterica* causes 1.4 million human illnesses, 19,000 hospitalizations, and 400 deaths in the United States annually (Scallan et al., 2011). Despite well-established control measures for preventing salmonellosis, the incidence of human salmonellosis has not decreased (Lee et al., 2015). *Listeria monocytogenes* is another important foodborne pathogen responsible for 19% of all deaths from foodborne illnesses in the USA (Scallan et al., 2011). Besides the consumption of contaminated foods, salmonellosis and listeriosis are zoonotic diseases that could be acquired by humans due to exposure to contaminated environments or infected animals.

A majority of dog owners rely on ready-to-feed dry dog food as the main diet for their pets. However, dry pet food contains ingredients of animal products, and these ingredients are at risk for *Salmonella* contamination (O'Bryan et al., 2015). During the production of dry dog foods, pet food ingredients are subjected to an extrusion process, where all the combined ingredients are heated under pressure to inactivate pathogens. However, flavor enhancers and fat are usually supplemented post-extrusion, and no additional treatments are proceeding to ensure the microbial safety of these ingredients (Thompson, 2008). In a survey conducted by the U.S. Food and Drug Administration (FDA), out of 185 pet food treats that were sampled, 65 (41%) were contaminated with *Salmonella* spp., including *S. Anatum* (19%), *S. Typhimurium* (14%), *S. Infantis* (10%), *S. Derby* (8%), and *S. Ohio* (8%) (White et al., 2003). Similarly, another prevalence study conducted during the period from 2007 to 2009 reported the isolation of *Salmonella* spp. from 6.1% of pet foods and treats and 7.1% of supplement-type pet products (Li et al., 2012). Recent multistate human outbreaks of salmonellosis linked to commercial contaminated dry dog foods in 2008 and 2012 and several recalls of *L.*

monocytogenes contaminated pet foods reported in the US during the last decade highlight the need for effective intervention strategies to control pathogens on these products. (FAO/WHO, 2003).

Plant essential oils are a group of natural molecules that have been historically used as food preservatives, flavor enhancers and dietary supplements to prevent food spoilage and maintain human health. The antimicrobial properties of several plant-derived antimicrobials (PDAs) have been documented (Kubo et al., 1993; Silva et al., 1996; Negi et al., 1999, 2003, 2005, 2010; Ahmad and Beg, 2001; Zeng et al., 2012). Some of the PDAs that have been reported to possess significant antimicrobial properties include trans-cinnamaldehyde (TC), eugenol (EG), thymol (TY), carvacrol (CR), and caprylic acid (CA). All these compounds have been shown to exert antimicrobial effects against Gram-positive and Gram-negative bacteria (Cosentino et al., 1999; Dorman and Deans, 2000; Mitsch et al., 2004).

Probiotic bacteria are defined as live microorganisms which when consumed in the foods, confer a health benefit on the host (FAO, 2001). Probiotic bacteria exert multiple benefits to the host, including protection against enteric pathogens (Candela et al., 2008; Fukuda et al., 2011), facilitating digestion and assimilation of nutrients (Sonnenburg et al. 2005; Yatsunenko et al. 2012) and potentiating host immune function (Olszak, T. et al. 2012). Pet food market offers several dry food products which claim to contain probiotics. However, Weese and Arroyo (2003) tested several commercial dog foods and reported that 26% of the tested products did not contain any relevant bacterial population, and none of the tested products contained all claimed strains. Thus, it is critical to investigate the viability of probiotics on dry dog food during long-term storage.

Betaine (*N,N,N*-trimethylglycine), a positively charged cationic amino acid isolated

from sugar beets, which has been found to improve the survival of the probiotic bacteria such as *L. plantarum* under dry environment (Kets and de Bont, 1994; Glaasker et al., 1996). Studies have shown that betaine uptake via betaine transporters significantly increased the survival of the bacteria in certain foods (Sleator et al., 1999; Sleator et al., 2003a, b; Smiddy et al., 2004). In addition, some *Lactobacillus* spp. contain hyperosmotic stress-activated betaine transporter systems (Glaasker et al., 1998; Obis et al., 1999; Wood et al., 2001), where researchers demonstrated that betaine is capable of improving the survivability of probiotic bacteria such as *Lactobacillus plantarum* under dry environment (Kets and de Bont, 1994; Glaasker et al., 1996).

Since the primary objective of dry pet food is to provide sufficient essential nutrients to meet the metabolic requirements of the pet, the use of probiotic bacteria as functional pet food compounds has gained more interest due to their beneficial effects on improving pet health and reducing the risk of gastrointestinal disorders. An important criterion for the selection of probiotic bacteria strain is their host species specificity, since is critical for these bacteria to colonize the host gut for imparting maximal health benefits (Fuller, 1989). However, most of the commercial probiotic products for dogs have been reported to be devoid of strains of canine origin (Beasley et al, 2006). Although abundant research has been conducted on isolating probiotic microorganisms from human and livestock origin and characterizing their beneficial effects, limited information exists on probiotics with canine origin (Groben et al, 1979; Beasley et al, 2006).

The overall objective of this dissertation was to investigate the efficacy of PDAs, including TC, CR, CA, TY, and EG, and probiotic bacteria for controlling *Salmonella* on dry dog food when applied as a spray coating. The specific objectives were:

1. To investigate the efficacy of CR, CA, TC, TY, and EG as a post-extrusion antimicrobial spray for reducing *Salmonella* Schwarzengrund on dry dog food.
2. To study the efficacy of PDAs in combination with probiotic bacteria for reducing *S. Schwarzengrund* on dry dog food, and determine the effect of betaine on the survivability of probiotic bacteria on dry dog food during long-term storage.
3. To determine the antimicrobial property of probiotic bacteria isolated from canine feces against *S. Schwarzengrund* and *L. monocytogenes*.

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Chapter II

Review of Literature

1. Salmonellosis in humans

Salmonella is a gram negative bacterium that causes gastrointestinal illness in both humans and animals (CDC, 2012). It is estimated that *Salmonella enterica* causes 1.4 million human illnesses, 19,000 hospitalizations, and 400 deaths in the United States annually (Scallan et al., 2011). The total cost associated with *Salmonella* illnesses was estimated to be several billion dollars annually (Frenzen et al., 1999; WHO, 2005). Despite well-established control measures for preventing salmonellosis, the incidence and severity of human salmonellosis have significantly increased (Lee et al., 2015). Most people infected with *Salmonella* develop diarrhea, fever and abdominal cramps within 12 to 72 hours after infection. Illness typically lasts 4 to 7 days, and most people recover without any treatment. However, children < 5 years old, older adults, and people with a compromised immune system are more likely to develop severe illness, including sepsis, joint infections, and meningitis.

Salmonellosis is a food-borne illness frequently associated with the consumption of contaminated foods such as meat, poultry, eggs, dairy, fresh produce, and processed foods (Barton et al., 2008; Cavallaro et al., 2011; CDC, 2013). Although the majority of *Salmonella* cases in humans are due to the consumption of contaminated food and water, approximately 11% of human salmonellosis cases are estimated to be attributable to contact with animals (Hale et al., 2012). Many animals, both domestic and wild, are colonized by *Salmonella* spp., usually harboring the bacterium in their gastrointestinal tracts with no apparent signs of illness (Marks et al., 2011). In animal-associated *Salmonella* infections, people are infected by physical contact with infected animals, contaminated animal feed, or contaminated environment (Santos et al., 2001).

2. *Salmonella* contamination in pet food

Dogs play an integral part in the lives of humans, providing security, labor, therapeutic support, and companionship. A report by the American Veterinary Medical Association indicated that approximately 43 million (~36%) households own dogs and 36 million (~30%) own cats in the United States (AVMA, 2012). Pets, especially dogs and cats live in close contact with their owners, sharing their homes and immediate environment. For example, 41% of dogs and 53% of cats were reported to share their owner's bed (APPMA, 2004). Studies conducted to determine the incidence of *Salmonella* in pets indicate a prevalence ranging from 0 to 36% in dogs and 1 to 8% in cats, with fecal shedding of *Salmonella* from infected animals observed for up to 6 weeks post-infection (Sanchez et al., 2002; Tupler et al., 2012). *Salmonella* infection can occur when a host consumed *Salmonella* contaminated foods; however, *Salmonella* also has the ability to cycle through a host into the environment and infect another host through cross-contaminated food, water or environment.

A majority of pet owners rely on ready-to-feed dry pet foods as the main diet for their pets. However, dry pet food contains ingredients of animal products, and these ingredients are at risk for *Salmonella* contamination (O'Bryan et al., 2015). During the production of dry pet foods, pet food ingredients are subjected to an extrusion process, where all the combined ingredients are heated under pressure to inactivate pathogens. However, flavor enhancers and fat are usually supplemented post-extrusion, and no additional treatments are proceeding to ensure the microbial safety of these ingredients (Thompson, 2008). In a survey conducted by the U.S. Food and Drug Administration (FDA), out of 185 pet food treats that were sampled, 65 (41%) were contaminated with *Salmonella* spp., including *S. Anatum* (19%), *S. Typhimurium* (14%), *S. Infantis* (10%), *S. Derby* (8%), and *S. Ohio* (8%) (White et al., 2003).

It was found that a total of 36% of the isolates were resistant to at least 1 antimicrobial, whereas 13% were resistant to 4 antimicrobials. Similarly, yet another prevalence study conducted during the period from 2007 to 2009 reported the isolation of *Salmonella* spp. from 6.1% of pet foods and treats, and 7.1% of supplement-type pet products (Li et al., 2012).

During January 2006 to December 2007, the Centers for Diseases Control and Prevention (CDC) collaborated with public health officials in Pennsylvania and the FDA to investigate a prolonged multistate outbreak of *Salmonella* Schwarzengrund infection linked to contaminated dry pet food. A total of 70 cases of *S. Schwarzengrund* infection were reported in 19 states, mostly in the northeastern part of the United States. The largest number of reported cases was in Pennsylvania (29 cases), followed by New York (9 cases) and Ohio (7 cases). The investigation revealed that 62% of patients in Pennsylvania owned one or more dogs. Among the 61 ill persons whose age was available, the median age was 3 years (range 1 month to 85 years), and 24 (39%) were aged less than 1 year. Of the 38 ill persons for whom clinical information was available, 15 (39%) experienced bloody diarrhea.

In 2012, another multi-state outbreak of dry pet food associated human salmonellosis was reported. From April to July 2012, there were 49 reported cases of human infections with *Salmonella* Infantis, where 42% of the infected patients were hospitalized. The outbreak resulted in a recall of 17 brands representing more than 30,000 tons of dry dog and cat food produced at a manufacturing facility in South Carolina. Follow up investigation revealed that human illnesses were due to direct contact with contaminated pet food and direct or indirect contact with infected animals or animal environment. In addition, 22 of 28 (79%) infected patients were found to be in contact with a dog within a week of infection. In 2011, *Salmonella* Infantis infection was reported in nine people in Canada, where epidemiological

investigations indicated exposure to contaminated pig ear treats as a potential risk factor (FDA, 2002). Following this outbreak, the FDA issued a public health advisory regarding *Salmonella* contamination of dog chews made from pig ears, rawhide and cow hooves (FDA, 2002).

In the United States, the FDA regulates pet foods, treats, and nutritional products. If *Salmonella* is present, these products are considered adulterated under the Federal Food, Drug, and Cosmetic (FDC) act. From 2008 to 2012, 54 recalls of pet foods (335 products) were made in the United States due to possible contamination with *Salmonella* spp., with 139 (41%) products being pet treats.

3. Factors associated with *Salmonella* contamination in pet food

Several factors are associated with *Salmonella* contamination in pet foods. During the pet food manufacturing process, poor sanitation practices, inadequate maintenance of equipment, lack of good manufacturing practices, poor ingredient control and handling, and poor pest control have been suggested to increase *Salmonella* contamination risks (Podolak et al., 2010). Cross-contamination could happen during transportation and receiving of the pet food ingredients. For example, a study by Fedorka-Cray and coworkers (1997) reported that trucks transporting animal feed were contaminated with *Salmonella* (Fedorka-Cray et al., 1997). Similarly, in a shared receiving area, a high-risk ingredient could cross-contaminate a low-risk one, and this is a concern because ingredients usually undergo different pathogen abatement steps. In addition, processing temperature and time, moisture and water activity, and macronutrients of the pet food may also play an important role in *Salmonella* contamination.

3.1 Processing temperature and time

During pet food manufacturing process, processing temperature and time are critical to inactivate pathogens, including *Salmonella* (van Schothorst and Brooymans, 1982; Jones, 2011). In pelleted animal feeds, the risk of microbial growth can be lowered by maintaining the temperature within the recommended range of 80°C to 85°C, and providing adequate airflow to the finished product to eliminate condensation during packaging and storage (Veldman et al., 1995; Jones and Richardson, 2004). Dry pet food production typically uses an extrusion process to manufacture diets instead of pelleting, which uses higher moisture and pressure. Okelo et al. (2006) examined time and temperature combinations that were most likely to eliminate pathogens on pet food, where they determined that an extruder exit temperature at 83°C, a mash feed moisture content at 285 g/kg, and a mean retention time of feed in the extruder barrel at 7s completely killed *S. Typhimurium* in the tested feed matrix post-extrusion. Jones and Richardson (2004) and Veldman et al. (1995) recommended that during thermal processing of dry pet food, whether by pelleting or extrusion, a minimum temperature of 110°C for 11 seconds and 85°C are required for reducing thermophilic and non-thermophilic pathogens, respectively.

3.2 Moisture and water activity

Apart from the processing temperature, moisture and water activity (a_w) also influence pathogen survival and growth in foods and feeds. Water activity is the amount of free water available for the growth of microorganisms, whereas moisture is the total amount of water in a matrix. It is well documented that *Salmonella* presents a serious hazard for low-moisture foods because of its ability to survive for a long period of time in extreme environments (Juven et al., 1984; Janning et al., 1994; Hiramatsu et al., 2005; Carrasco *et al.*, 2012). Janning and co-workers (1994) reported prolonged survival of *Salmonella* for up to 3 months

on anhydrous silica gel with a water activity as low as a_w of 0.2. Although the mechanisms behind the survival of *Salmonella* under low moisture conditions are fully clear, a number of adaptations, including formation of filaments, biofilm formation, modification of gene expression, and development of a viable but nonculturable (VBNC) state may contribute to the increased ability of the bacterium to persist under low moisture environments (Podolak et al., 2010; McMeechan et al., 2007). A water activity below 0.85 has been documented to limit the growth of pathogenic microorganisms, including *Salmonella* in foods (Beuchat et al., 2012)

3.3 Macronutrients

Pet food ingredients such as animal by-products are commonly treated by a rendering process aimed to separate fats from residual meat, organs, and bones. During rendering, by-product animal parts are ground, and heated to temperatures of 121–138°C for separating fats (Lambertini et al., 2015). Fats are subsequently filtered and used as pet food ingredients. Most supplemented fat in the pet foods is animal-derived oils or vegetable oils (Thompson, 2008). However, animal byproducts, including fats could be a potential source of pathogens. For example, Meeker et al. (2006) reported that animal by-products received by 17 rendering plants in the United States were found to be contaminated by a variety of pathogens, including *Salmonella* at a prevalence of 84%, *Campylobacter* at 30%, *Clostridium prefringens* at 71%, and *Listeria monocytogenes* at 8%. A survey by Franco (2005) revealed that approximately 25% of animal by-product samples tested were positive for *Salmonella*, at bacterial counts ranging from 0.03 to 1,100 MPN/g (mean 16.3 MPN/g). During pet food manufacturing process, since fat is usually applied post-extrusion, supplementation of

pathogen-contaminated fats could lead to contamination of dry pet foods (Lambertini et al., 2015).

4. Strategies to control *Salmonella* contamination in pet food

During the production of dry pet food, several treatments are applied to minimize the risk of pathogen contamination in food products (FEDIAF, 2010). For dry kibble products, extrusion step is commonly involved, where pet food ingredients are subjected to high temperatures above 90°C with high pressures of 34–37 atm under high-moisture conditions (FEDIAF, 2010; Zicker 2008). In pet food Hazard Analysis and Critical Control Point (HACCP) programs, the extrusion step is often the main critical control point for controlling microbial risks (Codex Alimentarius, 2003). Extrusion process involves injecting steam into a pre-mixed and pre-wetted mixture of ingredients, and injecting the mixture at high pressure through a small opening, thus conferring a determined shape to the extruded product. Heat, pressure, shear forces, and expansion have been attributed to contribute to the inactivation of pathogens that may be present in the ingredients (Latala, 2000). Guidelines by the American Feed Industry Association recommend that extrusion should take place for at least one second under wet heat at 22% moisture and 77°C to inactivate *Salmonella* (AFIA, 2010). On the other hand, Okelo et al. (2008) reported a reduction of at least 8 log CFU in *S. Typhimurium* in dry pet food at temperatures ranging from 83°C to 103°C. However, the effectiveness of heat treatment might be decreased if the target pathogens are heat-resistant bacteria. For example, *Salmonella* that survived a period of desiccation was observed to be less vulnerable to heat (Podolak et al., 2010; Spector and Kenyon, 2012), which is particularly important for dry pet food processing.

Good sanitation control in the processing plant is also critical to prevent post-processing cross-contamination. For example, pet food facility surfaces are routinely swabbed and tested for the presence of *Salmonella*. The storage condition of products in processing plants also affects the contamination of pathogens. Pet food products are usually stored for up to two weeks at room temperature prior to transportation (Lambertini et al., 2015). During this time, pathogen such as *Salmonella*, may contaminate the pet food products if the environment is favorable for growth. Additionally, *Salmonella* may also adapt to storage conditions and survive at temperatures ranging from near freezing (2°C) to up to 54°C (Beauchat, 2009; Podolak et al. 2010).

Pet food companies often test the finished product for contaminants such as *Salmonella*, and the product is held until laboratory testing results show no evidence of contamination. If the tested samples are negative for pathogen contamination, the product would be sealed in bags without the addition of antimicrobials. However, recontamination of the tested products may occur either at the processing plant or during household handling. Crane et al. (2010) indicated that the risk of recontamination at household exists for both canned wet food and dry kibble products (Crane et al., 2010).

Currently, chemical additives such as organic acids and formaldehyde have been used to prevent pathogen contamination in dry pet food. These chemicals control the growth of pathogens, including *Salmonella* by reducing the water activity and changing the pH of the pet food (Smyser and Snoeyenbos, 1979; Ha et al., 2000; and Ricke, 2005). However, potential health concerns of these chemicals to animals and workers of the pet food facility as well as the corrosive properties of these chemicals limit their applications. Heyse et al. (2015) reported that a cocktail of six naturally derived, lytic bacteriophages exerted broad-

scale effectiveness against *Salmonella* in dry pet food. The advantage of bacteriophage application is that they target and kill only specific bacterial hosts without showing corrosive effects on environmental surfaces or manufacturing equipment.

5. Probiotic bacteria

Probiotic bacteria are defined as live microorganisms which when consumed in appropriate amounts in foods, confer a health benefit on the host (FAO, 2001). Probiotics bacteria are classified as generally recognized as safe (GRAS) by the FDA. The possible beneficial health effects to humans include aiding lactose metabolism and food digestion, production of antimicrobials, anti-carcinogenic properties, immune system stimulation, enhancement of short-chain fatty acid production, anti-atherogenic effect, maintenance of epithelial integrity and barrier, and maintenance of well-balanced intestinal microbial community (Fuller, 1993; Lee and Salminen, 1995; Elmer et al., 1996, Surendran Nair et al., 2017). The term “probiotic” originates from the Greek *pro bios*, meaning “for life” or “in support of life,” which was used for the first time by Lilly and Stillwell (1965). Probiotic bacteria have a long history of safe consumption in fermented foods such as yogurt, and considerable interest exists in their use as food additives and supplements. Although several species of probiotic bacteria have been identified, a majority of them supplemented in human diets include *Lactobacilli* and *Bifidobacteria*.

The antimicrobial properties of probiotic bacteria have been extensively studied both *in vitro* and *in vivo* (Ogawa et al., 2002; Servin, 2004). The antimicrobial activity of probiotic bacteria generally results from the production of organic acids, hydrogen peroxide, strain-specific metabolites, bacteriocins, or non-lactic acid molecules (Servin, 2004). Bacteriocins are soluble antimicrobial peptides produced by probiotic bacteria, which can kill or inhibit

bacterial strains closely-related or non-related to producing bacteria (Yang et al., 2014). Lactic acid bacteria (LAB) are common probiotic bacteria that produce a variety of bacteriocins of different sizes, structures, physicochemical properties, and inhibitory range. For instance, nisin is a natural antimicrobial peptide produced by strains of *Lactococcus lactis* subsp. *lactis* inhibits Gram-positive and Gram-negative bacteria besides suppressing the outgrowth of spores of Bacilli and Clostridia (de Arauz et al., 2009). The major antimicrobial mechanisms of probiotic bacteria involved in reducing pathogens include inhibition of pathogen replication by producing antimicrobial substances, competition for limiting resources in the host, antitoxin effect, inhibition of virulence, antiadhesive and anti-invasive effects, and competitive exclusion by competition for binding sites or stimulation of epithelial barrier function (Surendran Nair et al., 2017).

5.1 Health benefits of probiotic bacteria in pet animals

The possible health effects of probiotic bacteria on pet animals have not been extensively examined, although some lactic acid bacteria strains have been documented to exert beneficial effects on the health of dogs. For example, Pasupathy and co-workers (2001) reported that supplementation of *L. acidophilus* improved food digestibility and growth parameters of puppies. Similarly, Benyacoub et al. (2003) demonstrated that dietary supplementation of *Enterococcus faecium* in puppy's diet enhanced immune function. In addition, some lactic acid bacteria have also been reported to improve the health status of dogs with gastrointestinal diseases. Sauter et al. (2006) reported that a probiotic cocktail of three different *Lactobacillus* spp. strains clinically improved the gut health of dogs with diarrhea. In the same study, authors observed a decrease in the numbers of Enterobacteriaceae and an increase in *Lactobacillus* spp. Moreover, enterococci are important

inhabitants of animal intestine, and are widely used in probiotic products. *Enterococcus faecium* is a probiotic documented to protect animals from diseases caused by *E. coli*, *Salmonella* spp. or *Clostridium* spp. (Maia et al., 2001). Studies have also shown that administration of *E. faecium* significantly decreased *Staphylococcus* spp. (Marcinaková et al., 2006) and *Clostridium* spp. (Vahjen and Männer, 2003) in dog's feces. Similarly, Stropfová et al. (2004) observed a reduction in the level of serum cholesterol and alanine aminotransferase after oral supplementation of a *Lactobacillus* strain to dogs with gastrointestinal diseases.

5.2 Probiotic bacteria in pet food

The primary objective of the dry pet food is to provide sufficient essential nutrients to meet the metabolic requirements of pet animals. In this regard, the use of probiotic bacteria as functional pet food ingredient has gained significant attention.

Recently, probiotic products in the forms of tablet, capsule, paste, and liquid are commercially available for use in dogs. Pet food market also offers several dry pet food products which claim to contain probiotics. However, currently no regulations exist on probiotic supplementation in pet animal diets. Weese and Arroyo (2003) tested several commercial dog foods that claimed to contain probiotics, but 26% of the tested products did not contain any relevant bacterial population and none of the tested products contained all claimed strains.

Probiotic bacteria are subjected to osmotic stress during dry pet food processing as a result of fluctuations in water activity, thereby reducing their survivability. However, betaine (*N,N,N*-trimethylglycine), a positively charged cationic amino acid isolated from sugar beets, has been found to improve the survival of the probiotic bacteria such as *L. plantarum* under

dry environment (Kets and de Bont, 1993; Glaasker et al., 1996). Studies have shown that betaine uptake via betaine transporters significantly increased the survival of bacteria in certain foods (Sleator *et al.*, 1999; Sleator *et al.*, 2003a, b; Smiddy *et al.*, 2004). In addition, some *Lactobacillus* spp. contain hyperosmotic stress-activated betaine transporter systems (Glaasker *et al.*, 1998; Obis *et al.*, 1999; Wood *et al.*, 2001), where researchers demonstrated that betaine is capable of improving the survivability of probiotic bacteria such as *L. plantarum* under dry environment (Kets and de Bont, 1993; Glaasker *et al.*, 1996).

Additionally, Biourge et al. (1998) evaluated the stability of probiotic bacteria in dry dog foods. In their study, probiotic *Bacillus* CIP 5832 was supplemented before or after the extrusion step during pet food manufacturing process. Although ~ 99% of the probiotics were killed during the extrusion process, ~ 75% of the probiotics survived on dog food when applied post-extrusion for up to 12 months of storage, which confirmed that the addition of probiotic bacteria to dry dog foods is practical, but has to be added after the extrusion process.

One important criterion for the selection of a probiotic bacterium is host species specificity, which is critical for ensuring that the beneficial characteristics of the probiotic are exerted in the host (Fuller, 1989). However, most of the commercial probiotic strains for dogs do not have a canine origin. Interest in canine origin probiotic strains has led to recent cultural studies directed towards the isolation of lactobacilli from dog feces. For example, Perelmutter et al. (2008) isolated a *L. murinus* strain from dog feces, which not only survived under lower pH and bile salts conditions, but also inhibited the growth of *E. coli* and *C. perfringens* *in vitro*. Another study by McCoy and Gilliland (2007) compared several *Lactobacillus* spp. in order to evaluate their possible use as probiotics, and suggested that *L.*

reuteri could potentially be used as a probiotic in dogs. Similar studies were performed by other researchers (Manninen et al., 2006; Beasley et al., 2006; Strompfová et al., 2006; Strompfová et al., 2004) and led to the identification of various lactic acid bacteria of canine origin that could be used as probiotics in dogs.

6. Plant-derived antimicrobials

Plant essential oils are a group of natural molecules that have been historically used as food preservatives, flavor enhancers and dietary supplements to prevent food spoilage and maintain human health. The antimicrobial properties of several plant-derived antimicrobials (PDAs) have been documented (Ahmad and Beg, 2001; Bhatt and Negi, 2012; Kubo et al., 1993; Negi et al., 1999, 2003, 2005, 2010; Silva et al., 1996; Zeng et al., 2012). A majority of active compounds in plant essential oils are secondary metabolites produced by plants as a defense against microorganisms such as bacteria, fungi and viruses (Kennedy and Wightman, 2011).

Plant extracts are commonly used as food preservative because they do not exert deleterious effects that are usually associated with synthetic chemicals (VanWyk and Gericke, 2000). Moreover, Ali et al. (2005) and Ohno et al. (2003) demonstrated that PDAs generally do not induce resistance in targeting bacteria. The major groups of PDAs include polyphenols, flavonoids, alkaloids, lectins, and tannins (Cowan, 1999; Geissman, et al., 1963). Plant-derived antimicrobials are considered as environmental-friendly antimicrobials due to their low mammalian cytotoxicity and quick biodegradability in soil and water (Isman, 2000).

A major mechanism behind the antimicrobial effect of PDAs is to attack the bacterial pathogens by damaging the cell wall and compromising membrane integrity, thereby leading to leakage of cellular contents and cell death (Burt, 2004). When PDAs are used in foods, the

presence of fat, sugars, and proteins could influence their antimicrobial efficacy (Gutierrez et al., 2008; Cava-Roda et al., 2010; Kyung, 2012). In addition, the antimicrobial efficacy of PDAs may be influenced by the extrinsic factors such as temperature, water activity and atmospheric composition (Gould, 1989). The PDAs investigated for reducing *Salmonella* contamination in this M.S. thesis include trans-cinnamaldehyde (TC), carvacrol (CR), thymol (TY), eugenol (EG), and caprylic acid (CA).

6.1 *Trans-cinnamaldehyde (TC)*

Trans-cinnamaldehyde (TC) is a GRAS-status ingredient present in the bark extract of cinnamon (*Cinnamomum zeylandicum*). Trans-cinnamaldehyde possesses a wide margin of safety with no reported genotoxic and mutagenic effects (Adams et al., 2004). The antimicrobial properties of TC against wide range of Gram-negative and -positive bacteria, including *Clostridium botulinum* (Bowles and Miller, 1993), *C. difficile* (Mooyottu et al., 2014), *S. aureus* (Bowles et al., 1995, Huang et al., 2014), *Vibrio spp.*, (Brackman et al., 2008) and *E. coli* O157:H7 (Baskaran et al., 2010, 2013) have been reported. Trans-cinnamaldehyde was also found to reduce *S. Typhimurium* DT104 with little inhibition towards *Lactobacilli* and *Bifidobacteria in vitro* (Si et al., 2006). Previous researches from our laboratory found that in-feed supplementation of TC significantly decreased *S. Enteritidis* colonization in chickens without adversely affecting bird performance and feed palatability (Kollanoor-Johny et al., 2012). Besides the deleterious effect exerted on the bacterial membrane, TC acts by depleting intracellular ATP by inhibiting ATPase supported energy metabolism along with inhibition of glucose uptake and its utilization (Gill and Holley, 2004; Oussalah et al., 2006; Negi, 2012).

6.2 *Carvacrol (CR)*

Carvacrol (CR), a major component in oregano oil obtained from *Origanum vulgare* (*Lamiaceae*), is also listed as GRAS by the FDA. Oregano oil and the active component carvacrol possess significant antibacterial activity against various Gram-positive and Gram-negative bacteria, including *Helicobacter pylori*, *Staphylococcus aureus*, *E. coli*, *Streptococcus pneumoniae*, *Salmonella mutans*, *Enterobacter sakazakii*, *Haemophilus influenza* and methicillin-resistant *S. aureus* (Chun et al., 2005; Hersch-Martinez et al., 2005; Botelho et al., 2009). Further, CR is inhibitory on spore outgrowth of *C. perfringens* (Juneja and Friedman, 2007)

Besides the antibacterial property, CR was found to possess various pharmacologic actions, including anti-inflammatory, anti-diarrheal, anti-cancer, anti-adipogenic and neuroprotective effects (Baser et al., 2008; Hotta et al., 2010; Yu et al., 2012; Hotta et al., 2010; Kim et al., 2013). Carvacrol was shown to exhibit strong anti-oxidant and anti-inflammatory properties by reducing the accumulation of free-radicals and decreasing the synthesis of inflammatory mediators (Hotta et al., 2010).

6.3 Thymol (TY)

Thymol (TY) is another major ingredient other than CR in oregano oil, and it is also classified as GRAS by the FDA. The oil of thyme has been found effective against bacterial and fungal infections of the gastrointestinal and genitourinary tracts (Blumenthal et al., 2000; Chun et al., 2005). Ilhak and Guran et al. (2014) reported that TY significantly reduced *S. Typhimurium* and *L. monocytogenes* in fish patties. A study by Si et al. (2006) found that supplementation of TY effectively inactivated *S. Typhimurium* DT 104 and *E. coli* O157: H7 in pigs without deleteriously affecting the natural intestinal microflora of the animals. In addition, *in vitro* experiments conducted by our laboratory demonstrated that TY

significantly reduced the survival of *S. Enteritidis* in chicken cecal contents as well as inhibited the attachment and invasion of *S. Enteritidis* in chicken oviduct epithelial cells (Kollanoor Johny et al., 2010; Upadhyaya et al., 2013).

6.4 Eugenol (EG)

Eugenol (EG) is yet another natural molecule present as an active ingredient in the oil from cloves (*Eugenia caryophyllis*) (Ali et al., 2005). The antibacterial activities of clove oil and eugenol have been documented by many researchers (Stecchini et al., 1993; Menon and Garg, 2001; Suhr and Nielsen, 2003; Ali et al., 2005). Eugenol also serves as an antioxidant (Ogata et al., 2000), and is widely used in the food industry.

6.5 Caprylic acid (CA)

Caprylic acid (octanoic acid, CA) is an eight-carbon medium chain fatty acid present in breast milk, bovine milk, goat milk and coconut oil (Sprong et al., 2001; Jensen, 2002). It is a GRAS-status, food-grade compound approved by the FDA. Previous research indicated that supplementation of CA through feed reduced *Campylobacter jejuni* carriage in broiler chickens (Solis de los Santos et al., 2008, 2009). Similarly, prior research conducted in our laboratory revealed that in-feed supplementation of CA at 0.7% and 1% significantly reduced *S. Enteritidis* colonization in broiler chickens, without adversely affecting body weight, feed intake, cecal endogenous flora in birds (Kollanoor Johny et al., 2009).

7. Hypothesis

The hypothesis of this research was that CR, CA, TC, TY, and EG, and probiotic bacteria decrease *Salmonella* on in dry pet food when applied as a spray coating. The specific objectives of this thesis were:

1. To investigate the efficacy of CR, CA, TC, TY, and EG as a post-extrusion antimicrobial spray for reducing *Salmonella* Schwarzengrund on dry dog food.
2. To study the efficacy of PDAs in combination with probiotic bacteria for reducing *Salmonella* Schwarzengrund on dry dog food, and to determine the effect of betaine on the survivability of probiotic bacteria on dry pet food during long-term storage.
3. To determine the antimicrobial property of probiotic bacteria isolated from canine feces against *Salmonella* Schwarzengrund and *Listeria monocytogenes*.

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Chapter III

Efficacy of Plant-Derived Antimicrobials for Controlling *Salmonella* on Dry Pet Food

Abstract

Salmonella is a major human pathogen in the United States, with contaminated food products as the major source of infection. However, contaminated pet food and contact with infected companion animals can potentially transmit salmonellosis to humans. Recent multistate human outbreaks of salmonellosis linked to commercial contaminated dry dog foods underscore the need for controlling the pathogen in pet foods for protecting pet health and human health. In this study, the efficacy of five GRAS status, plant-derived antimicrobials (PDAs), namely trans-cinnamaldehyde (TC), carvacrol (CR), thymol (TY), eugenol (EG), and caprylic acid (CA) applied as a vegetable oil or chitosan based antimicrobial spray on dry pet food for reducing *Salmonella* Schwarzengrund was investigated. Three hundred gram portions of a commercial dry dog food were inoculated with a two-strain mixture of nalidixic acid (NA) resistant *S. Schwarzengrund* (~ 6 log CFU/g), followed by a spray treatment with 0%, 0.5%, 1% or 2% of TC, CR, TY, EG or CA in combination with 5% vegetable oil or 1% chitosan as a carrier. The control and treated dog food samples were stored at 25°C for 28 days. On days 0, 1, 3, 5, 7, 14, 21, and 28, *Salmonella* on pet food was enumerated. All PDAs at 1% and 2% applied in vegetable oil or chitosan reduced *S. Schwarzengrund* by at least ~2 log CFU/g on day 3 of storage when compared to control ($P < 0.05$). No significant reductions in *Salmonella* were observed on feed sprayed with only vegetable oil or chitosan ($P > 0.05$). Overall, 2% TC in vegetable oil or chitosan was the most effective treatment, where at least 3 to 3.5 log CFU/g reduction in pathogen counts was observed during storage ($P < 0.05$). Results suggest that the aforementioned PDAs could potentially be used as an antimicrobial spray to reduce *Salmonella* on dry dog food. However, further studies on the acceptance of PDA-treated dry food by dogs are needed.

1. Introduction

Salmonella spp. is a major foodborne pathogen that causes an estimated 1.2 million human illnesses, 19,000 hospitalizations and 450 deaths annually in the United States. (CDC, 2011). Salmonellosis is also a zoonotic disease that can occur due to exposures other than the consumption of contaminated food. For example, salmonellosis in humans has been reported from handling contaminated pet food (Freeman et al, 2013; Jackson et al., 2013). *Salmonella* contaminated pet food leads to infections in companion animals, where the infected animals shed *Salmonella* in the feces and saliva for prolonged time, thereby making them a viable carrier for the pathogen (Apanavicius et al, 2007; Singh et al., 2007). Further, dogs and cats could shed *Salmonella* asymptomatically for 3–6 weeks, and up to 3 months (Imanish and Rostein, 2014). Infection in humans could be linked to direct contact with contaminated pet foods, exposure to cross-contaminated human food products, direct or indirect contact with infected pets or house environment (Lambertini et al., 2012). From 2007 to 2012, two major outbreaks of human salmonellosis have been linked to contaminated pet food products, where more than 100 people were reported ill from 20 different states, and about 40% of the infected patients were one year of age or younger (CDC, 2008; CDC, 2011; Li et al., 2012). In light of these outbreaks, the US Food and Drug Administration conducted a nationwide survey in 2012 to determine *Salmonella* prevalence on dry pet foods, pet treats and supplements (FDA, 2012).

Currently, chemicals such as organic acids and formaldehyde are used for decontamination of animal feeds, including pet food (Jones, 2011). However, they are found to be minimally effective in reducing pathogen load (Carrique-Mas et al., 2007). Thus, there is a critical need for identifying novel strategies for inactivating *Salmonella* on dry pet foods.

Plant-derived antimicrobials (PDAs) are a group of natural plant compounds that have traditionally been used as food preservatives and flavor enhancers. In the past decade, the use of PDAs as effective antimicrobials has gained significant attention due to their non-toxic nature, increasing concern over the safety of synthetic chemicals, and emerging antibiotic-resistant microorganisms (Salamci et al., 2007). Carvacrol (CR) and thymol (TY) are phenolic isomers present as active ingredients in oregano oil (*Origanum glandulosum*). Trans-cinnamaldehyde (TC) is an aromatic aldehyde obtained from the bark extract of cinnamon (*Cinnamomum zeylandicum*). Eugenol (EG) is another polyphenolic compound that is a component of clove oil (*Eugenia caryophyllis*). Caprylic acid (CA) is an eight-carbon medium chain fatty acid present in bovine milk, goat milk and coconut oil (Sprong et al., 2001; Jensen, 2002). All the aforementioned compounds are classified as GRAS (generally recognized as safe) by the FDA (Arrebola et al., 1994; Leriche and Carpentier, 1995; Venkitanarayanan et al., 2013). Previous studies conducted in our laboratory revealed that these natural molecules possess significant antimicrobial properties against *Salmonella* (Upadhyaya et al., 2013; Kollanoor Johny et al., 2010; Venkitanarayanan et al., 2013).

Chitosan (CH) is a biodegradable, GRAS-status polymer derived from the deacetylation of chitin, a natural polysaccharide present as the main component of exoskeletons of crustaceans (Kumar, 2000). Chitosan possesses antimicrobial properties against Gram-positive and Gram-negative bacteria (No et al., 2002; Sagoo et al., 2002). In addition, chitosan is used as an antimicrobial carrier coating or film on foods due to its emulsification and gelation properties (Knorr, 1984; No et al., 2002). In the pet food industry, vegetable oil is commonly used as a carrier and diluent of additives in dry pet foods (Aldrich, 2012). Therefore, this study investigated the efficacy of aforementioned PDAs in

combination with 5% vegetable oil or 1% chitosan as an antimicrobial spray for reducing *Salmonella* on dry dog food.

2. Materials and Methods

2.1 Preparation of bacterial culture

Two strains of *Salmonella enterica* Seroovar Schwarzengrund (CVM 19633 and DBS-GA-F25499) obtained from BEI resources (Manassas, VA) were used in this study. All bacteriological media were purchased from Difco (Becton Dickinson, Sparks, MD). *Salmonella* strains were pre-induced for resistance to 50 µg/ml of nalidixic acid (NA; Sigma Aldrich, St. Louis, MO) for selective enumeration. To prepare the inoculum, 100 µl of each NA-resistant *S. Schwarzengrund* strain was cultured separately in 10 ml tryptic soy broth (TSB) containing 50 µg/ml of NA, and incubated at 37°C overnight. After incubation, each *S. Schwarzengrund* culture was centrifuged at 3,600 \times g for 15 min at 4°C. The pellet of each *Salmonella* strain was washed twice and resuspended in 10 ml of 0.1% peptone water, and 0.1 ml of the resuspension was spread plated onto xylose lysine desoxycholate (XLD) agar plates containing 50 µg/ml of NA (XLD+NA). After incubation at 37°C for 24 h, 10 ml of 0.1% peptone water was added onto the XLD+NA plate containing colonies of each *Salmonella* strain, and the agar surface was gently washed to collect the bacteria (Beuchat and Mann, 2011). Equal portions of the two *S. Schwarzengrund* cultures were combined and used as the inoculum ($\sim 8 \log$ CFU/ml). The bacterial count of the two-strain cocktail was confirmed by plating 0.1 ml portions of appropriate dilutions on XLD+NA plates, followed by incubation at 37°C for 24-48 h.

2.2 Preparation of PDA treatments

All PDAs (TC, CR, EG, TY, and CA) and low molecular weight chitosan (~5 to 15 kDa) were purchased from Sigma-Aldrich (99% purity, SAFC grade; Sigma-Aldrich). Pure vegetable oil was procured from Fisher Scientific (Asheville, NC). To prepare 1% chitosan solution, 1 g chitosan was dissolved in sterile deionized water containing 0.1% acetic acid (Sigma Aldrich), heated at 60°C, and stirred for 6 h to fully dissolve chitosan (Chen et al., 2012; Wu and Zivanovic., 2008; Martinez-Camacho et al., 2010). Subsequently, each PDA was added to 5% vegetable oil or 1% chitosan solution at the desired concentrations, and the solution was vortexed thoroughly for proper mixing of the PDAs.

A commercially available dry dog food was purchased from a local pet store. Prior to the experiment, duplicate 10 g portions of dog food were placed in a sterile WhirlPak bag (Sigma-Aldrich) containing 100 ml of cysteine selenite broth and incubated at 37°C for 48 h. The enriched culture was streaked on XLD plates and incubated at 37°C for 48 h, and observed for typical *Salmonella* colonies to determine the presence of any inherent *Salmonella* spp. on dry dog food.

For each treatment, 300 g portions of dry dog food were spray inoculated with 15 ml of *S. Schwarzengrund* cocktail culture to obtain ~ 6 log CFU/g of the pathogen using an air atomizer (Master air brush, Eco kit-17, TCP global, San Diego, CA) in a biosafety cabinet. Following inoculation, dog food was placed for 1 h in a biosafety cabinet to facilitate bacterial attachment. Ten milliliters of the aforementioned PDA treatments prepared in 5% vegetable oil or 1% chitosan were sprayed onto the inoculated dry dog food to obtain final concentrations of 0%, 0.5%, 1%, and 2% (vol/wt) of each PDA. Dry dog food inoculated with *S. Schwarzengrund*, but not subjected to any PDA spray treatment served as the baseline. Moreover, vegetable oil and 1% chitosan without any PDA were included as controls to test

if they exerted any antimicrobial effect on *S. Schwarzengrund*. After treatment, 10 g portions of dry dog food were transferred to a sterile WhirlPak bag and stored at 25°C for 28 days. On days 0, 1, 3, 5, 7, 14, 21, and 28, a volume of 20 ml of neutralizing broth (Sigma-Aldrich) was added to each bag containing 10 g of PDA-treated or untreated dry dog food, and pummeled in a stomacher (Stomacher 400 Circulator, Seward, Davie, FL) for 1 min. The dog food homogenate was serially diluted (1:10) in 0.1% peptone buffer, and 0.1 ml aliquots from appropriate dilutions were surface plated on duplicate XLD-NA plates, and incubated at 37°C for 24-48h. In addition, 1 ml of the homogenate was enriched in 50 ml of cysteine selenite broth at 37°C for 48 h. Following enrichment, the culture was streaked on XLD-NA plates, incubated at 37°C for 48 h, and observed for typical *Salmonella* colonies.

2.3 Water activity and pH measurement

The pH and water activity of dog food from all treatments was measured on each sampling day, as previously described (Ruth et al., 2016). Briefly, pH was determined at 25°C by weighing 1 g portions of dry dog food from each treatment, pulverizing with mortar and pestle and hydrating with 2.5 ml distilled water, and the pH value of each sample was measured using a pre-calibrated pH meter (Horiba, Baltimore, MD). For water activity measurement, 4 g portions of dry dog food from each treatment was ground and the water activity of each sample was determined using a water activity meter (Rotronic, Hauppauge, NY) as per the manufacture's instructions.

2.4 Statistical analysis

A completely randomized design was used with a 5 x 4 x 8 factorial treatment structure. The factors included 5 PDAs (CR, CA, TC, TY, and EG), 4 concentrations (0%, 0.5%, 1%, and 2%) and 8 time points (days 0, 1, 3, 5, 7, 14, 21, and 28). The data were analyzed using

the PROC-MIXED procedure of the Statistical Analysis Software (SAS Institute Inc., Cary, NC). Triplicate samples were assayed on each sampling day, and the study was replicated twice. Differences among the means were analyzed at $P < 0.05$ using Fisher's least significance difference test with appropriate corrections for multiple comparisons

3. Results

3.1 Effect of PDAs in combination with vegetable oil on *S. Schwarzengrund*

Figures 1A-E show the effect of various PDAs at 0%, 0.5%, 1%, and 2% applied in in vegetable oil on *Salmonella* survival on dry dog food. On day 0, ~ 5 to 5.5 log CFU/g of *Salmonella* was recovered from treated and control samples. In all samples, *Salmonella* counts gradually decreased over the 28-day storage period. On day 28, ~ 4.0 log CFU/g of *Salmonella* was present on control samples. Vegetable oil did not significantly affect the survival of *Salmonella* as compared to control throughout the storage period ($P > 0.05$). However, all PDAs at 1% and 2% significantly reduced the survival of *Salmonella* on dry dog food in a concentration dependent manner ($P < 0.05$). Dry dog food treated with 0.5% of each aforementioned PDA decreased *Salmonella* populations by approximately 0.4 to 2.0 log CFU/g as compared to untreated control and vegetable oil control on day 28; TC was the most effective treatment followed by TY, CA, EG, and CR. Among the PDAs, TC and TY at 2% exerted the greatest antimicrobial effect against *Salmonella* with ~ 3.0 log CFU/g and 2.5 log CFU/g reduction when compared to control on day 28, respectively (Fig. 1C-D).

3.2 Effect of PDAs in combination with 1% chitosan on *S. Schwarzengrund*

Salmonella counts recovered from untreated (control) and 1% chitosan control samples on day 28 ranged from 3.2-3.8 log CFU/g. All PDA treatments sprayed in 1% chitosan exhibited a similar inhibitory effect on *Salmonella* survival on dry dog food as observed in combination

with vegetable oil (Fig. 2A-E). Treatment with 1% chitosan by itself did not significantly reduce *Salmonella* counts on dog food ($P > 0.05$); however, PDAs at 0.5%, 0.1%, and 0.2% in combination with 1% chitosan decreased the pathogen throughout the storage period ($P < 0.05$). Treatments containing PDAs at 0.5% in combination with 1% chitosan resulted in approximately 1.3 to 2.4 log CFU/g reduction in *Salmonella* counts on day 28 compared to control, where maximum reduction (~ 2.4 log CFU/g reduction) was observed with 0.5% TC. Treatment containing TC at 2% was most effective, where *Salmonella* population was decreased to as low as 0.5 log CFU/g on day 14.

3.3 Effect of PDA treatments on pH and water activity

Table 1 shows the pH and water activity of dry dog food treated with PDAs in combination with vegetable oil, where it can be observed that the PDAs at 0.5%, 1%, and 2% in combination with vegetable oil did not significantly affect these parameters. Although PDAs in combination with 1% chitosan did not significantly change the pH values of dog food samples, these treatments significantly increased the water activity as compared to untreated control (Table 2).

4. Discussion

Approximately 43 million households in the United States own at least one dog, and majority of these pet owners rely on dry pet food as the primary source of nutrition for their pets (AVMA, 2013). However, two multi-state outbreaks of human salmonellosis linked to contaminated dry dog food and numerous recalls of contaminated pet foods and treats highlight the need for an effective strategy to control *Salmonella* on dry pet food. Since *Salmonella* would not survive under the extrusion conditions applied during dry pet food manufacturing process, the presence of *Salmonella* is usually due to post-extrusion

contamination in the processing plant. Therefore, the present study investigated the efficacy of the PDAs in combination with vegetable oil or 1% chitosan for potential application as a post-extrusion spray treatment for reducing *Salmonella* contamination on dry dog food.

Results revealed that all PDAs especially at 1% and 2% levels in combination with vegetable oil or 1% chitosan significantly reduced *Salmonella* counts on dry dog food (Fig. 1 and Fig 2). Among the various PDAs, TC was found to be the most effective treatment against *Salmonella*, regardless of its combination with vegetable oil or 1% chitosan. In addition, vegetable oil and 1% chitosan, which were used as a carrier of the PDAs did not exert any significant inhibitory effect on *Salmonella*.

Water activity is one of the critical factors that affect the survivability of pathogens on dry pet foods. In our study, dry dog food sprayed with PDAs at 0.5%, 1%, and 2% in vegetable oil did not significantly change the water activity as compared to that of untreated control ($P > 0.05$). Although all PDAs applied in combination with chitosan increased the water activity of dog food (0.49-0.73) ($P < 0.05$) compared to control (0.27), the PDA treatments exerted significant inhibitory effect on *Salmonella*, where pathogen counts were found to be consistently lower than that on control samples (Table 2). Himathongkham et al. (1999) and Koutsoumanis et al. (2004) noted that a water activity above 0.90 was required for the growth of *Salmonella*, which was not observed in any of the samples in the current study. Further, Oni et al. (2006) reported that rehydration of dry dog food with up to 35-50% of additional water may be needed to support the growth of *Salmonella* on dry dog food.

All PDA treatments at 0.5% in combination with 1% chitosan were generally found to be more effective in reducing *Salmonella* than their combination with vegetable oil. For instance, the PDAs at 0.5% with vegetable oil decreased *Salmonella* on dog food by ~ 0.4 to

2.0 log CFU/g (Fig. 1A-E), whereas the same concentration of PDAs sprayed in 1% chitosan yielded 1.3 to 2.4 log CFU/g reduction in *Salmonella* counts (Fig. 2A-E) compared to controls on day 28. Similarly, on day 28, ~ 1 log CFU/g reduction in *Salmonella* populations was observed on dog food sprayed with 0.5% CR in vegetable oil (Fig. 1A); however, 0.5% CR in combination with 1% chitosan decreased *Salmonella* counts by ~ 1.8 log CFU/g (Fig. 2A). Likewise, TY at 0.5% with vegetable oil resulted in a reduction of ~ 1 log CFU/g *Salmonella* on day 28 (Fig. 1D), whereas 0.5% TY with 1% chitosan decreased pathogen load by ~ 1.7 log CFU/g (Fig. 2D). The increased antimicrobial efficacy of PDAs with chitosan observed in this study concurs with the that by Wang et al. (2011), who observed synergistic antimicrobial effects between chitosan and a variety of antimicrobials, including essential oils. Similarly, Anacarso et al. (2011) reported that a combination of chitosan with essential oils produced greater anti-listerial activity than chitosan alone on vegetables and fruits.

The hydrophobicity of PDAs allows them to target the lipid-containing bacterial cell membrane and makes the membrane more permeable, leading to leakage of ions and other cell contents (Sikkema et al., 1995; Cox et al., 2000; Ultee et al., 2002). On the other hand, chitosan has also been documented to weaken the membrane barrier properties of the outer membrane of Gram-negative bacteria, where the interaction between the positively charged chitosan molecules and negatively charged microbial cell walls plays a critical role in its antimicrobial activity (Helander et al., 2001). Thus, the combination of PDAs and chitosan could be more detrimental on bacteria than PDAs or their combination with vegetable oil.

Results of the current study suggest that all tested PDAs, especially at 1 and 2% in combination with 5% vegetable oil or 1% chitosan could potentially be used as an

antimicrobial spray to reduce *Salmonella* on dry pet foods. However, follow-up investigations on the palatability of PDA-treated pet food and large scale efficacy studies under commercial settings are warranted.

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Table 1. Water activity and pH of dry dog food treated with plant-derived antimicrobials in combination with vegetable oil.

Treatments	a_w ¹	pH ²
Control	0.30±0.004 ^a	5.89±0.015 ^a
Vegetable oil	0.30±0.005 ^a	5.81±0.026 ^a
TC 0.5%	0.29±0.006 ^a	5.92±0.032 ^a
TC 1%	0.31±0.008 ^a	5.80±0.067 ^a
TC 2%	0.31±0.009 ^a	5.84±0.061 ^a
TY 0.5%	0.31±0.003 ^a	5.82±0.031 ^a
TY 1%	0.31±0.006 ^a	5.83±0.057 ^a
TY 2%	0.32±0.004 ^a	5.80±0.050 ^a
CR 0.5%	0.29±0.008 ^a	5.82±0.037 ^a
CR 1%	0.28±0.003 ^a	5.80±0.043 ^a
CR 2%	0.29±0.001 ^a	5.80±0.067 ^a
CA 0.5%	0.31±0.004 ^a	5.85±0.057 ^a
CA 1%	0.31±0.007 ^a	5.70±0.099 ^a
CA 2%	0.32±0.004 ^a	5.76±0.040 ^a
EG 0.5%	0.32±0.014 ^a	5.84±0.032 ^a
EG 1%	0.31±0.001 ^a	5.72±0.080 ^a
EG 2%	0.31±0.012 ^a	5.84±0.084 ^a

^{1,2} Values represent the mean ± SEM of three samples.

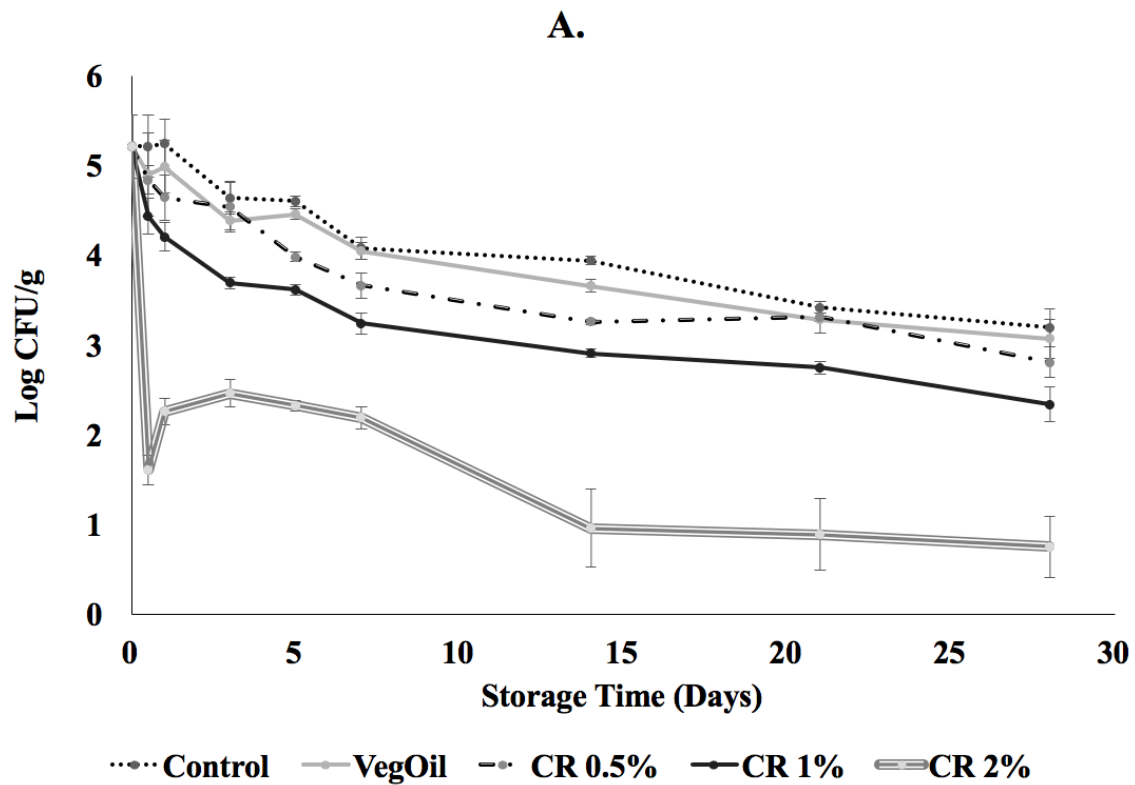
Table 2. Water activity and pH of dry dog food treated with plant-derived antimicrobials in combination with 1% chitosan.

Treatments	a_w ¹	pH ²
Control	0.27±0.004 ^h	5.89±0.014 ^a
Chitosan 1%	0.70±0.018 ^a	5.83±0.043 ^a
TC 0.5%	0.57±0.007 ^e	5.76±0.055 ^a
TC 1%	0.53±0.004 ^f	5.90±0.040 ^a
TC 2%	0.49±0.009 ^g	5.79±0.040 ^a
TY 0.5%	0.70±0.005 ^a	5.81±0.072 ^a
TY 1%	0.67±0.003 ^b	5.82±0.041 ^a
TY 2%	0.65±0.006 ^c	5.79±0.026 ^a
CR 0.5%	0.65±0.010 ^c	5.90±0.005 ^a
CR 1%	0.64±0.003 ^c	5.74±0.089 ^a
CR 2%	0.62±0.006 ^d	5.88±0.041 ^a
CA 0.5%	0.64±0.003 ^c	5.84±0.071 ^a
CA 1%	0.63±0.002 ^{cd}	5.75±0.072 ^a
CA 2%	0.62±0.005 ^d	5.74±0.045 ^a
EG 0.5%	0.56±0.006 ^e	5.74±0.078 ^a
EG 1%	0.56±0.004 ^e	5.86±0.080 ^a
EG 2%	0.55±0.005 ^{ef}	5.78±0.068 ^a

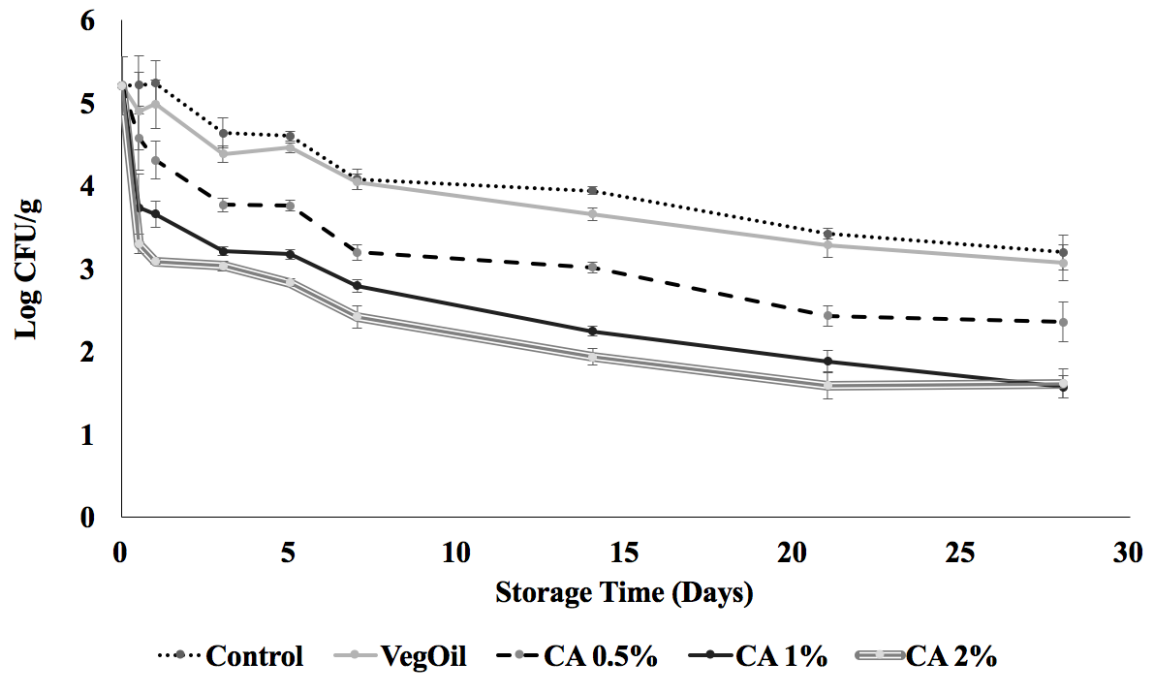
^{1,2} Values represent the mean ± SEM of three samples.

* represent the mean significantly differed from the control ($P < 0.05$)

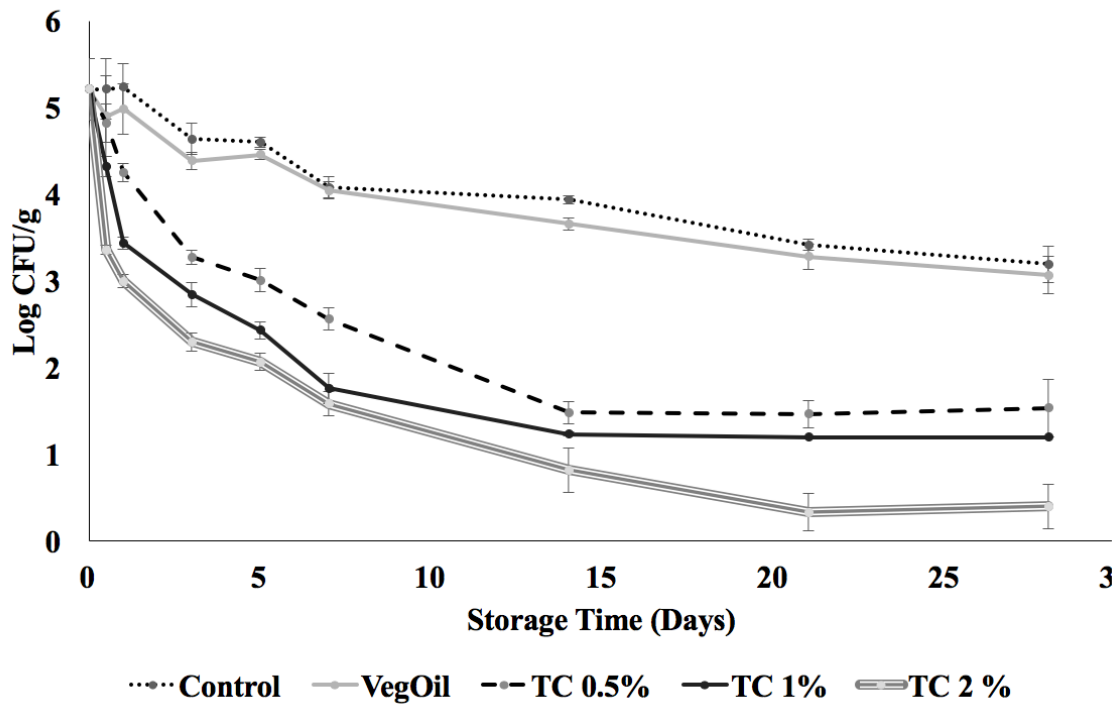
Figure 1. Effect of plant-derived antimicrobials in combination with vegetable oil on *Salmonella* Schwarzengrund survival on dry dog food. Data are mean \pm SEM obtained from 2 separate experiments with triplicate samples on each sampling point (days 0, 1, 3, 5, 7, 14, 21, and 28). Error bar indicates SEM (n=6). A-E show the effect of carvacrol (CR), caprylic acid (CA), trans-cinnamaldehyde (TC), thymol (TY), and eugenol (EG), respectively.



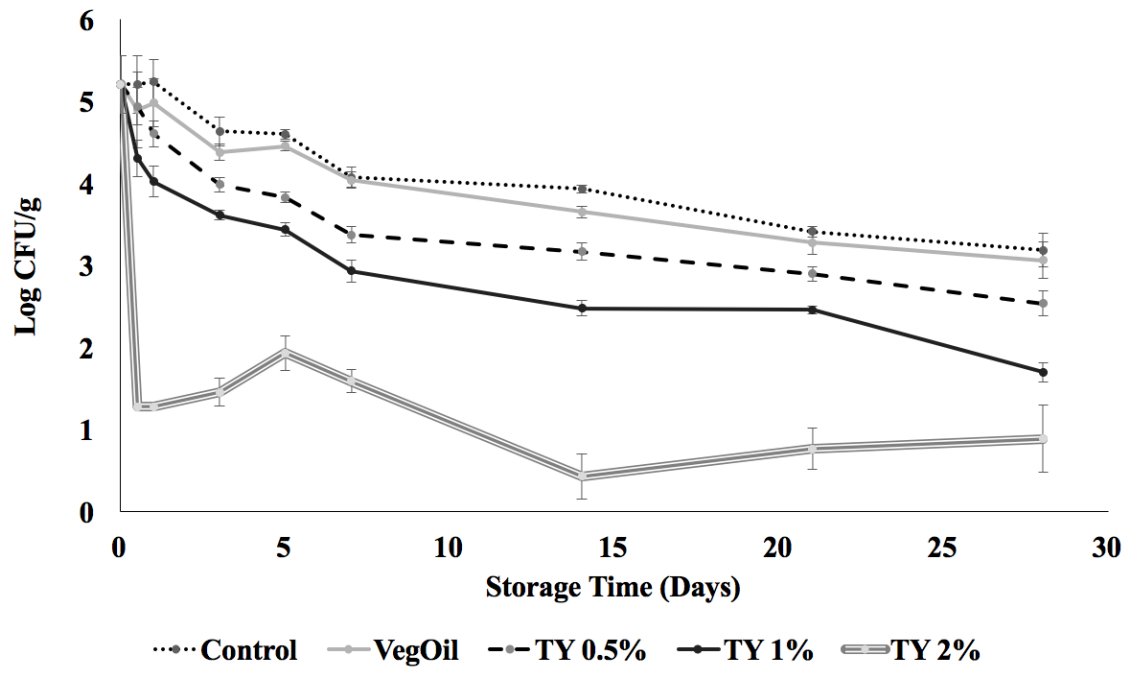
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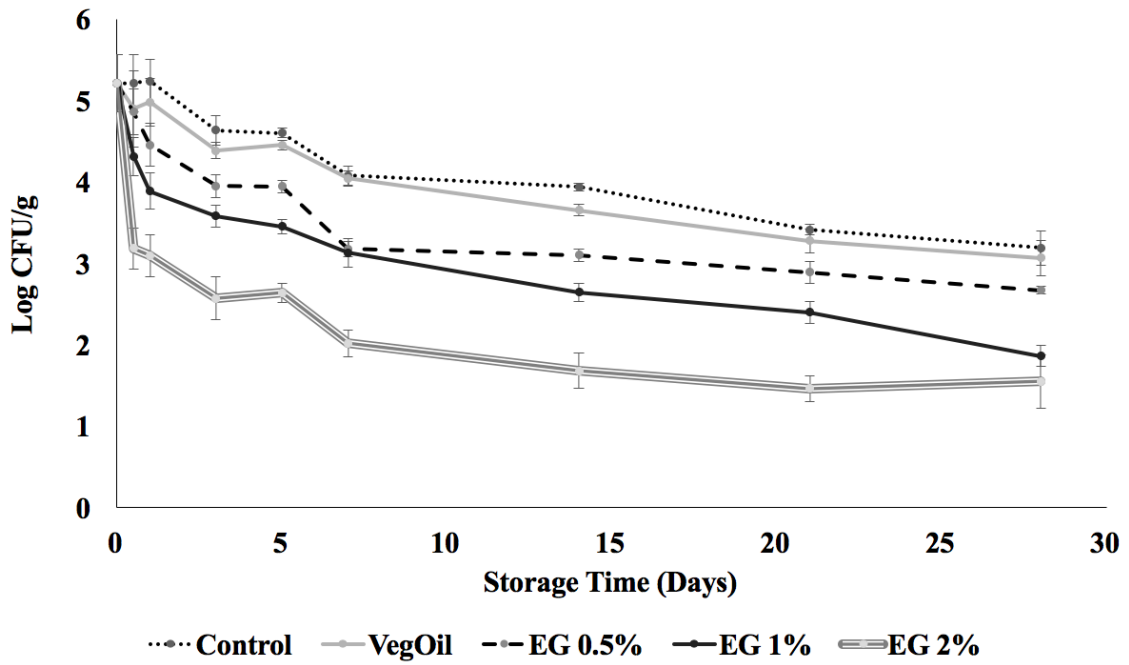
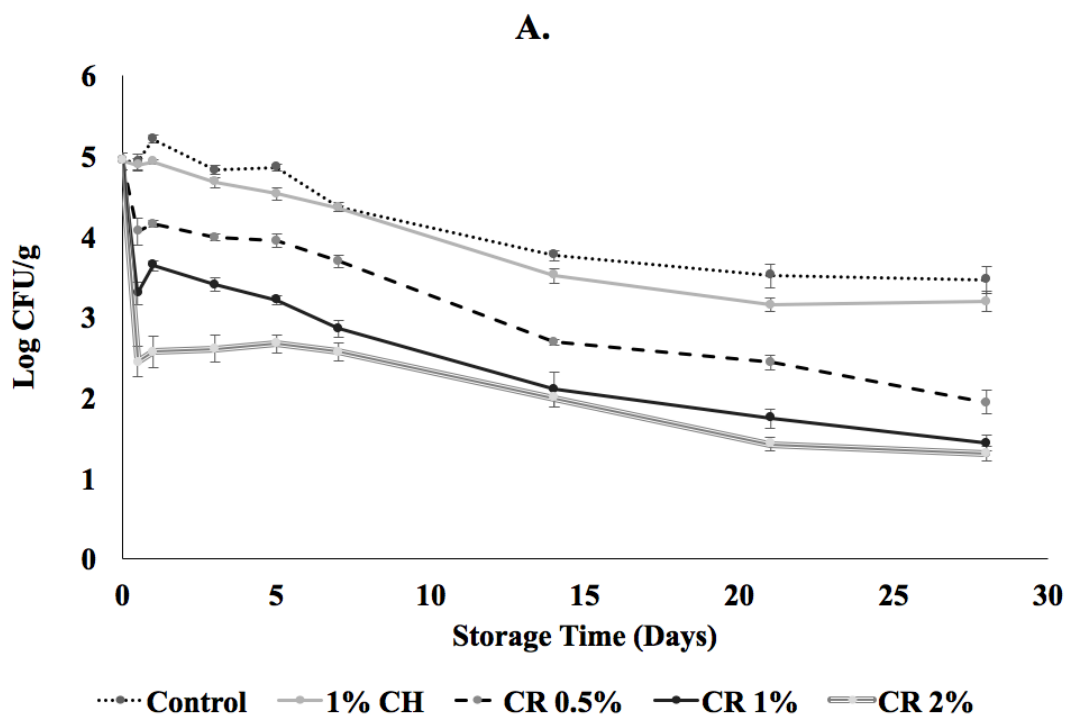
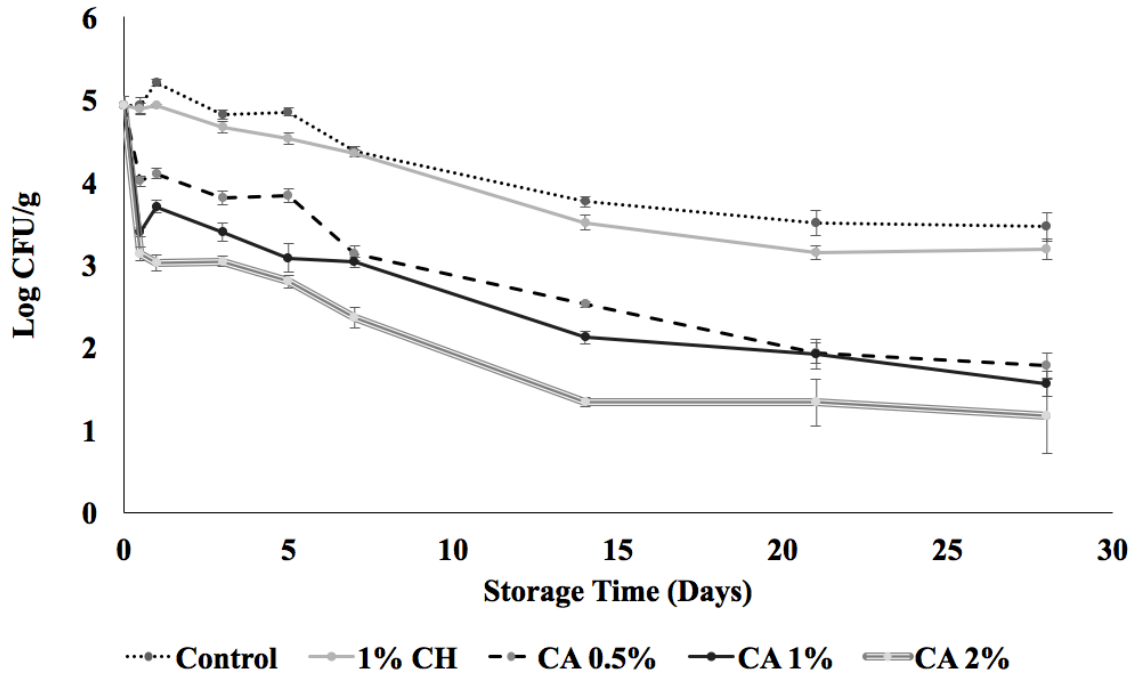


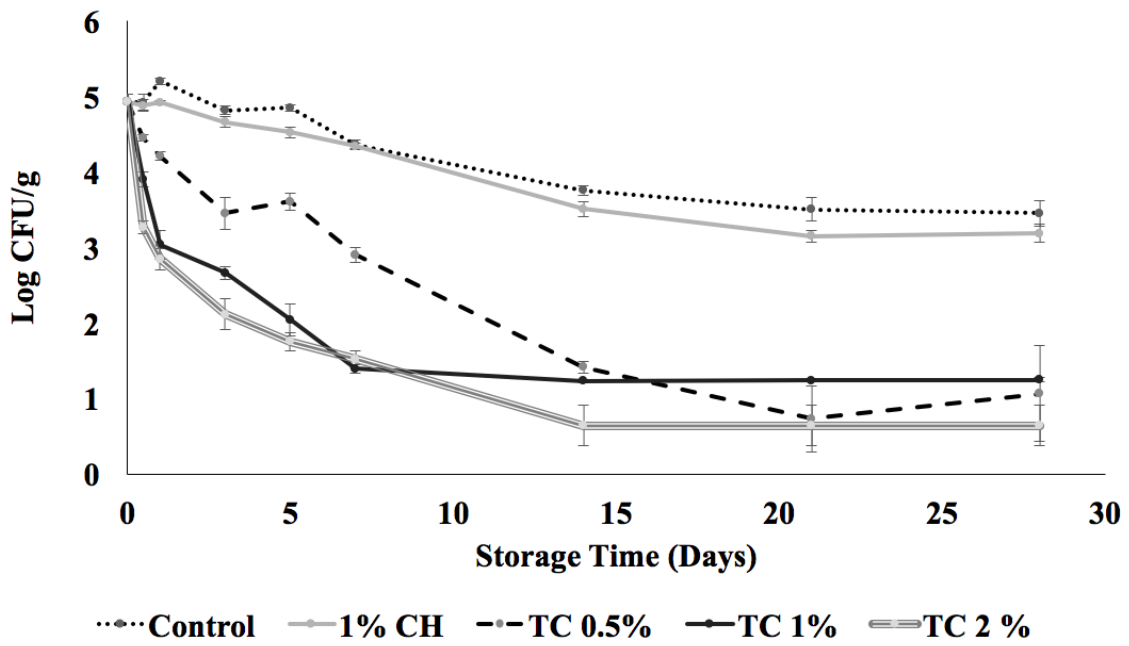
Figure 2. Effect of plant-derived antimicrobials in combination with 1% chitosan on *Salmonella* Schwarzengrund on dry dog food. Data are mean \pm SEM obtained from 2 separate experiments with triplicate samples on each sampling point (days 0, 1, 3, 5, 7, 14, 21, and 28). Error bar indicates SEM (n=6). A-E show the effect of carvacrol (CR), caprylic acid (CA), trans-cinnamaldehyde (TC), thymol (TY), and eugenol (EG), respectively.

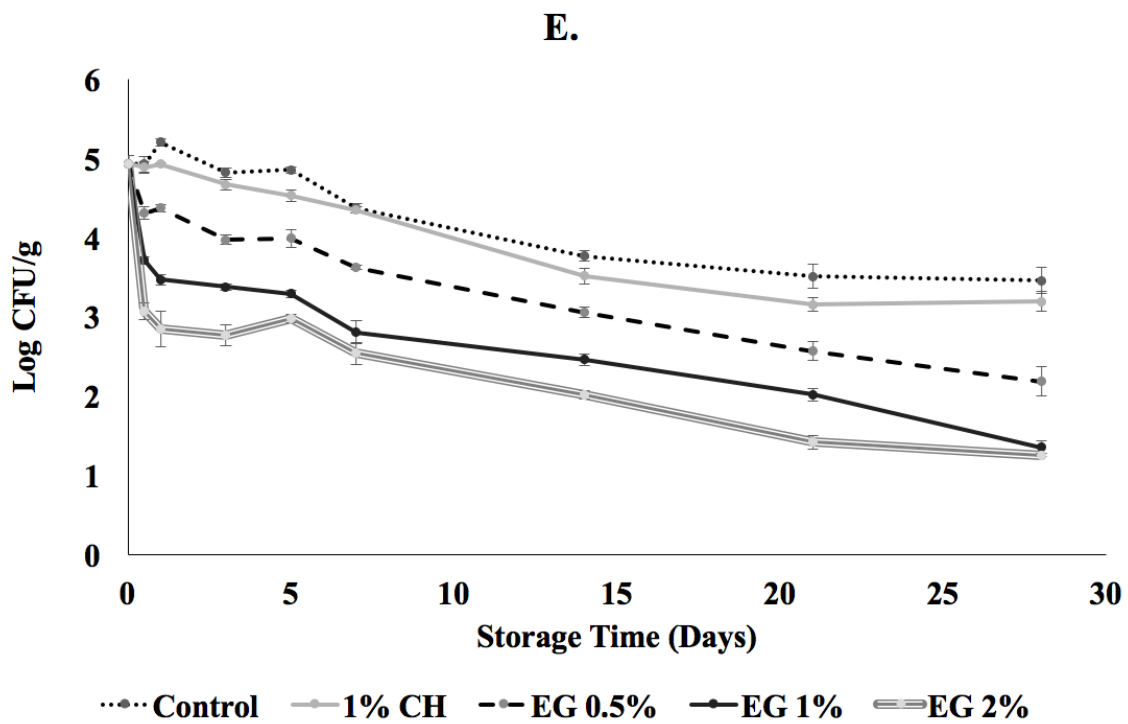
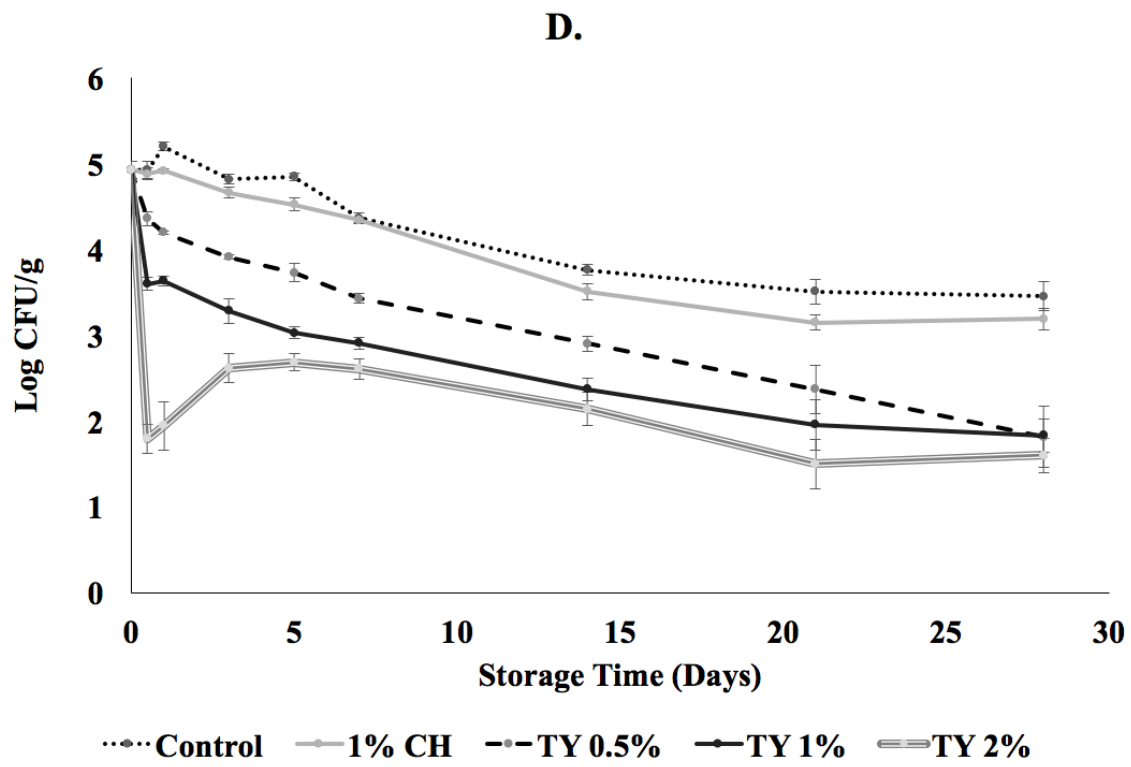


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Chapter IV

Survival of Probiotic Bacteria and their Antimicrobial Effect against *Salmonella* on Dry Dog Food

Abstract

In this study, the long-term survival of three probiotic bacteria, namely *Lactobacillus plantarum* (NRRL B-4496), *L. casei* (ATCC 334), and *L. brevis* (NRRL B-3365), on dry dog food was determined in the presence and absence of betaine and potassium chloride (KCl). In addition, the efficacy of *L. plantarum* with or without thymol (TY), a phytochemical derived from the oil of thyme, for reducing *Salmonella* Schwarzengrund on dry dog food was investigated. The three lactobacilli were grown separately in De Man-Rogosa-Sharpe (MRS) broth with or without 0.1% or 0.02% betaine (vol/vol) in the presence or absence of 0.8 mM KCl at 37°C for 24 h. Each probiotic bacterial culture was sprayed onto dry dog food (~ 8 log CFU/g) and stored at 25°C for 24 weeks. In weeks 0, 1, 2, 4, 8, 12, 18, and 24, the probiotic bacterial populations on dog food were enumerated on MRS agar plates. Moreover, 300 g portions of dry dog food inoculated with a two-strain mixture of nalidixic acid (NA) resistant *S. Schwarzengrund* (~ 6 log CFU/g) were sprayed with *L. plantarum* alone or in combination with 0.5%, 1%, or 2% of TY, and stored at 25°C for 4 weeks. On days 0, 1, 3, 5, 7, 14, 21, and 28, surviving *S. Schwarzengrund* populations on dog food were enumerated on Xylose lysine deoxycholate agar + NA agar plates.

All three probiotic bacteria survived on dry dog food during the entire storage period, but *L. plantarum* demonstrated the highest survivability on dog food, followed by *L. brevis* and *L. casei*. Betaine at 0.02% with 0.8 M KCl increased the survival of three lactobacilli on dry dog food compared to that on control ($P < 0.05$). In addition, TY (0.5%, 1%, and 2%) combined with *L. plantarum* decreased *S. Schwarzengrund* populations on dog food as compared to controls ($P < 0.05$). Results suggest that *L. plantarum* alone or combined with TY could potentially be used as a functional antimicrobial on dry pet food.

1. Introduction

In light of multiple outbreaks of human salmonellosis linked to contaminated pet foods (CDC, 2008; CDC, 2011; Li et al., 2012), there exists a significant public health concern on the microbiological safety of pet foods and treats. Although the pet food industry employs good manufacturing practices and chemical based interventions for reducing pathogens on pet foods (CDC, 2011), the use of probiotics as food additives for improving pet food safety and pet health is not common. Probiotics are defined as live microorganisms which when consumed in appropriate amounts confer a health benefit on the host (FAO/WHO, 2002). The US Food and Drug Administration has classified probiotic microorganisms as generally recognized as safe (FDA/FAO/WHO, 2001). During the last several decades, probiotic bacteria have been increasingly used for enhanced nutrition and health purposes in humans (Hoesl et al., 2005; Rautava et al., 2005), with probiotic containing products reaching an approximate annual market value of \$ 6 billion in the world (Hoesl et al., 2005). Lactic acid bacteria such as lactobacilli and bifidobacteria are most commonly used as probiotics in food products (Kailasapathy et al., 2000; Schrezenmeir and de Vrese, 2001). Probiotic bacteria exert numerous benefits to the host, including nutrient digestion and assimilation (Sonnenburg et al. 2005; Yatsunenko et al. 2012), potentiating host immune function (Isolauri et al., 2001), anti-cancer and anti-atherogenic effects (Nair et al., 2017), and protection against enteric pathogens (Candela et al., 2008; Fukuda et al., 2011). In light of increasing evidence on the potential implication of gut dysbiosis in gastro-intestinal disorders, including diarrheal diseases, ulcerative colitis, inflammatory bowel diseases, and life style diseases such as obesity and type 2 diabetes mellitus, human consumption of probiotic containing products has been steadily increasing worldwide (Grover et al., 2012).

As pet owning households are rising in the US, there is considerable demand for high-quality pet diets by pet owners. (Brady and Palmeri, 2007). Currently 7% of pet foods and supplements contain probiotics, and the use of probiotic microorganisms in pet foods is expected to grow in the future (Courage, 2014). In this regard, research on the use of probiotic bacteria as functional pet food ingredients for improving animal health has gained significant interest. For example, *Lactobacillus acidophilus* has been found to improve the intestinal microbial balance by reducing the populations of clostridia, and stimulate the immune response by increasing the IgG concentration in dogs (Baillon et al., 2004). Similarly, a multi-species probiotic product containing *L. acidophilus* MA 64/4E, *L. farciminis*, *Bacillus subtilis*, *B. licheniformis*, and *Pediococcus acidilactici* was reported to decrease the duration of acute diarrhea in dogs (Herstad et al., 2010).

Although substantial market potential exists for developing pet foods and supplements containing probiotics, the viability of probiotic bacteria on pet foods, especially dry pet food products containing a low water activity, during long-term storage is a concern. For example, Weese and Arroyo (2003), while evaluating 19 commercial pet food products that claimed to have probiotic supplementations, found that 26% of the tested products did not contain any relevant probiotic bacterial population, and none of the tested products contained all claimed probiotic strains.

Under a dry environment, the ionic strength of a medium rises subjecting bacteria to osmotic stress due to reduced water activity. Bacteria under such circumstances respond to low water activity by accumulating osmolytes or compatible solutes such as betaine (Brown, 1976; Sheehan et al., 2007). Studies have shown that betaine uptake via betaine transporters significantly increased the survival of bacteria under dry environment (Sleator et al., 1999;

Sleator et al., 2003 a, b; Smiddy et al., 2004; Dreux et al., 2008; Burgess et al., 2016). In addition, some *Lactobacillus* spp. contain hyperosmotic stress-activated betaine transporter systems (Glaasker et al., 1998; Obis et al., 1999; Wood et al., 2001), where betaine accumulation has been documented to enhance the survivability of bacteria under a dry environment (Kets et al., 1993; Glaasker et al., 1996).

The objective of this study was to determine the effect of betaine on the survival of *L. plantarum*, *L. casei*, and *L. brevis* on dry dog food during long term storage, for potential application as a functional feed additive to improve pet food safety and animal health. In addition, the efficacy of *L. plantarum* in combination with thymol, a phytochemical derived from the oil of thyme, for reducing *S. Schwarzengrund* on dry dog food was determined.

2. Materials and Methods

2.1 Effect of betaine on the survival of probiotic bacteria on dry dog food

All bacteriological media were obtained from Difco, Becton Dickinson (Sparks, MD). *Lactobacillus plantarum* (NRRL B-4496), *L. casei* (ATCC 334), and *L. brevis* (NRRL B-3365) were procured from the USDA-ARS culture collection (Peoria, IL). Betaine and potassium chloride (KCl) (99% purity) were procured from Sigma Aldrich (St. Louis, MO).

To prepare the probiotic inoculum, *L. plantarum*, *L. casei* and *L. brevis* (~ 5 log CFU/ml) was cultured separately in 10 ml of MRS broth containing 0%, 0.1% or 0.02% betaine (vol/vol) with or without 0.8 M KCl at 37°C for 24 h (Glaasker et al., 1996). After incubation, each probiotic culture was centrifuged at 3,600 \times g for 15 min at 4°C. The pellet of each probiotic culture was washed twice and resuspended in 10 ml of 0.1% peptone water (pH 7). The bacterial population in each probiotic culture was confirmed by plating 0.1 ml portions of appropriate dilutions on MRS agar plates followed by incubation at 37°C for 48 h.

The various treatment groups included in this experiment are listed in Table 1. For each treatment, 300 g portions of a commercially available dry dog food were spray inoculated with each probiotic bacterium to obtain $\sim 8 \log$ CFU/g using an air atomizer (Master air brush, Eco kit-17, TCP global, San Diego, CA). The inoculated dog food was air-dried for 1 h in a biosafety cabinet to facilitate bacterial attachment. Following this, 10 g portions of dog food were transferred to sterile WhirlPak bags (Sigma-Aldrich) and stored at 25°C for 24 weeks. In weeks 0, 1, 2, 4, 8, 12, 18, and 24, a volume of 20 ml of neutralizing broth (Sigma-Aldrich) was added to each bag containing 10 g of dry dog food and pummeled in a stomacher (Stomacher 400 Circulator, Seward, Davie, FL) for 1 min. The dog food homogenate was serially diluted (1:9) in phosphate buffer saline (PBS, pH 7.0), and 0.1 ml aliquots from appropriate dilutions were surface plated on duplicate MRS agar plates, and incubated at 37°C for 48 h before counting colonies.

2.2 Efficacy of probiotic bacteria combined with thymol for reducing *S. Schwarzengrund* on dry dog food

The *Lactobacillus* spp. which showed the highest survival on dry dog food at the end of 24-week storage was selected to determine its efficacy in reducing *Salmonella* on dog food, by applying alone or in combination with 0.5%, 1%, or 2% TY (w/w) (99% purity, SAFC grade; Sigma-Aldrich). Two strains of *Salmonella enterica* Serovar Schwarzengrund (CVM 19633 and DBS-GA-F25499) obtained from BEI resources (Manassas, VA) were used in this study. *Salmonella* Schwarzengrund was pre-induced for resistance to 50 µg/ml of nalidixic acid (NA; Sigma Aldrich) for selective enumeration (Ebers et al., 2009). To prepare the inoculum, 100 µl of an overnight culture of each NA-resistant *Salmonella* strain was cultured separately in 10 ml tryptic soy broth (TSB) containing 50 µg/ml of NA, and incubated at 37°C for 24 h.

After incubation, each *Salmonella* culture was centrifuged at $3,600 \times g$ for 15 min at 4°C. The pellet of each *S. Schwarzengrund* strain was washed and resuspended in 10 ml of 0.1% peptone water, and 0.1 ml of the suspension was spread plated on xylose lysine desoxycholate agar containing 50 µg/ml of NA (XLD + NA). After incubation at 37°C for 24 h, 10 ml of 0.1% peptone water was added onto the XLD+NA plate containing colonies of each *Salmonella*, and the agar surface was gently washed to collect the bacteria (Beuchat and Mann, 2011). Equal portions of the two *S. Schwarzengrund* cultures were combined and was used as the inoculum ($\sim 8 \log$ CFU/ml). The bacterial count in the two-strain cocktail was confirmed by plating 0.1 ml portions of appropriate dilutions on XLD+NA plates, followed by incubation at 37°C for 24-48 h.

Prior to the experiment, 10 g of dry dog food in duplicate was enriched in 100 ml of cysteine selenite broth and incubated at 37°C for 48 h. The enriched culture was streaked on XLD agar plate to determine the presence of any inherent *Salmonella* spp. on dry dog food. For each treatment, 300 g portions of dry dog food were spray-inoculated with the two-strain NA-resistant cocktail of *S. Schwarzengrund* to obtain $\sim 6 \log$ CFU/g using an air atomizer (Master air brush, Eco kit-17, TCP global). The inoculated dog food was then air-dried for 1 h in a biosafety cabinet for facilitating bacterial attachment. Ten milliliters of *L. plantarum* culture (CFU/ml) were sprayed onto the *Salmonella*-inoculated dry dog food to obtain $\sim 8 \log$ CFU/g, followed by spraying 10 ml of TY solutions to obtain final concentrations of 0%, 0.5%, 1%, and 2% (wt/wt) on dog food. Dry dog food inoculated with *S. Schwarzengrund*, but not subjected to TY and *L. plantarum* treatment served as the control. Since TY was dissolved in vegetable oil before spraying on the pet food, vegetable oil at the final concentration of 5% was included as a diluent control.

After the spray treatment, 10 g portions of treated dog food were transferred to sterile WhirlPak bags and stored at 25°C for 28 days. On days 0, 1, 3, 5, 7, 14, 21, and 28, a volume of 20 ml of neutralizing broth was added to each bag containing 10 g of pet food and pummeled in a stomacher for 1 min. The pet food homogenate was serially diluted (1:10) in PBS, and 0.1 ml aliquots from appropriate dilutions were surface plated on duplicate XLD+NA and MRS agar plates, and incubated at 37°C for 48 h. In addition, 1 ml of neutralizing broth was added to 20 ml of cysteine selenite broth, and incubated at 37°C for 48 h. Following enrichment, the culture was streaked on XLD-NA plates, incubated at 37°C for 48 h, and the plates were observed for *Salmonella* colonies.

2.3 Water activity and pH measurement

Water activity and pH of the dog food samples were measured, as described by Ruth et al. (2016). In the probiotic survival experiment, water activity and pH of the samples were measured on day 0 and in week 24, whereas in the *Salmonella* inactivation study, water activity and pH were determined on days 0 and 28. Briefly, pH was determined at 25°C by weighing 1 g portions of dry dog food from each treatment, pulverizing with a mortar and pestle and hydrating with 2.5 ml distilled water, using pre-calibrated pH meter (Horiba, Baltimore, MD). As per the manufacture's specifications, 4 g portions of dry dog food from each treatment were ground, and the water activity of each sample was determined using a water activity meter (Rotronic, Hauppauge, NY).

2.4 Statistical analysis

A completely randomized design was used with a factorial treatment structure. In the probiotic bacterial survival experiment, the factors included 6 treatments (0%, 0.02%, and 0.1% of betaine with or without 0.8M KCl) and 8 time points (week 0, 1, 2, 4, 8, 12, 18, and

24). In the *Salmonella* inactivation experiment, the factors included 2 probiotic treatments (with and without *L. plantarum*) x 4 TY concentrations (0%, 0.5%, 1%, and 2%) x 8 time points (day 0, 1, 3, 5, 7, 14, 21, and 28). Data were analyzed using the PROC-Genmode procedure of the Statistical Analysis Software (SAS Institute Inc., Cary, NC). Each experiment had triplicate samples, and was repeated twice. Differences among the means were analyzed at $P < 0.05$ using Fisher's least significance difference test with appropriate corrections for multiple comparisons

3. Results

3.1 Effect of betaine on the survival of *Lactobacillus* spp. on dry dog food

The effect of betaine on the survival of *L. plantarum*, *L. casei*, and *L. brevis* on dry dog food is shown in Figure 1A, 1B, and 1C, respectively. All three *Lactobacillii* survived on dry dog food during the entire storage period of 24 weeks. Among the three tested lactobacillii, *L. plantarum* demonstrated the highest survivability (8.30 log CFU/g), followed by *L. brevis* (5.60 log CFU/g) and *L. casei* (2.04 log CFU/g) at the end of 24 weeks of storage (Fig. 1A-C). On week 24, ~ 8.69 log CFU/g of *L. plantarum* was recovered in control samples, where only a minimal reduction of ~ 0.3 log CFU/g in bacterial population was observed at the end of storage period (Fig. 1A). However, compared to control, all treatments except 0.1% betaine and 0.02% betaine + KCl yielded lower *L. plantarum* counts ($P < 0.05$), although the magnitude of reduction was less than 1 log CFU/g. On the contrary, when *L. plantarum* was incubated with 0.02% betaine and 0.8M KCl, the bacterial population (~8.67 log CFU/g) recovered at the end of 24 week storage was greater than that of control (~8.30 log CFU/g) ($P < 0.05$) (Fig. 1A). In addition, compared to control a significantly greater population of ~ 1.92 log CFU/g and 1.40 log CFU/g of *L. brevis* and *L. casei*, respectively were recovered at

the end of week 24, when these probiotic cultures were incubated with 0.02% betaine + 0.8M KCl (Fig. 1B and 1C).

3.2 Effect of *L. plantarum* alone or combined with TY on the survival of *S. Schwarzengrund* on dry dog food

Since *L. plantarum* showed the highest survival on dry dog food during the 24-week storage, it was selected for subsequent anti-*Salmonella* study. Table 2 shows the effect of *L. plantarum* alone or in combination with TY at 0%, 0.5%, 1%, and 2% on *S. Schwarzengrund* on dry dog food. On day 0, ~ 5.7 log CFU/g of *Salmonella* was recovered from all the dog food samples. On day 28, ~ 3.9 log CFU/g of *Salmonella* was recovered from the control dog food samples. Vegetable oil control did not significantly affect the survival of *Salmonella* throughout the storage period as compared to control ($P > 0.05$). Spraying of dog food with *L. plantarum* alone reduced *S. Schwarzengrund* by ~ 0.8 log CFU/g compared to control ($P < 0.05$). Similarly, compared to control, TY at 0.5%, 1%, and 2% significantly reduced the survival of the *Salmonella* on dog food in a concentration dependent manner, with 2% TY decreasing *Salmonella* by ~ 2.0 log CFU/g on day 28 ($P < 0.05$). However, the inhibitory effect of *L. plantarum* and TY on *S. Schwarzengrund* was increased when both treatments were applied together on dog food. As observed in Table 2, spraying with *L. plantarum* in combination with TY at 0.5%, 1% and 2% reduced *S. Schwarzengrund* counts on dog food by 2.0, 2.4 and 2.6 log CFU/g on day 28 ($P < 0.05$).

3.3 Water activity and pH of pet food

Table 3 and Table 4 show the water activity and pH, respectively of dry dog food treated with and without betaine and KCl and various *Lactobacillus* spp. None of the treatments had

any effect on the water activity and pH of dry dog food as compared to control on day 0 and at the end of six months of storage ($P > 0.05$).

The water activity and pH of dog food samples in the *Salmonella* inactivation study are shown in Table 5, where TY at 0.5%, 1%, and 2% did not significantly affect the pH and the water activity of the dry dog food; however, *L. plantarum* alone or in combination with TY at 0.5%, 1%, and 2% increased the water activity of samples compared to control ($P < 0.05$).

4. Discussion

A majority of dog owners rely on dry food as the primary source of nutrition for their pets (AVMA, 2013). However, dry dog food associated human outbreaks of salmonellosis and several recalls of pathogen contaminated pet foods highlight the need for an effective strategy to control *Salmonella* in these products. Recently, there is an increased interest to supplement probiotic bacteria as functional ingredients in dry pet foods; however, the viability of probiotics under the low water activity encountered in dry dog food, especially during long-term storage time is uncertain. Therefore, we investigated the viability of three *Lactobacillus* spp. on dry dog food during a 24-week storage period. In addition, the effect of betaine, a common compatible solute, in enhancing the survival of probiotic bacteria on dry dog food was evaluated in the presence and absence of KCl.

Viability studies of three *Lactobacillus* spp. on dry dog food during the 24-week storage period revealed that the three probiotic species differed in their survivability, with the highest survival rate for *L. plantarum*, followed by *L. brevis* and *L. casei*. It was also observed that betaine at 0.02% in combination with 0.8 M KCl significantly enhanced the survival of all three *Lactobacillus* spp. on dry dog food, although the effect was minimal in *L. plantarum* compared to the other two species. This could be attributed to the fact that *L. plantarum* was

able to persist under the dry environment on dog food even without the presence of betaine and KCl; hence, the effect of betaine was not marked. We included a treatment combining betaine with KCl (0.8 M) since it has been documented that the presence of KCl creates a moderately high osmolarity medium and stimulates betaine uptake by *L. plantarum* and *L. acidophilus* (Hutkins et al., 1987). Additionally, Glaasker and co-workers (1996) reported that when the osmolarity of a growth medium was raised by adding KCl, betaine uptake rates by *L. plantarum* increased from 15 nmol/min to 80 nmol/min. Similarly, Kets et al. (1996) observed that *L. plantarum* cultivated under an osmotic stress of NaCl along with betaine showed increased survival after air-drying and subsequent vacuum desiccation to a low water activity environment.

Since *L. plantarum* demonstrated the highest survival rate compared to *L. casei* and *L. brevis*, the antimicrobial activity of *L. plantarum* alone or in combination with TY against *S. Schwarzengrund* on dry dog food was determined. Results revealed that although *L. plantarum* or TY by itself was able to reduce *S. Schwarzengrund* counts on dog food compared to control, the combination of *L. plantarum* with TY exerted a significantly greater antimicrobial activity against *Salmonella* (Table 2). However, TY had no significant inhibitory effect on *L. plantarum*.

Probiotics inhibit the growth of pathogens through competition for nutrients or binding sites, or by production of antimicrobial substances (Casey et al., 2004; Möndel et al., 2009). On the other hand, TY being strongly hydrophobic, targets the lipid-containing bacterial cell membrane and makes it more permeable with leakage of ions and other cell contents (Sikkema et al., 1995; Cox et al., 2000; Ultee et al., 2002). The enhanced inhibitory effect of TY-*L. plantarum* combination on *Salmonella* could be attributed to the reciprocal interaction

of the phytochemical with *L. plantarum*. Since phytochemicals and probiotics exert their antimicrobial effects by different mechanisms (Shipradeep et al., 2012), their combination could be more inhibitory on *Salmonella*. However, research delineating synergistic interactions between probiotics and phytochemicals is scanty, and additional research is necessary to elucidate the interactive mechanisms behind their augmented antimicrobial effect.

Water activity of dry pet food is another critical factor affecting the survival of pathogens. In this study, although results showed an increased water activity (~ 0.58) of probiotic-treated dog food compared to control, the probiotic alone or combined with TY still effectively inhibited *Salmonella* growth. This could be due to the fact a water activity of 0.58 is not high enough to support *Salmonella* growth on dry pet food. Previously, Himathongkham et al. (1999) and Koutsoumanis et al. (2004) suggested that a water activity above 0.90 was required for the growth of *Salmonella*. Moreover, Oni et al. (2016) reported that rehydration of dry dog food with up to 35-50% of additional water may be needed to support the growth of *Salmonella* on dry dog food.

In conclusion, this study suggests that lactobacilli can differ in their ability to survive on dry pet food, and they should be screened for long-term viability before application on pet food. Moreover, betaine and KCl could potentially be used for enhancing probiotic bacterial viability on dry pet food. Further, Biourge et al. (1998), while evaluating the stability of a probiotic *Bacillus* spp. on dry dog food during manufacturing process, observed that ~ 75% of the probiotic bacterium survived on the dog food when applied at the post-extrusion stage, which suggests that supplementation of probiotic bacteria to a dry dog food product is potentially feasible. However, additional large-scale studies, especially under a commercial

setting and nutritional and palatability analyses of probiotic and TY supplemented dry pet food are warranted.

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Table 1. Treatment groups.

Group Abbreviation	Composition of the group Betaine in MRS broth (wt/vol)
Control	Probiotic alone
KCl control	Probiotic + KCl
0.02% B	Probiotic + 0.02% Betaine
0.1% B	Probiotic + 0.1% Betaine
0.02% B + KCl	Probiotic + 0.02% Betaine + KCl
0.1% B + KCl	Probiotic + 0.1% Betaine + KCl

Table 2. Effect of *Lactobacillus plantarum* in combination with thymol on *Salmonella* Schwarzengrund on dry dog food¹

Treatments ²	Day 0	Day 1	Day 3	Day 5	Day 7	Day 14	Day 21	Day 28
Control	5.74±0.02 ^a	5.55±0.03 ^a	5.14±0.02 ^a	4.75±0.05 ^a	4.48±0.02 ^a	4.29±0.03 ^a	4.24±0.05 ^a	3.93±0.05 ^a
VegOil	5.74±0.02 ^a	5.52±0.01 ^a	4.90±0.02 ^{ab}	4.60±0.03 ^{ab}	4.27±0.08 ^{ab}	4.01±0.07 ^{ab}	3.98±0.09 ^{ab}	3.93±0.03 ^a
Probiotic	5.74±0.02 ^a	5.34±0.06 ^a	4.87±0.03 ^b	4.53±0.04 ^{bc}	4.21±0.06 ^b	3.65±0.01 ^b	3.41±0.02 ^c	3.15±0.02 ^{ab}
Probiotic + VegOil	5.74±0.02 ^a	5.41±0.02 ^a	5.06±0.02 ^a	4.48±0.03 ^{bc}	4.22±0.07 ^b	3.75±0.01 ^b	3.56±0.01 ^{bc}	2.99±0.07 ^b
TY 0.5%	5.74±0.02 ^a	5.20±0.02 ^{ab}	4.84±0.03 ^b	4.45±0.05 ^c	4.16±0.02 ^b	3.73±0.01 ^b	3.37±0.02 ^c	2.92±0.10 ^b
TY 1%	5.74±0.02 ^a	4.41±0.09 ^c	4.17±0.04 ^d	4.04±0.02 ^d	3.83±0.01 ^c	3.36±0.01 ^c	3.07±0.02 ^d	2.49±0.02 ^c
TY 2%	5.74±0.02 ^a	2.67±0.04 ^d	3.06±0.05 ^f	3.03±0.05 ^f	2.96±0.07 ^d	2.74±0.06 ^d	2.32±0.04 ^e	1.84±0.08 ^d
Probiotic + TY0.5%	5.74±0.02 ^a	5.13±0.02 ^b	4.71±0.01 ^c	4.51±0.03 ^{bc}	3.83±0.01 ^c	3.31±0.01 ^c	2.22±0.02 ^e	1.90±0.03 ^d
Probiotic + TY 1%	5.74±0.02 ^a	4.45±0.02 ^c	4.03±0.06 ^e	3.71±0.06 ^e	3.08±0.10 ^d	2.59±0.09 ^d	1.50±0.06 ^f	1.46±0.10 ^e
Probiotic + TY 2%	5.74±0.02 ^a	1.46±0.10 ^e	1.86±0.03 ^g	1.89±0.04 ^g	2.02±0.08 ^e	1.96±0.04 ^e	1.30±0.00 ^g	1.30±0.00 ^f

¹Data are mean ± SEM obtained from 2 separate experiments with triplicate samples on each sampling point (Day 0, 1, 3, 5, 7, 14, 21, and 28).

² Treatments: Control: dog food inoculated with *Salmonella* only; VegOil: *Salmonella* inoculated dog food treated with 5% vegetable oil; Probiotic: *Salmonella* inoculated dog food sprayed with *L. plantarum*; Probiotic + VegOil: *Salmonella* inoculated dog food sprayed with *L. plantarum* and vegetable oil; TY 0.5%-2%: *Salmonella* inoculated dog food sprayed with TY at 0.5%, 1%, or 2%; Probiotic + TY0.5%-2%: *Salmonella* inoculated dog food sprayed with *L. plantarum* and TY at 0.5%, 1%, or 2%.

^{a-g} differed significantly in the same column.

Table 3. Water activity of the dry dog food subjected to different probiotic treatments¹.

Treatments ²	<i>L. plantarum</i>		<i>L. casei</i>		<i>L. brevis</i>	
	Day 0	Month 6	Day 0	Month 6	Day 0	Month 6
Control	0.61±0.031 ^a	0.38±0.005 ^a	0.61±0.029 ^a	0.38±0.002 ^a	0.62±0.027 ^a	0.39±0.003 ^a
KCl control	0.65±0.017 ^a	0.39±0.007 ^a	0.59±0.004 ^a	0.38±0.005 ^a	0.65±0.018 ^a	0.36±0.012 ^a
0.02% B	0.63±0.027 ^a	0.37±0.012 ^a	0.63±0.026 ^a	0.39±0.007 ^a	0.63±0.023 ^a	0.38±0.002 ^a
0.1% B	0.59±0.004 ^a	0.39±0.004 ^a	0.61±0.003 ^a	0.37±0.012 ^a	0.64±0.010 ^a	0.38±0.005 ^a
0.02% B + KCl	0.65±0.023 ^a	0.38±0.002 ^a	0.62±0.006 ^a	0.41±0.009 ^a	0.65±0.020 ^a	0.39±0.007 ^a
0.1% B + KCl	0.64±0.012 ^a	0.41±0.009 ^a	0.64±0.014 ^a	0.41±0.017 ^a	0.62±0.002 ^a	0.37±0.012 ^a

¹ Data are mean ± SEM obtained from triplicate samples on each sampling point (day 0 and 6 month).

²Treatments: Control: dog food inoculated probiotics only; KCL control: probiotics incubated with KCL and inoculated on the dog food; 0.02% B: probiotics incubated with 0.02% betaine and inoculated on the dog food; 0.1% B: probiotics incubated with 0.1% betaine and inoculated on the dog food; 0.02% B + KCL: probiotics incubated with 0.02% betaine + KCL and inoculated on the dog food; 0.1% B + KCL: probiotics incubated with 0.1% betaine + KCL and inoculated on the dog food

^a differed significantly in the same column.

Table 4. pH of dry dog food treated with different probiotic treatments¹.

Treatments	<i>L. plantarum</i>		<i>L. casei</i>		<i>L. brevis</i>	
	Day 0	Month 6	Day 0	Month 6	Day 0	Month 6
Control	5.90±0.008 ^a	5.87±0.025 ^a	5.90±0.008 ^a	5.87±0.025 ^a	5.90±0.008 ^a	5.87±0.002 ^a
KCl control	5.84±0.042 ^a	5.81±0.006 ^a	5.88±0.029 ^a	5.87±0.041 ^a	5.85±0.041 ^a	5.83±0.029 ^a
0.02% B	5.84±0.036 ^a	5.83±0.032 ^a	5.88±0.030 ^a	5.86±0.043 ^a	5.83±0.029 ^a	5.83±0.047 ^a
0.1% B	5.85±0.035 ^a	5.83±0.059 ^a	5.83±0.032 ^a	5.83±0.029 ^a	5.83±0.029 ^a	5.74±0.061 ^a
0.02% B + KCl	5.84±0.042 ^a	5.83±0.029 ^a	5.86±0.020 ^a	5.85±0.022 ^a	5.83±0.029 ^a	5.83±0.047 ^a
0.1% B + KCl	5.83±0.047 ^a	5.83±0.056 ^a	5.85±0.041 ^a	5.83±0.029 ^a	5.90±0.009 ^a	5.87±0.025 ^a

¹ Data are mean ± SEM obtained from triplicate samples on each sampling point (day 0 and 6 month).

²Treatments: Control: dog food inoculated probiotics only; KCL control: probiotics incubated with KCL and inoculated on the dog food; 0.02% B: probiotics incubated with 0.02% betaine and inoculated on the dog food; 0.1% B: probiotics incubated with 0.1% betaine and inoculated on the dog food; 0.02% B + KCL: probiotics incubated with 0.02% betaine + KCL and inoculated on the dog food; 0.1% B + KCL: probiotics incubated with 0.1% betaine + KCL and inoculated on the dog food

^a differed significantly in the same column.

Table 5. Water activity and pH of dry dog food treated with *Lactobacillus plantarum* in combination with thymol.

Treatments ²	water activity		pH	
	Day 0	Day 28	Day 0	Day 28
Control	0.28±0.003 ^b	0.27±0.00 ^b	5.84±0.060 ^a	5.83±0.033 ^a
VegOil	0.27±0.003 ^b	0.27±0.006 ^b	5.83±0.029 ^a	5.83±0.060 ^a
Probiotic	0.58±0.003 ^a	0.45±0.031 ^a	5.84±0.042 ^a	5.83±0.048 ^a
Probiotic + VegOil	0.57±0.006 ^a	0.50±0.030 ^a	5.83±0.048 ^a	5.91±0.048 ^a
TY 0.5%	0.31±0.015 ^b	0.31±0.007 ^b	5.85±0.039 ^a	5.90±0.009 ^a
TY 1%	0.31±0.007 ^b	0.31±0.006 ^b	5.77±0.076 ^a	5.81±0.036 ^a
TY 2%	0.32±0.018 ^b	0.29±0.034 ^b	5.81±0.038 ^a	5.90±0.009 ^a
Probiotic + TY0.5%	0.59±0.014 ^a	0.44±0.025 ^a	5.83±0.048 ^a	5.83±0.048 ^a
Probiotic + TY 1%	0.59±0.012 ^a	0.44±0.014 ^a	5.77±0.033 ^a	5.78±0.031 ^a
Probiotic + TY 2%	0.59±0.019 ^a	0.47±0.028 ^a	5.84±0.042 ^a	5.76±0.043 ^a

¹ Data are mean ± SEM obtained from triplicate samples on each sampling point (day 0 and day 28).

²Treatments: Control: dog food inoculated with *Salmonella* only; VegOil: *Salmonella* inoculated dog food treated with 5% vegetable oil; Probiotic: *Salmonella* inoculated dog food sprayed with *L. plantarum*; Probiotic + VegOil: *Salmonella* inoculated dog food sprayed with *L. plantarum* and vegetable oil; TY 0.5%-2%: *Salmonella* inoculated dog food sprayed with TY at 0.5%, 1%, or 2%; Probiotic + TY0.5%-2%: *Salmonella* inoculated dog food sprayed with *L. plantarum* and TY at 0.5%, 1%, or 2%.

^{a-b} differed significantly in the same column.

Figure 1. Effect of betaine on the survival of probiotic bacteria on dry dog food. Data are mean \pm SEM obtained from 2 separate experiments with triplicate samples at each sampling point. Error bar indicates SEM (n=6). A-C show the effect of betaine on *L. plantarum*, *L. casei*, and *L. brevis*, respectively.

Figure 1A *Lactobacillus plantarum*

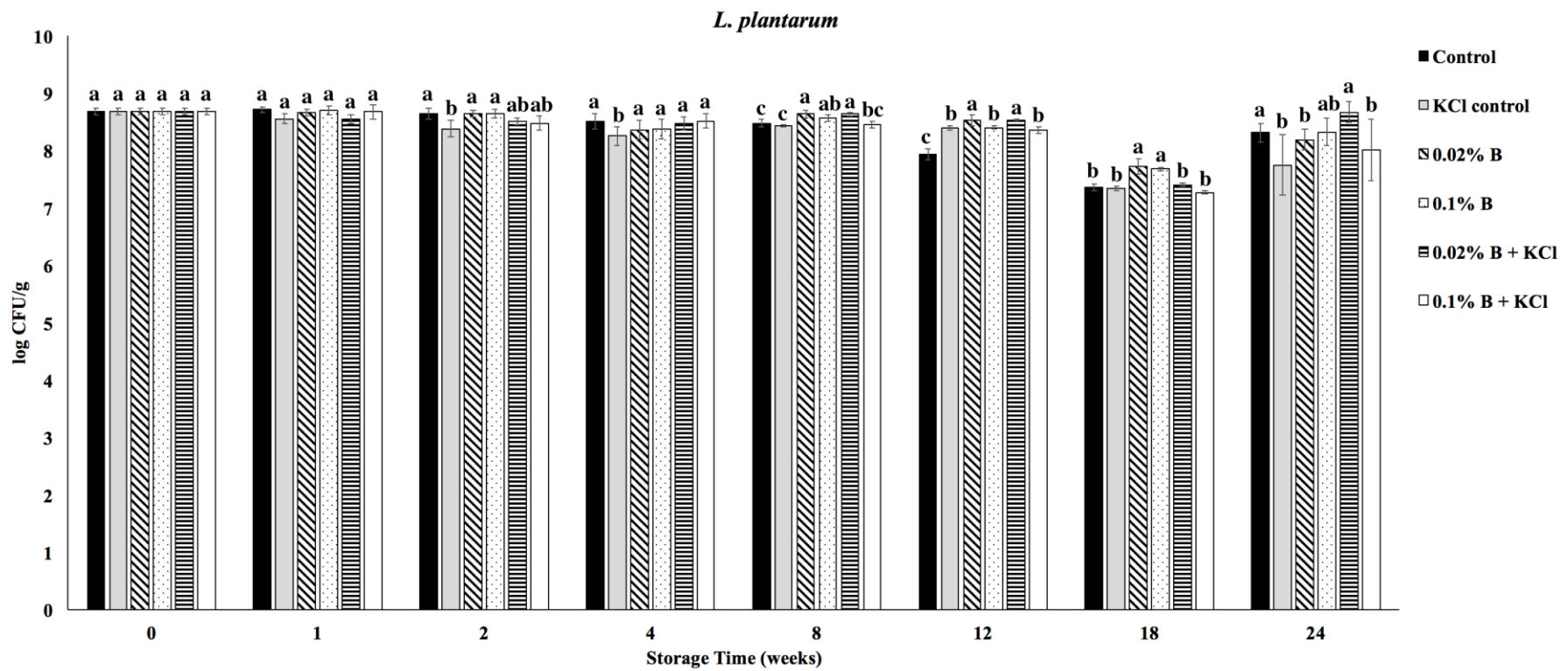


Figure 1B *Lactobacillus casei*

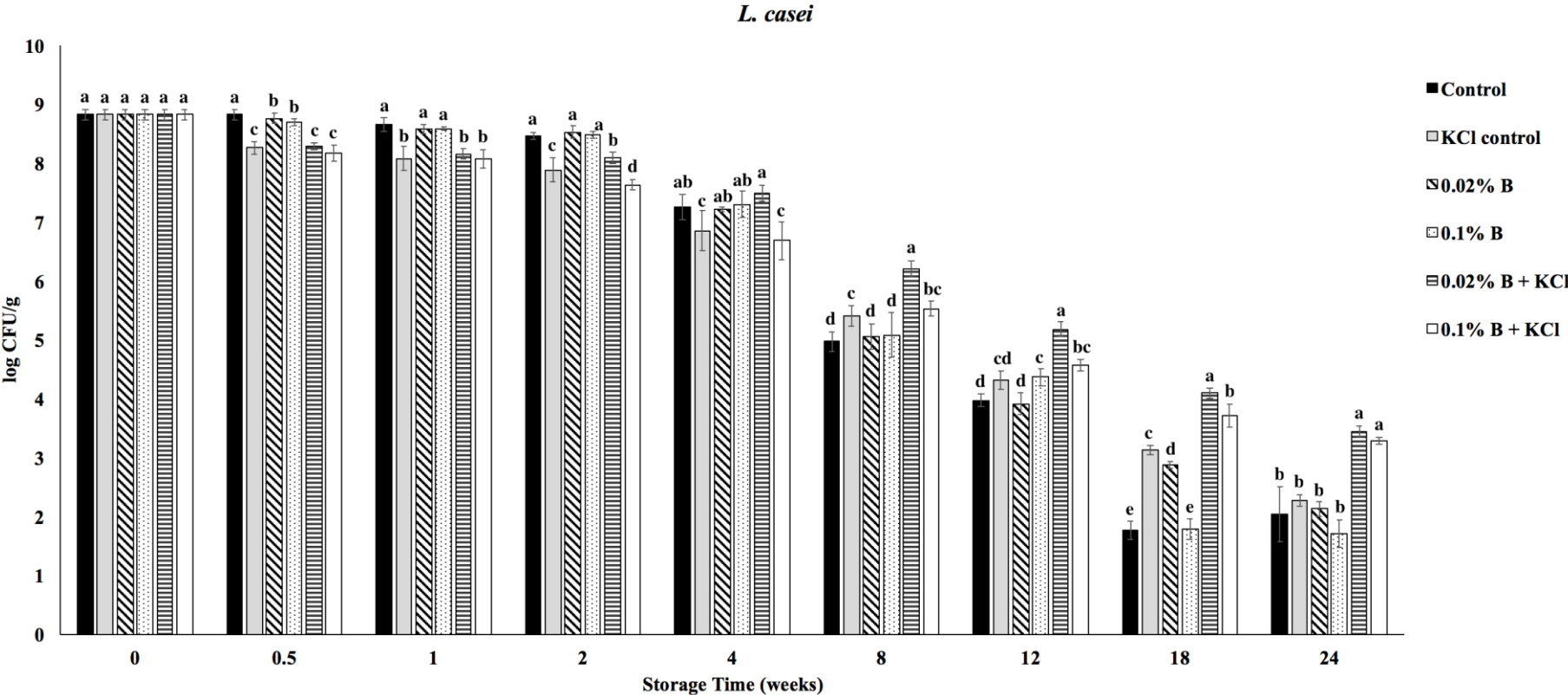
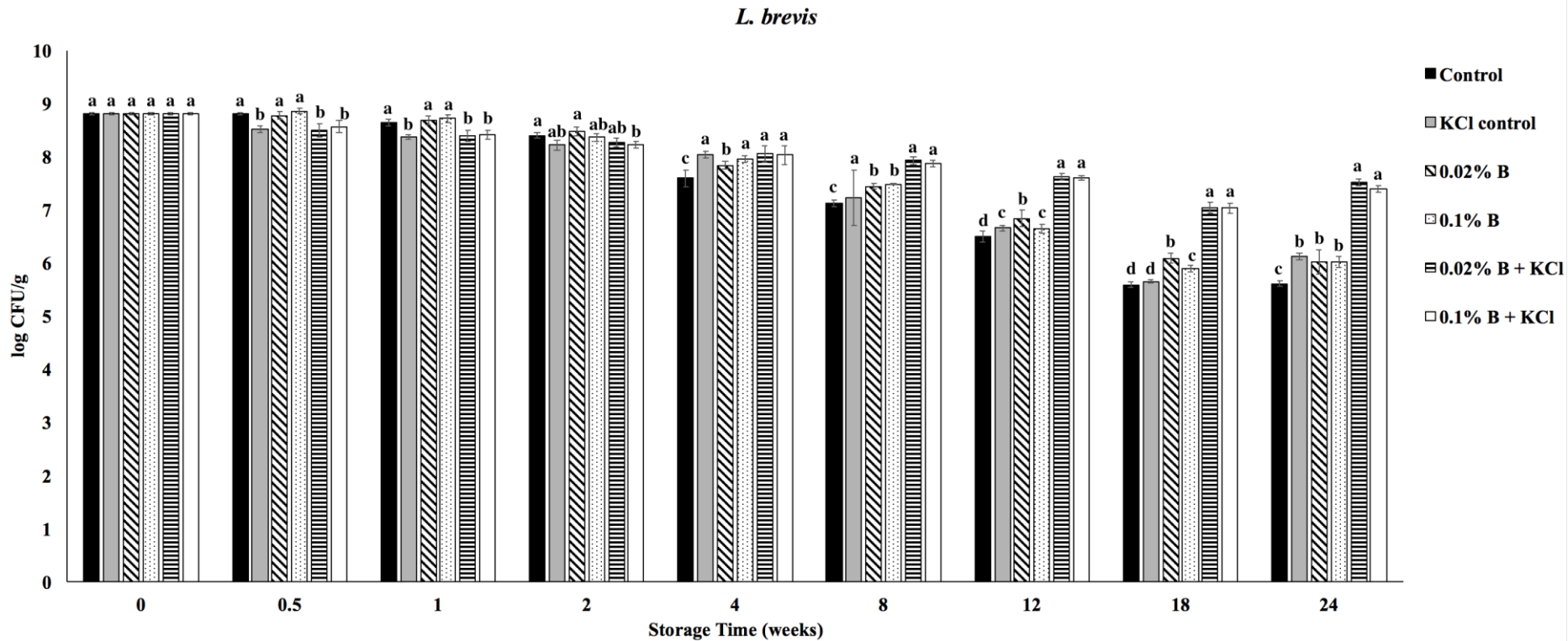


Figure 1C *Lactobacillus brevis*



Chapter V

A Pilot Study on Isolation of Probiotic Bacteria from Canine Feces with Potential Antimicrobial Activity against *Salmonella* Schwarzengrund and *Listeria monocytogenes*

Abstract

The use of probiotic bacteria as functional pet food ingredients has gained significant interest due to their beneficial effects on improving pet health and reducing the risk of gastrointestinal disorders. However, compared to human probiotics microorganisms, relatively limited information exists on the antimicrobial activity of probiotic bacteria originating from canines. *Salmonella* spp. and *Listeria monocytogenes* are two important pathogens that are significant to human and pet health. The objective of this study was to isolate lactic acid bacteria (LAB) from canine feces with potential antimicrobial activity against *Salmonella* spp. and *L. monocytogenes*. Five hundred and twenty presumptive LAB cultures were isolated from 70 canine feces samples, and their antimicrobial property against *Salmonella* Schwarzengrund and *L. monocytogenes* was determined. The LAB isolates that exerted strong inhibitory effect against both pathogens were identified by matrix assisted laser desorption ionization (MALDI-TOF) and species-specific PCR.

Among the 520 tested cultures, 52 isolates exerted potential antimicrobial effect against both target pathogens. Out of these 52 isolates, eight LAB isolates demonstrated strong inhibition against *S. Schwarzengrund* and *L. monocytogenes*. Of the eight LAB isolates, five isolates were identified as *Lactobacillus plantarum* and two isolates as *Pediococcus pentosaceus* by MALDI-TOF analysis and PCR, whereas one isolate was unidentified. Results suggest that the isolated LAB cultures could potentially be used as probiotics in dogs, however, follow up studies are needed to elucidate the mechanisms behind their antagonistic effect.

1. Introduction

Salmonella is a major foodborne pathogen that causes an estimated 1.2 million human illnesses, 19,000 hospitalizations and 450 deaths annually in the United States (CDC, 2011). *Listeria monocytogenes* is another important foodborne pathogen responsible for 19% of all deaths from foodborne illnesses in the USA (Scallan et al., 2011). Besides the consumption of contaminated foods, salmonellosis and listeriosis are zoonotic diseases that could be acquired by exposure to contaminated environment or infected animals. It has been suggested that infected dogs could be reservoirs of these pathogens, where animals shed the bacteria in their feces and saliva for prolonged period of time, thereby leading to human illnesses (Verma, 2007; Apanavicius et al, 2007). In addition, contaminated pet food has been linked to two major outbreaks of human salmonellosis involving more than 100 people from 20 different states (CDC, 2008; CDC, 2012; Li et al., 2012). Similarly, several recalls of *L. monocytogenes* contaminated pet foods have been reported in the US during the last decade (Anonymous, 2016, 2017; FDA, 2017). These outbreaks and recalls raise concerns on the microbiological safety of pet foods, highlighting the need for effective intervention strategies to control pathogens on these products.

Probiotics are defined as live microorganisms which when consumed in appropriate amounts confer a health benefit on the host (WHO, 2002). The US Food and Drug Administration has classified probiotic microorganisms as generally recognized as safe (FAO, 2001). Lactic acid bacteria, especially *Lactobacillus* spp., and Bifidobacteria are the most commonly used probiotic bacteria, since they are considered as integral and desirable members of the intestinal microflora (Kailasapathy et al., 2000; Schrezenmeir and de Vrese, 2001; Socol et al., 2010). Probiotic bacteria exert multiple health benefits to the host,

including improved nutrient digestion and assimilation, potentiating host immune function, and protection against enteric pathogens (Sonnenburg et al. 2005; Socol et al., 2010; Fukuda et al., 2011; Olszak et al. 2012; Hill et al., 2014).

With steady increase in the population of pet dogs and cats in the US, the use of probiotic bacteria as a functional pet food component has gained significant interest due to their beneficial effects on pet health, especially in reducing the risk of gastrointestinal disorders (Bunešová et al, 2012). Currently probiotic products in the form of tablet, capsule, and liquid are commercially available for use in dogs. An important criterion for the selection of probiotic bacteria are their host species specificity, since it is critical for these bacteria to colonize the host gut for imparting maximal health benefits (Fuller, 1989). However, most of the commercial probiotic products for dogs have been reported to be devoid of strains of canine origin (Beasley et al, 2006). Although abundant research has been conducted on isolating probiotic microorganisms from human and livestock origin, and characterizing their beneficial effects, limited information exists on probiotics with canine origin (Groben et al, 1979; Beasley et al, 2006). Therefore in this study, we isolated lactic acid bacteria from canine feces and evaluated their antimicrobial effect against *S. Schwarzengrund* and *L. monocytogenes* for potential use as a canine health supplement or as an additive for enhancing the microbiological safety of pet foods.

2. Material and Methods

2.1 Isolation of Lactic Acid Bacteria (LAB)

Samples of freshly voided feces of 70 healthy dogs of different breeds of both sexes ranging from 7 months to 18 years (median age 5.8 years) were collected from different canine kennels located in Connecticut. All samples were collected in sterile WhirlPak bags

(Sigma-Aldrich, St. Louis, MO), stored at 4°C, and processed within 4 h after collection.

Isolation of LAB from canine feces was conducted, as previously described by Beasley et al. (2005). Briefly, 50 g portions of each dog feces sample were mixed with 100 ml of phosphate buffered saline (PBS, pH 7.0) and pummeled in a stomacher for 2 min (Stomacher 400 Circulator, Seward, Davie, FL). The feces homogenate was then serially diluted (1:10) in PBS and 0.1 ml aliquots from 10^0 to 10^{-8} dilutions were spread-plated on *Lactobacillus* Selective Agar not supplemented with acetic acid (LBS; Becton Dickinson Microbiology System, Cockeysville, MD), and incubated at 30°C for 48 h. Following incubation, 10 presumptive LAB colonies from each LBS agar plate representing each dog feces sample were randomly transferred to 10 ml of de Man, Rogosa and Sharpe broth (MRS, Becton Dickinson Microbiology System) containing 20% glycerol, and stored at -80°C for further analysis.

2.2 Antimicrobial activity

Isolated LAB were tested for their antimicrobial activity against *S. Schwarzengrund* and *Listeria monocytogenes* using a previously published protocol (Toure et al., 2003; Shokryazdan et al., 2014; Tharmaraj and Shah, 2009). Two strains of *S. Schwarzengrund* (CVM 19633 and DBS-GA-F25499) obtained from BEI resources (Manassas, VA), and two strains of *L. monocytogenes*, including ATCC 19115 and Scott A from our laboratory culture collection were used in this experiment. A volume of 2 µl of an overnight culture of each presumptive LAB (~ 8 log CFU/ml) was spotted on a MRS agar plate, kept at room temperature for 30 min until the plates dried, and subsequently incubated at 37°C for 18 h. After colony development, the plates were overlaid with 10 ml of semi-solid brain heart infusion agar tempered at 45°C (0.8% agar; Sigma Aldrich) that was seeded with an

overnight culture of the aforementioned target pathogen ($\sim 7 \log$ CFU/ml), and incubated at 37°C for 48 h. Gentamicin (Sigma-Aldrich) was used as a positive control (Pesavento et al., 2010). After incubation, the diameter of inhibition zone around the presumptive LAB colonies was measured from the outer edge of each colony to the outer edge of the clear zone by using a digital caliper (sensitivity: $\text{mm} \pm 0.01$; Fisher Scientific, Waltham, MA). Based on the zone of inhibition, the antimicrobial activity of each presumptive LAB isolate was classified as strong ($> 19 \text{ mm}$ zone), intermediate (15 to 18 mm zone), and low ($< 14 \text{ mm}$ zone).

2.3 Matrix assisted laser desorption ionization (MALDI-TOF) identification

Based on the results of the antimicrobial assay, presumptive LAB isolates that exerted a strong inhibition ($>19 \text{ mm}$ zone) against the two pathogens were subjected to MALDI-TOF identification (Pavlovic et al., 2013). Each presumptive LAB isolate was streaked on a MRS agar plate and incubated at 37°C for 48 h. The isolates were then subjected to automated MALDI-TOF analysis by selecting three single colonies from each streaked MRS plate using MALDI Biotyper, version 4.1.70 (Bruker, Bremen, Germany). The spectra of the presumptive isolates were analyzed and compared to the reference spectra in the database (Mycobacteria Library, version 3.0). A logarithmic score ranging from 0 to 3 was generated for each tested colony corresponding to the similarity of reference spectral patterns, and interpreted as per manufacturer's instructions (MALDI Biotyper MSP Identification Standard Method 1.1). Based on the scores, the isolate identification was classified as high confidence level species identification ($> \text{score of } 2.3$), species identification ($> \text{score of } 2.0$), genus identification (a score of 1.7-1.9), and no identification (a score of < 1.7).

2.4 Polymerase chain reaction (PCR) identification

The presumptive LAB isolates that were identified by MALDI-TOF analysis were further confirmed by traditional species-specific PCR (Satokari et al, 2001). Total chromosomal DNA was isolated from an overnight culture of each LAB isolate using DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD). The quality and quantity of the DNA samples were determined using a spectrophotometer (Nanodrop 2000, Wilmington, DE). Specific primers for detecting each presumptively identified LAB were selected using genomic sequences obtained from Genbank (National Center for Biotechnology Information; NCBI) and Primer 3 software (NCBI). The sequences of the selected primers are provided in Table 1. The PCR was performed in a total volume of 25 µl reaction with Platinum SuperFi PCR Master Mix reagents (Thermo Scientific, Halethorpe, MD) and ~ 50 ng of template DNA in a thermocycler (C1000 Touch Thermal Cycler, Bio-rad, Hercules, CA) with heating at 95°C for 2 min, 35 cycles of 95°C for 30 s, annealing at 60°C for 30 s, and an extension 72°C 1 min, with final cooling at 4°C. The amplified DNA was visualized in a 2% agarose gel stained with ethidium bromide (Sigma Aldrich) and a 100-bp DNA ladder (Invitrogen, Carlsbad, CA). For each PCR reaction, appropriate positive and negative controls were included.

2.5 Statistical analysis

The antimicrobial activity assay had triplicate samples per LAB isolate, and the experiment was replicated three times. In addition, MALDI-TOF measurement was conducted once with triplicate samples of each LAB isolate, and the PCR assay was repeated three times with one sample of DNA from each LAB isolate. The data from the antimicrobial activity experiment were analyzed using the PROC-GLM procedure of SAS version 9.3 (SAS

Institute, Cary, NC). The mean results were compared using the least significant difference test, and differences were considered significant at $P < 0.05$.

3. Results

3.1 Antimicrobial activity of presumptive LAB isolates

Of the 70 examined canine fecal samples, 52 (74.2%) contained presumptive LAB with an average bacterial population of 6.8 ± 1.9 log CFU/g. A total of 520 presumptive LAB cultures isolated from 52 dog feces samples (10 pure cultures/feces sample) were tested for their antimicrobial activity against the two strains of each *S. Schwarzengrund* and *L. monocytogenes*. Among the 520 tested cultures, 52 isolates exerted potential antimicrobial effect against both target pathogens. Out of these 52 isolates, 12, 4, and 36 LAB exhibited strong, intermediate and low inhibition against *S. Schwarzengrund*, respectively (Table 2). Similarly, out of the 52 isolates, 8, 8, and 36 LAB demonstrated strong, intermediate and low inhibition against *L. monocytogenes*, respectively (Table 2).

The 8 presumptive LAB cultures that exerted a strong inhibitory effect against both pathogens were isolated from four individual dog feces samples (isolates 24-2, 24-4, 38-1, 38-10, 41-7, 41-9, 42-1, and 42-3). Zones of inhibition ranging from 25.1 ± 0.7 mm to 27.5 ± 1.0 mm were observed when the 8 LAB cultures were tested against *L. monocytogenes*, and 23.6 ± 0.7 to 27.3 ± 1.0 mm against *S. Schwarzengrund*. There was no significant difference between the inhibitory effects of these LAB isolates against two strains of *S. Schwarzengrund* and *L. monocytogenes*.

3.2 MALDI-TOF measurement

The eight presumptive LAB isolates that exerted the strongest inhibitory effect against both *L. monocytogenes* and *S. Schwarzengrund* were identified by MALDI-TOF

measurement, and the results are shown in Table 3. Out of the eight analyzed isolates, five cultures with a MALDI-TOF score of > 2.0 were identified as *L. plantarum* (isolates 38-1, 38-10, 41-9, 42-1, and 42-3), two cultures as *Pediococcus pentosaceus* with a MALDI-TOF score > 2.0 (isolates 24-2 and 24-4), and one culture had a Maldi-Tof score < 1.69 , which was unidentifiable (isolate 41-7).

3.3 PCR identification

Results of the MALDI-TOF analysis were further confirmed by conventional species-specific PCR. Based on the MALDI-TOF identification, primers that targeted species specific genes in the isolates were used. For the identification of *L. plantarum*, primers specific for *recA* gene that encodes the enzyme RecA involved in ATP hydrolysis, and the bacteriocin *plnF* gene encoding plantaricin precursor peptides were selected. Similarly, species-specific *recA* gene and *gyr* genes were used to identify *P. pentosaceus*, in which *recA* encodes an enzyme that catalyzes ATP hydrolysis and *gyr* encodes the enzyme gyrase. *Lactobacillus plantarum* strain NRRL B-3365 and *P. pentosaceus* strains ATCC 3316 and ATCC 43200 were used as positive controls in the PCR assay. Figure 1 shows the results of gel electrophoresis of PCR products from *L. plantarum* (Fig. 1A and 1B) and *P. pentosaceus* (Fig. 1C and 1D). The primers for *recA* and *plnF* amplified a DNA fragment of 171 bp and 114 bp, respectively from the chromosomal DNA of *L. plantarum* isolates, and in the positive control isolate. Similarly, the primers for *recA* and *gyr* amplified a fragment of 72 bp and 114 bp, respectively from the DNA of *P. Pentosaceus* isolates and in the positive control. These results indicate that the PCR detection concurred with the MALDI-TOF analysis, where 5 isolates were identified as *L. plantarum* (38-1, 38-10, 41-9, 42-1, and 42-3) and 2 as *P. pentosaceus* (24-2 and 24-4).

4. Discussion

Due to accurate detection, short turnaround time, low sample volume requirement, and low reagent costs, MALDI-TOF method is increasingly used for rapid identification of microorganisms (Chiu, 2014). Therefore, in this study MALDI-TOF method was used to identify the presumptive LAB isolates from canine feces before confirmation by species-specific PCR. As observed in Table 3, the results from MALDI-TOF analysis matched with those of the PCR, thereby underscoring the reliability of MALDI-TOF for microbial identification.

In the current study, 52 presumptive LAB isolates from 35 dog feces samples exhibited low to strong antimicrobial property against *S. Schwarzengrund* and *L. monocytogenes*. Of these 35 LAB isolates, eight isolates demonstrated a strong inhibitory effect (zone of inhibition > 19 mm) against both *S. Schwarzengrund* and *L. monocytogenes*, where five of them were identified as *L. plantarum*. Several species of LAB, including *Lactobacillus* spp. have been found in different parts of the canine gastrointestinal tract (Batt et al., 1991; Benno et al., 1992). Similarly, a variety of *Lactobacillus* spp., including *L. plantarum* (Grześkowiak et al., 2013; Kim et al., 2016), *L. fermentum* (Mitsuoka et al., 1976; Beasley et al., 2006), *L. salivarius* (Fujisawa and Mitsuoka, 1996; Beasley et al., 2006), *L. reuteri* (Tzortzis et al., 2004; Beasley et al., 2006), and *L. mucosae* (Tzortzis et al., 2004; Beasley et al., 2006) have been previously reported in canine feces. However, in this study no other *Lactobacillus* spp. besides *L. plantarum* was identified from the feces samples. This may be due to the fact that by selecting only eight of the 52 presumptive LAB isolates that exhibited a strong inhibitory effect against the two pathogens, we could have excluded other LAB species that might have been present in the feces samples.

Of the remaining presumptive three LAB isolates, two of them were identified as *P. pentosaceus*, and one isolate being unidentifiable. Although a literature search did not reveal any scientific report on *P. pentosaceus* as a part of the canine gut microbiota, *P. acidilactici* has been found effective in treating digestive disorders in dogs afflicted with parvovirus infection (Lin, 2006). In addition, *P. pentosaceus* is used as an ingredient in several commercially available probiotic supplements for dogs. The isolate which was unidentifiable by MALD-TOF and PCR was found to be a Gram positive rod on Gram's staining, and further characterization by biochemical methods and genome sequencing is currently underway.

The antimicrobial properties of probiotic bacteria have been extensively studied both *in vitro* and *in vivo* (Ogawa et al., 2002; Servin, 2004), and their antimicrobial activity is attributed to the production of organic acids, hydrogen peroxide, antimicrobial metabolites, bacteriocins, and non-lactic acid antimicrobial molecules (Servin, 2004). In the current study, although the eight LAB isolates demonstrated potent antimicrobial property against the two target bacteria, further studies are needed to elucidate the mechanisms behind their antagonistic effect before recommending potential applications of these beneficial bacteria.

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Table 1. Species specific primers for PCR analysis.

Target organism	Gene	Primers	Sequences	Amplicon Size
<i>L. plantarum</i>	<i>recA</i>	Forward	5'-CTTAGATGACGCGTTGGGTG-3'	171 bp
		Reverse	5'-TAAACGGGGTCTAGTGCGTT-3'	
	<i>plnF</i>	Forward	5'-CTATTTCAGGTGGCGTTTTTC-3'	113 bp
		Reverse	5'-GTGGATGAATCCTCGGACAG-3'	
<i>P. pentosaceus</i>	<i>recA</i>	Forward	5'-CTATTGACTTGGTCGTTATTGATTCC-3'	72 bp
		Reverse	5'-CCCCCATCTCTCCATCAATTT-3'	
	<i>gyr</i>	Forward	5'-TTCACAAACGGCTGAGGTTG-3'	101 bp
		Reverse	5'-TCAAACGCACCAAACGCATA-3'	

Table 2. Antimicrobial activity (zone of inhibition*, mm) of presumptive LAB isolated from canine feces samples against *S. Schwarzengrund* and *L. monocytogenes*.

Isolate Code	<i>L. monocytogenes</i>		<i>S. Schwarzengrund</i>	
	ATCC19115TM	Scott A	CVM 19633	DBS_GA_F25499
1-1	9.8±0.9	8.7±0.9	9.8±0.9	9.5±0.5
1-8	8.0±0.2	8.0±0.3	7.9±0.4	7.7±0.3
3-6	6.7±0.3	7.1±0.3	6.7±0.5	6.9±0.4
3-7	7.5±0.3	7.7±0.3	6.7±0.5	7.0±0.5
7-5	8.1±0.4	8.3±0.3	12.8±0.5	12.7±0.8
7-7	8.8±0.3	9.1±0.3	10.3±0.3	10.5±0.4
9-2	7.5±0.3	7.8±0.2	10.0±0.6	10.2±0.3
9-5	9.3±0.4	9.1±0.4	13.7±0.7	13.3±0.7
11-8	8.5±0.6	8.8±0.3	12.4±0.7	12.6±0.3
11-10	15.7±0.4	15.4±0.3	17.5±0.5	17.1±0.5
12-7	13.2±0.9	13.4±0.6	11.1±0.8	11.4±0.8
14-5	13.8±0.5	13.9±0.4	11.9±0.3	12.0±0.2
14-8	10.3±0.6	10.9±0.4	7.8±0.8	8.0±0.5
18-2	13.0±0.3	13.2±0.5	11.3±0.6	11.4±0.3
20-2	14.2±0.6	14.0±0.8	12.1±0.9	12.7±0.2
20-9	15.0±0.9	15.2±0.6	8.5±0.3	8.4±0.3
24-2	25.7±0.4	25.1±0.7	27.3±1.0	26.3±1.8
24-4	27.4±0.9	27.0±1.0	23.6±0.7	24.1±0.9
25-3	13.8±0.5	13.9±0.5	11.8±0.4	12.0±0.2
26-5	15.8±0.6	15.5±0.4	13.1±0.8	12.9±0.8
28-3	8.1±0.6	8.3±0.5	5.8±0.8	6.0±0.3
32-3	13.4±0.5	12.8±0.3	7.6±0.3	8.0±0.6
33-4	10.4±0.3	10.1±0.7	5.5±0.4	6.0±0.2
35-3	9.6±0.7	10.3±0.3	8.0±0.7	8.3±0.2
35-6	11.7±0.6	11.7±0.6	10.0±0.7	10.1±0.4
36-6	19.2±0.6	19.4±0.3	16.5±0.9	16.1±0.6
37-9	15.5±0.4	15.0±0.4	13.4±0.3	13.7±0.5
38-1	26.4±0.8	26.9±0.8	24.5±1.0	25.3±0.9
38-10	26.8±0.5	27.5±0.4	24.6±0.8	24.8±0.9
39-3	11.6±0.3	11.7±0.6	6.3±0.5	6.5±0.4
40-8	12.8±0.3	12.5±0.1	11.0±0.5	11.2±0.4
41-7	25.8±0.5	26.0±0.9	26.0±1.0	25.1±0.9
41-9	26.0±0.3	26.0±0.3	25.0±0.5	25.1±0.8
42-1	25.6±0.4	26.5±0.5	24.1±0.6	24.2±0.7
42-3	25.6±0.6	26.1±0.9	24.1±1.0	26.1±1.0
44-7	13.2±0.4	12.6±0.2	11.5±0.3	11.7±0.4
45-10	14.3±0.3	14.2±0.6	12.1±0.3	12.0±0.1
52-2	8.3±0.2	8.1±0.6	7.7±0.3	7.9±0.3
52-3	11.9±0.5	11.7±0.6	7.5±0.2	7.8±0.2
55-1	19.5±0.7	19.2±0.6	15.2±0.6	15.2±0.2

58-1	14.3±0.8	14.2±0.5	13.4±0.8	13.1±0.2
59-1	7.1±0.2	7.2±0.3	8.7±0.2	8.4±0.1
59-6	21.1±0.3	20.2±0.1	17.7±0.4	17.4±0.2
61-8	13.1±0.8	13.5±0.5	13.1±0.8	13.1±0.2
61-10	13.4±0.3	13.1±0.8	11.6±0.4	11.5±0.3
62-5	19.8±0.5	19.2±0.6	17.8±0.3	17.8±0.2
63-5	10.5±0.4	10.4±0.3	7.7±0.6	7.5±0.2
66-9	12.0±0.8	12.3±0.3	16.5±0.7	16.8±0.4
68-2	14.3±0.9	14.2±0.9	17.2±0.5	17.2±0.5
68-3	14.0±0.9	14.3±0.3	16.5±0.7	16.6±0.2
70-5	11.2±0.4	11.6±0.3	10.5±0.6	10.4±0.4
70-7	13.5±0.5	13.7±0.3	12.2±0.4	12.1±0.6
Gentamycin	15.1±1.1	15.0±1.1	16.0±0.3	16.0±0.3

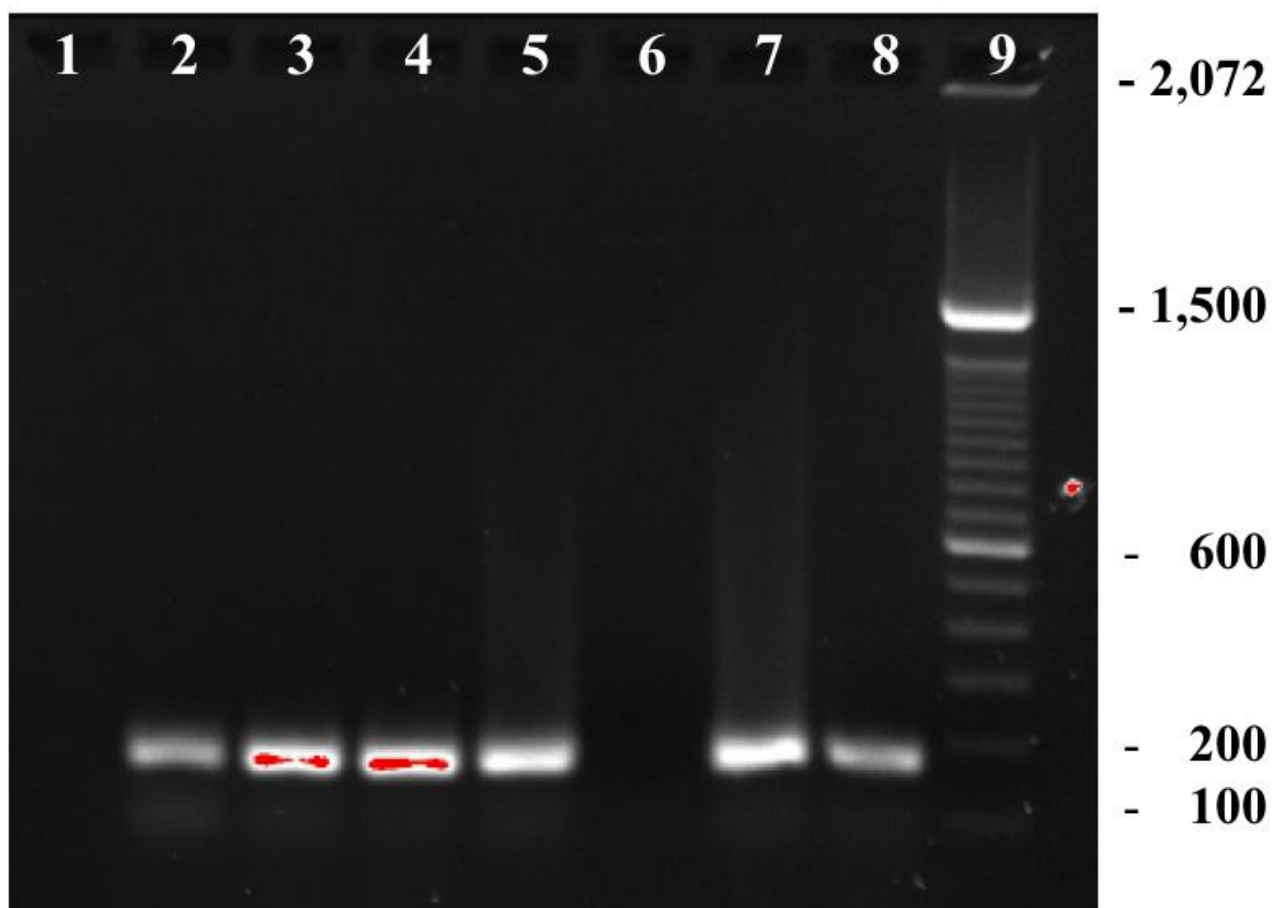
* Values represent mean and standard deviation.

Table 3. Identification of the eight LAB isolates with strong inhibitory effect against *S. Schwarzengrund* and *L. monocytogenes* using MALDI-TOF and PCR methods

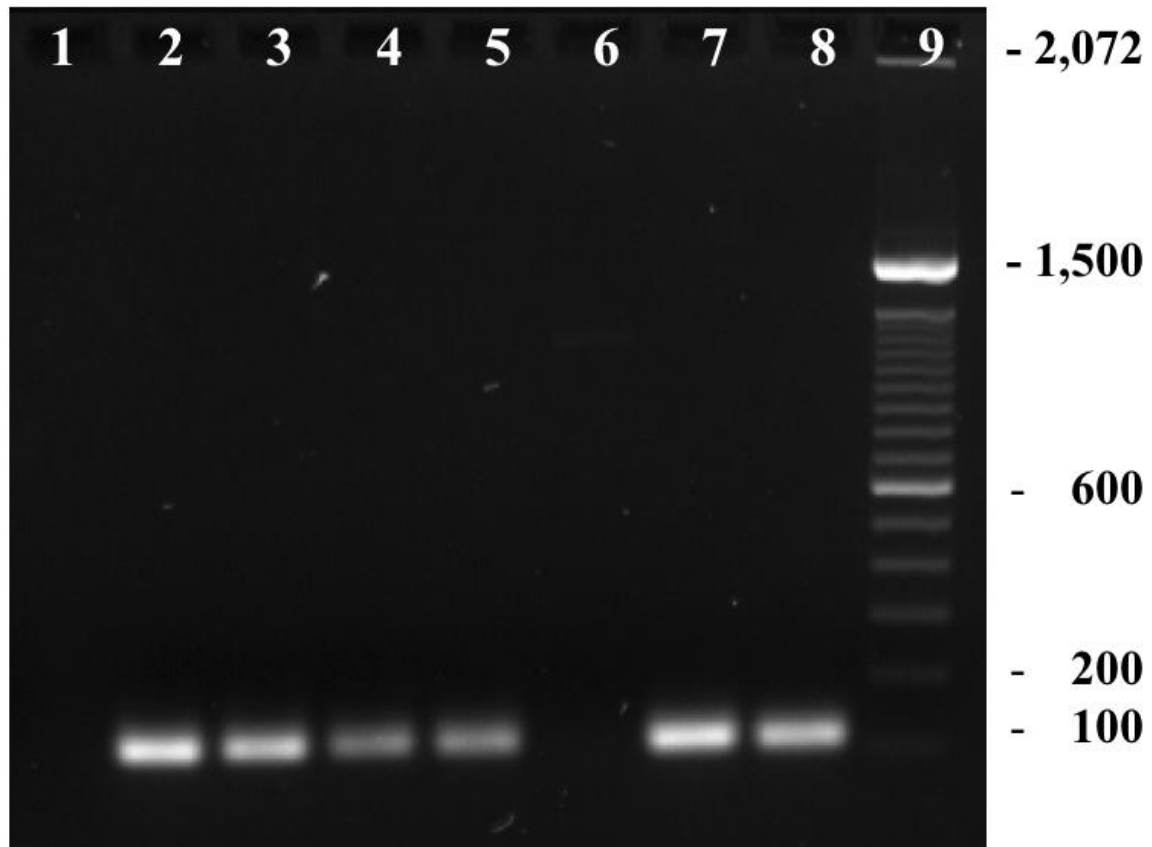
Isolate code	Dog breed	Age	MALDI-TOF	PCR
24-2	Beagle	4	<i>P. pentosaceus</i>	<i>P. pentosaceus</i>
24-4			<i>P. pentosaceus</i>	<i>P. pentosaceus</i>
38-1	Maltese	9	<i>L. plantarum</i>	<i>L. plantarum</i>
38-10			<i>L. plantarum</i>	<i>L. plantarum</i>
41-7	Labrador retriever	5.5	Unknown	Unknown
41-9			<i>L. plantarum</i>	<i>L. plantarum</i>
42-1	Labrador retriever	7.5	<i>L. plantarum</i>	<i>L. plantarum</i>
42-3			<i>L. plantarum</i>	<i>L. plantarum</i>

Figure 1. Identification of the eight LAB isolates with strong inhibitory effect against *S. Schwarzengrund* and *L. monocytogenes* using PCR. (1A) Detection of *L. plantarum* by targeting *recA* gene; (1B) Detection of *L. plantarum* by targeting *plnF* gene; (1C) Detection of *P. pentosaceus* by targeting *recA* gene; (1D) Detection of *P. pentosaceus* by targeting *gyr* gene

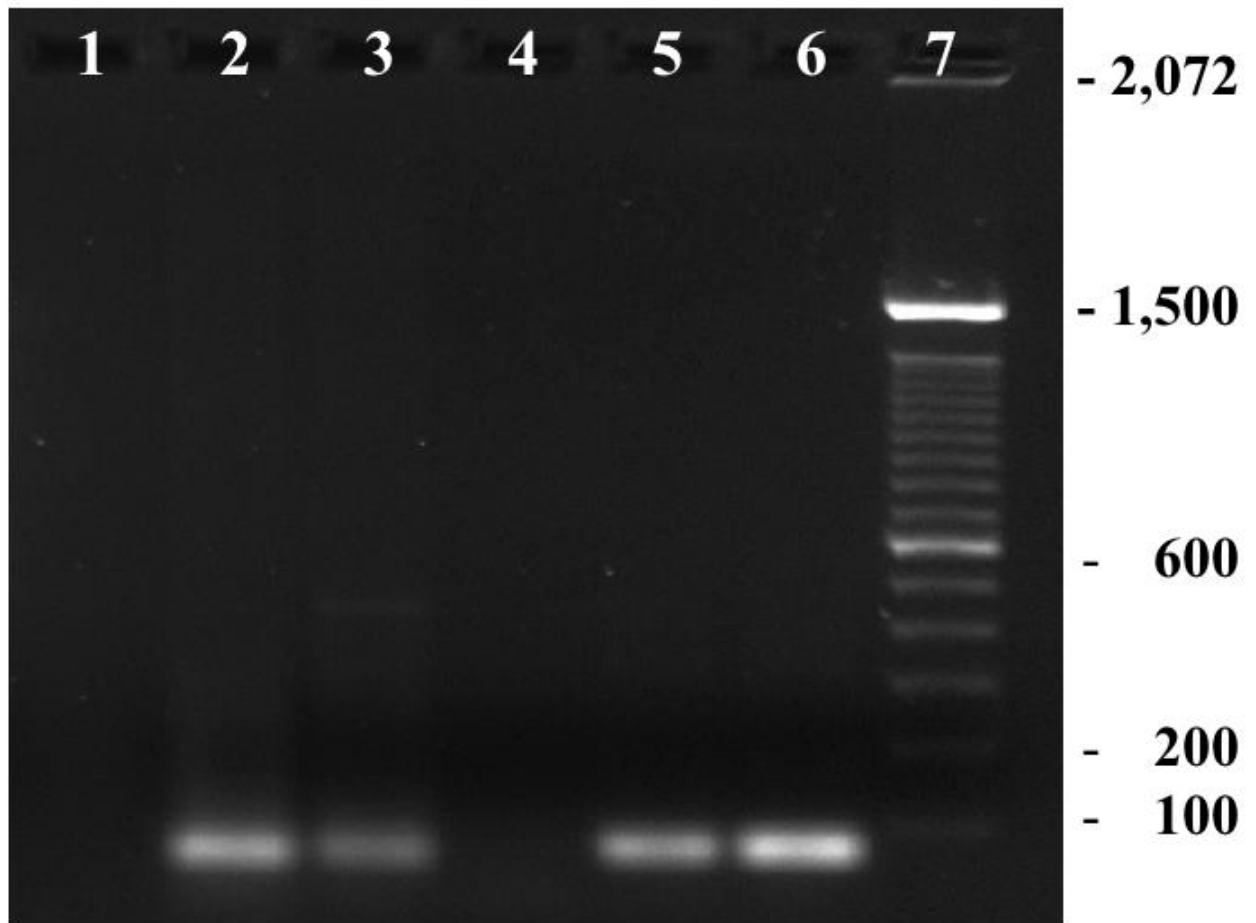
(1A) Detection of *L. plantarum* by PCR targeting *recA* gene. Lane 1: Negative control (no template DNA); Lane 2: Positive control *L. plantarum* strain NRRL B-3365; Lane 3: isolate 42-3; Lane 4: isolate 42-1; Lane 5: isolate 41-9; Lane 6: isolate 41-7; Lane 7: isolate 38-10; Lane 8: isolate 38-1; Lane 9: 100 bp ladder.



(1B). Detection of *L. plantarum* by PCR targeting *plnF* gene. Lane 1: Negative control; Lane 2: Positive control *L. plantarum* strain NRRL B-3365; Lane 3: isolate 42-3; Lane 4: isolate 42-1; Lane 5: isolate 41-9; Lane 6: isolate 41-7; Lane 7: isolate 38-10; Lane 8 isolate 38-1; Lane 9: 100 bp ladder.



(1C). Detection of *P. pentosaceus* by PCR targeting *recA* gene. Lane 1: Negative control; Lane 2: Positive control *P. pentosaceus* ATCC 3316; Lane 3: Positive control *P. pentosaceus* ATCC 43200; Lane 4: isolate 41-7; Lane 5: isolate 24-4; Lane 6: isolate 24-2; Lane 7: 100 bp ladder.



(1D). Detection of *P. pentosaceus* by PCR targeting *gyr* gene. Lane 1: Negative control; Lane 2: Positive control *P. pentosaceus* ATCC 3316; Lane 3: Positive control *P. pentosaceus* ATCC 43200; Lane 4: isolate 41-7; Lane 5: isolate 24-4; Lane 6: isolate 24-2; Lane 7: 100 bp ladder.

