Determining the Probiotic Potential of Turkey Gut-Derived *Lactobacillus* against Multidrug-Resistant *Salmonella enterica* serovar Heidelberg in Turkey Poults

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Dedication

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Abstract

Foodborne infections of enteric origin constitute a significant segment of diseases that affect humans. Despite many control strategies adopted, foodborne infections invite public health concerns worldwide. Among the foodborne outbreaks in the United States, non-typhoidal salmonellosis ranks very high among its competitors. Some of the major serotypes of *Salmonella* that cause non-typhoidal foodborne infections include *Salmonella enterica* serovar Enteritidis, *Salmonella enterica* serovar Typhimurium, and *Salmonella enterica* serovar Heidelberg. Poultry and poultry products are significant contributors of foodborne salmonellosis in humans. *Salmonella* colonizes the cecum of poultry and results in fecal excretion of the pathogen to the environment. Also, the pathogen can invade internal organs such as liver and spleen, and could be deposited in eggs during its formative stage. Among the *Salmonella* serotypes, *Salmonella enterica* serovar Heidelberg has emerged to cause multiple foodborne outbreaks associated with poultry products, recently. The pathogen has acquired resistance to commonly used antibiotics such as gentamicin, streptomycin, tetracycline, and ampicillin.

Antimicrobial interventional strategies targeting the control of *Salmonella enterica* serovar Heidelberg is an emergent need. Given the situation that the bacteria are multidrug resistant (MDR), and that the Food and Drug Administration (FDA) has issued restrictions on the use of medically important antibiotics in animal agriculture, alternative strategies that can control the pathogen are required. Probiotics could be an alternative to antibiotics, as they can competitively exclude pathogens from attaching to intestinal

epithelium thereby maintaining normal gut microbiota. Among the many probiotics, host-derived *Lactobacillus* could play a major role as they have an intrinsic affinity towards the host epithelium. In this thesis project, we investigated the potential of turkey gut-derived *Lactobacillus ingluviei* and *Lactobacillus salivarius* as an alternative strategy to control MDR *Salmonella enterica* serovar Heidelberg in turkey poults. We designed two objectives: 1. to determine the effect of supplementation of turkey gut-derived *Lactobacillus salivarius* and *Lactobacillus ingluviei* in combination against MDR *Salmonella enterica* serovar Heidelberg colonization in turkey poults, and 2. to determine the probiotic qualities of turkey gut-derived *Lactobacillus salivarius* and *Lactobacillus ingluviei* isolates *in vitro.*

At first, we determined the efficacy of *Lactobacillus* of turkey-gut origin (*Lactobacillus salivarius* and *Lactobacillus ingluviei*) against MDR *Salmonella enterica* serovar Heidelberg colonization in commercial straight-run Hybrid Converter turkey poults. Three independent experiments were conducted. The treatment groups were negative control (-*S. enterica* serovar Heidelberg, -*Lactobacillus*), *S. enterica* serovar Heidelberg control (+*S. enterica* serovar Heidelberg, -*Lactobacillus*), and a treatment group (+*S. enterica* serovar Heidelberg, +*Lactobacillus*), with at least nine birds in each treatment group per experiment. *Lactobacillus* (8 log10 CFU/gallon of water) was supplemented through drinking water continuously for 14 days. Poults were challenged with the 2011 turkey outbreak strain of *Salmonella enterica* serovar Heidelberg (5.0 log10 CFU/ml) on day 7, and *Salmonella enterica* serovar Heidelberg counts were

determined on days 2 and 7 post-inoculation. Results indicated that the treatments significantly reduced MDR *Salmonella enterica* serovar Heidelberg in the cecum, liver, and spleen of turkey poults compared to the *Salmonella enterica* serovar Heidelberg control in two of the three experiments conducted (P<0.05).

In the follow-up study, we determined the qualities of *Lactobacillus ingluviei* and *Lactobacillus salivarius* to be considered as potential probiotics, *in vitro*. Although turkey gut-derived, any potential probiotic strain will face a series of physiological challenges until efficient colonization in the cecum, once administered orally. These obstacles include the low acid environment in the gizzard, and the detergent action of bile and bile salts, before its colonization in the lower part of the intestine. Once these obstacles are traversed, the probiotic strain should have the ability to colonize strongly to the intestinal epithelium for performing colonization resistance against invading pathogens. In this process, potential probiotic strains will induce strong antimicrobial property. Our studies indicated that both *Lactobacillus* exerted significant resistance to low pH and bile salts (P<0.05). Our cell culture studies indicated that the tested *Lactobacillus* isolates had high adhesion to model avian intestinal epithelial cells, validating the *in vivo* studies. Moreover, the cell-free extracts of *Lactobacillus salivarius* and *Lactobacillus ingluviei* showed high antimicrobial activity separately against three major serotypes of *Salmonella*, namely, *Salmonella enterica* serovar Enteritidis, *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Heidelberg (P<0.05). We also tested the antibiotic susceptibility of the *Lactobacillus* isolates.

Lactobacillus salivarius and *Lactobacillus ingluviei* were sensitive to a variety of commonly used antibiotics for human therapy.

The overall results indicated that turkey gut-derived *Lactobacillus* (*Lactobacillus salivarius* and *Lactobacillus ingluviei*) could be an effective strategy to reduce MDR *Salmonella enterica* Heidelberg colonization in turkeys, potentially improving the microbiological safety of turkey products.

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Chapter 1

Literature review

1.1 Foodborne illness

Despite the multitude of control efforts, foodborne infections remain one of the leading causes of human enteric disease in the United States and worldwide. According to the Centers for Disease Control and Prevention (CDC), every year, 1 in 6 Americans, suffers from foodborne illnesses (CDC, 2015). Annually, an estimated 128,000 people are hospitalized, and 3000 die due to foodborne illnesses caused by pathogenic microbes (CDC, 2015). Among the microbes, different types of bacteria such as *Salmonella* spp., *Clostridium perfringens*, *Campylobacter*, *Listeria* (Mead et al., 1999, CDC, 2015), viruses such as Norovirus, and parasites such as Toxoplasma (Mead et al., 1999) contribute to over 250 different types of foodborne illnesses.

1.2. Foodborne *Salmonella*

Among the bacterial infectious agents, *Salmonella* species ranks high among its competitors such as *Campylobacter* and *Escherichia coli*. Non-typhoidal salmonellosis is a major foodborne illness in the United States caused by *Salmonella* serotypes such as *Salmonella enterica* serovar Enteritidis, *Salmonella enterica* serovar Typhimurium, and *Salmonella enterica* serovar Heidelberg (Altekruse et al., 1997). Discovered and named after Dr. Salmon about 125 years ago (CDC, 2015) *Salmonella* spp. has been differentiated to more than 2500 serotypes (D'Aoust, 1994). These pathogens together cause 1.2 million illness cases and nearly 450 deaths annually (CDC, 2015). Although a

self-limiting disease in healthy individuals, children below 5 years of age, elderly, and immunocompromised individuals are more susceptible to *Salmonella* infections (CDC, 2015; Tauxe, 1991) resulting in significant mortality, morbidity, and economic loss. Considering these alarming reports, the US Department of Health and Human Services has set a goal of reducing *Salmonella* incidence by 25% by 2020 (Jackson et al., 2013).

1.3. *Salmonella* in poultry

Salmonella spp. can colonize the intestinal tract of poultry (Barrow et al., 1988). In chickens, the pathogen colonizes the cecum predominantly, further contaminating the environment, chicken carcass, and eggs at or after lay. There is mounting evidence that contaminated poultry products, especially eggs and meat are epidemiological sources for *Salmonella* (Humphrey and Jorgensen, 2006; Marcus et al., 2007; White et al., 2007; CDC, 2010; USDA 2012).

1.3.1. *Salmonella* serotypes in meat

Poultry meat and eggs can harbor *Salmonella* and can cause gastrointestinal disease in humans (Stock and Stoll, 2000; Foley et al., 2011). Poultry meat, including the whole carcass, cut-up parts, and processed meats are significant sources of several *Salmonella* serotypes that can cause disease in humans. In an early Canadian study, *Salmonella* was detected from 73.7% turkey carcasses, and 38.2% chicken carcasses (Lammerding et al.,

1998). Later, Logue et al. (2001) studied the incidence of *Salmonella* in two turkey processing plants in the Midwestern US. Surface swabs were collected from poultry carcasses pre-chill and post-chill. Samples were also collected from the chill water. The overall incidence of *Salmonella* was found to be 16.7% after enrichment, and more positive samples were observed in pre-chill than post chill. Major serotypes recovered were *Salmonella enterica* serovar Senftenberg, *Salmonella enterica* serovar Agona, *Salmonella enterica* serovar Heidelberg, and *Salmonella enterica* serovar Hadar. Jorgenson et al. (2002) studied the prevalence of *Salmonella* in 241 whole raw chicken samples purchased from retail shops in the United Kingdom at two different winter seasons of 1998/1999 and 1999/2000. The study found that *Salmonella* was present in 25% of the chicken samples. Among these, 19% of *Salmonella* was detected from both inside and outside of the chicken packages. The predominant serotypes detected were *Salmonella enterica* serovar Indiana, *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Hadar (Jorgenson et al., 2002). Roy et al. (2002) detected *Salmonella* from 569 samples (11.99%) in 4745 samples collected from poultry liver and yolk sac, chicken ground meat, rinse water from spent hens and broilers, hatchery fluff and drag samples from poultry environment during 1999/2000 at Pacific Northwest. Out of the 97 positive samples serotyped, *Salmonella enterica* serovar Heidelberg (25.77%), *Salmonella enterica* serovar Kentucky (21.64%), *Salmonella enterica* serovar Montevideo (11.34%), *Salmonella enterica* serovar Hadar (5.15%), and *Salmonella enterica* serovar Enteritidis (5.15%) were the major serotypes isolated. Likewise, the incidence of *Salmonella* in several poultry products obtained from a local butcher shop in

Belgium revealed that 60% of the samples were contaminated with *Salmonella* of 10 different serotypes. Most prominent serotypes isolated in the study were *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Hadar (Antunes et al., 2003). In a study conducted in Spain to isolate *Salmonella* from 198 samples of chicken meat for sale in retail outlets, it was reported that the pathogen was isolated from 35.83% of the samples where the predominant serovars were *Salmonella enterica* serovar Enteritidis (47.88%), *Salmonella enterica* serovar Hadar (25.35%) and serotype 4, 12: b :-(II) (19.71%) (Dominguez et al., 2012). In yet another study conducted in Maryland, USA, Cui et al. (2004) reported 61% of organic and 44% of conventional chickens were contaminated with *Salmonella*. Between the years, 2002 to 2006, *Salmonella* was isolated from 59.7% ground turkey, 36.9% chicken breast, and 3.4% pork chops among retail meat outlets in the United States (Zhao et al., 2008).

Frozen chicken nuggets, strips, and eggs are found to be the main factors that cause *Salmonella enterica* serovar Heidelberg infection in Canada (Currie et al., 2005). Bohaychuk et al. (2006) detected *Salmonella* in 30% of raw chicken legs and 800 meat, and poultry products collected from a retail market in Alberta, Canada. In addition to *Salmonella*, other major pathogens such as *Campylobacte*r spp, Shiga toxin–producing *Escherichia coli* and *Listeria monocytogenes* were isolated as well. In a Portugal study, Antunes et al. (2003) found *Salmonella* in 60 samples of poultry products obtained from local shops and canteens and detected 10 different serotypes of *Salmonella* in 60% of samples, and identified *Salmonella enterica* serovar Enteritidis and *Salmonella enterica*

serovar Hadar as more prevalent. Jason et al. (2013) studied the link between different *Salmonella* serotypes, and various foods, including poultry, by analyzing outbreaks that occurred between 1998 and 2008. The study found that eggs and poultry meat acted as vehicles in more than 80% cases of *Salmonella* outbreaks caused by *Salmonella enterica* serovar Enteritidis, *Salmonella enterica* serovar Heidelberg, and *Salmonella enterica* serovar Hadar. In another epidemiological study, Chittick et al. (2006) analyzed the national foodborne outbreak data from 1973 through 2001 and found that, among 6633 outbreaks of known etiology, 184 (3%) were contributed by *Salmonella enterica* serovar Heidelberg. Among these, 3 outbreaks were due to egg consumption, 17 cases were related to consumption of foods prepared using eggs, 25 cases were related to poultry, and 8 were due to consumption of food containing both poultry and eggs. Even though hydrogen sulfide production is an important determinant for isolation of *Salmonella*, a study conducted by Lin et al. (2014) isolated hydrogen sulfide negative *Salmonella* in retail meats. Out of 82 *Salmonella* strains that were isolated from 113 chicken and 204 pork samples, 49 were hydrogen sulfide positive, and 33 were negative. *Salmonella enterica* serovar Derby (40%) and *Salmonella enterica* serovar Heidelberg (30%) were the predominant hydrogen sulfide negative serotypes isolated (Lin et al., 2014).

Foley et al. (2007) had observed that serovars *Salmonella enterica* Senftenberg and *Salmonella enterica* serovar Hadar have become more prevalent in poultry, compared to *Salmonella enterica* serovar Enteritidis, and *Salmonella enterica* serovar Typhimurium. *Salmonella enterica* serovar Heidelberg was reported to be more isolated from clinical

cases and suggested to be virulent than other serovars. The study concluded that among the top 10 serovars of *Salmonella* associated with human infections, the majority were from swine and poultry, including *Salmonella enterica* serovar Heidelberg. In a different study conducted by Salina et al. (2007) revealed high *Salmonella* contamination in processed poultry products. In this study, 480 pre-chill and post-chill poultry carcasses, and the chill water from entry, and exit point were enriched, and analyzed using automated BAX system, and culture methods to detect *Salmonella*. About 88.4% of pre-chill, and 84.1% post-chill carcasses were found to be positive for the pathogen. In addition, 92% of the samples collected from entry points were found to be positive for *Salmonella*, whereas none was identified at the exit point. The predominant serotypes isolated were *Salmonella enterica* serovar Kentucky (59.5%) and *Salmonella enterica* serovar Typhimurium (17.8%) (Salina et al., 2007). Lestari et al. (2009) studied the prevalence of *Salmonella* isolated from 141 conventionally raised, and 53 organically raised chicken carcasses from 27 retail stores located in Baton Rouge, Louisiana. Recovery rates were similar. Twenty-two % of the conventionally raised chicken was found to be positive for *Salmonella* whereas 20.8% organic chicken was found to be positive for *Salmonella*. Out of the eight serotypes isolated, predominant ones were *Salmonella enterica* serovar Kentucky, *Salmonella enterica* serovar Hadar and *Salmonella enterica* serovar Enteritidis (Lestari et al., 2009).

1.3.2. *Salmonella* serotypes in eggs

Eggs and related production environment can be a significant source of *Salmonella*. According to Hennessey et al. (2004), outside home egg consumption is the main factor that caused *Salmonella* outbreaks. Since *Salmonella*, especially *Salmonella enterica* serovar Enteritidis, *Salmonella enterica* serovar Heidelberg, and *Salmonella enterica* serovar Typhimurium, has the capability to colonize the intestine of birds, and invade internal organs such as liver, spleen, ovary, and oviduct (Gast 2004), those that are invaded and colonized the reproductive tract are found to be deposited in different regions of eggs even though no relationship was established between the intensity of pathogen deposited in eggs, and the presence of pathogen in reproductive tissues (Gast et al. 2004; 2007; 2011). Similarly, Barnhart et al. (1991) detected *Salmonella* from the ovaries collected from commercial layer hens at the time of slaughter. In addition to *Salmonella* that gets deposited from the reproductive tract, eggs that meet feces contaminated with *Salmonella* may also result in the penetration of pathogen through egg shells (Schoeni et al., 2005). Although *Salmonella enterica* serovar Enteritidis are more implicated in egg borne outbreaks (reviewed in Upadhyaya et al., 2015), emerging *Salmonella* such as *Salmonella enterica* serovar Heidelberg can penetrate the vitelline membrane, and can survive inside the egg albumen even at 42°C when inoculated in low numbers (Gantois et al., 2008), and can even multiply inside the yolk on the first day if stored at warm temperatures (Gast et al., 2005). Even though *Salmonella* can penetrate the outer shell, and vitelline membrane of eggs, proper storage at refrigerated condition can hinder this effect (Gast et al., 2007).

Poppe et al. (1991) studied the prevalence of *Salmonella* among commercial egg-producing flocks in Canada. Environmental samples, including fecal and egg belt, collected from 295 randomly selected commercial flocks revealed 152 positive samples (52.9%) with more *Salmonella* detected in the egg belt samples. Some of the major serovars detected were *Salmonella enterica* serovar Heidelberg (20%), *Salmonella enterica* serovar Infantis (6.1%), *Salmonella enterica* serovar Hadar (5.8%), and *Salmonella enterica* serovar Schwarzengrund.

1.3.3. Antibiotic-resistant *Salmonella*

White et al. (2001) isolated ceftriaxone-resistant *Salmonella enterica* serovar Heidelberg from retail meat; ceftriaxone is the drug of choice for treating salmonellosis in children (White et al., 2001). Ceftiofur-resistant *Salmonella enterica* serovar Heidelberg was isolated from retail chicken in Canada that had similar etiology in humans (Dutil et al., 2010). Jorgenson et al. (2002) reported that 70% of *Salmonella* isolated from 241 whole carcasses collected from retail stores in England were resistant to least one antibiotic, and 46% were resistant to more than one antibiotic. In a Portugal study, Antunes et al. (2003) detected 10 different serotypes of *Salmonella* from 60% of chicken samples, of which 50% were resistant to nalidixic acid, and enrofloxacin. In a Maryland study (Cui et al., 2004), all *Salmonella enterica* serovar Typhimurium isolates obtained from retail chicken were resistant to more than five antimicrobials, whereas those isolated from organic chicken were resistant to more than 17 antimicrobials. Out of

the 569 samples positive for *Salmonella* (N=4745), Roy et al. (2002) reported 92 samples collected from various environmental sources with resistance towards erythromycin, lincomycin and penicillin antibiotics, whereas all being susceptible to sarafloxacin and ceftiofur. In a different study, Salina et al. (2007) found high *Salmonella* contamination in processed poultry products with 79.8% of the isolates resistant to at least one antimicrobial agent whereas 53.4% were resistant to more than one antimicrobial agent, among the 92% isolates tested positive for the pathogen from the chill water from the entry point.

1.3.4. *Salmonella enterica* serovar Heidelberg – an emerging MDR *Salmonella* serotype in poultry

Salmonella enterica serovar Heidelberg is one of the most important serotypes associated with poultry and poultry-derived foods, including meat and eggs (Snoeyenbos et al., 1969; CDC, 1986; Mahoney et al., 1990; Bokanyi et al., 1990; Schoeni et al., 1995; Layton et al., 1997; Gast et al. 2004; Hennessy et al., 2004; Scharff, 2012, Jackson et al., 2013). More recently, in 2011, two nationwide outbreaks were reported linking contaminated broiled chicken livers and ground turkey to human infections (Folster et al., 2011). In the former case, 190 illnesses caused by the pathogen were reported from 6 states in the US. Thirty-four states were involved when *Salmonella enterica* serovar Heidelberg resulted in 136 cases of illness linked to the consumption of ground turkey. In 2013, multidrug-resistant *Salmonella enterica* serovar Heidelberg infections were linked

to contaminated chicken products from a leading farm in California (CDC, 2015). In 2014, yet another outbreak caused by *Salmonella enterica* serovar Heidelberg was reported from a Tennessee Correctional Facility epidemiologically linked to the consumption of mechanically separated chicken (CDC, 2014).

Salmonella enterica serovar Heidelberg is a highly invasive serotype of *Salmonella* in humans. This serotype is estimated to cause 84,000 enteric illnesses within United States characterized by invasive, systemic infections, and mortality (Gast et al., 2004; Zhao et al., 2008; CDC, 2011; Foley et al., 2011; Young et al., 2012; Amand et al., 2013). In addition to its invasion property, *Salmonella enterica* serovar Heidelberg isolates have high antibiotic resistance potential. Recent reports on failures in antimicrobial therapy against the pathogen indicate that the strains are acquiring resistance to several antibiotics, including third generation cephalosporins such as ceftiofur and ceftriaxone (Dutil et al., 2010, Crump et al., 2011) that are medically important in treating *Salmonella enterica* serovar Heidelberg in children and pregnant women where other options are limited (Dutil et al., 2010; reviewed by Amand et al., 2013).

1.4. Preharvest intervention strategy for *Salmonella* control

In poultry, including turkeys, antibiotics are used to treat specific bacterial infections, and as growth promoters (Rabsch et al., 2003). However, the emergence of MDR strains of

the pathogens has alarmed the scientific community to explore alternative antimicrobial strategies (Shea, 2003; Bywater, 2005; Kollanoor Johny et al., 2009; 2010a, b; 2012 a, b; 2013; Nair and Kollanoor-Johny, 2016; Nair et al., 2016). This is specifically important in poultry because cecal carriage of *Salmonella enterica* serovar Heidelberg could result in horizontal transmission of the pathogens, contamination of eggshells with feces and carcass contamination during slaughter. Therefore, reducing the populations of *Salmonella enterica* serovar Heidelberg in poultry cecum would reduce contamination of meat and eggs. A variety of approaches for reducing the colonization of *Salmonella* in broiler chickens has been explored, however, research on the use of alternative approaches to control *Salmonella enterica* serovar Heidelberg in turkeys are scanty. In broilers and layers, these approaches include feeding competitive exclusion bacteria, bacteriophages, organic acids, oligosaccharides, antibiotics, and vaccination (Fernandez et al., 2000; Spring et al., 2000; Byrd et al., 2001; Stern et al., 2001; Fernandez et al., 2002; Chadfield and Hinton, 2003; Heres et al., 2004; Fiorentin et al., 2005; Higgins et al., 2007; Methner et al., 1997; Dueger et al., 2001; Khan et al., 2003; Inoue et al., 2008).

In this thesis, turkey-gut derived (host-specific) *Lactobacillus*-based probiotic cultures are investigated to control the cecal colonization of *Salmonella enterica* serovar Heidelberg in turkey poults.

1.5. Probiotics

1.5.1. Definitions

Probiotics are beneficial microbes that can contribute to gut health (Pagnini et al., 2009; Gartz et al., 2010). The word "probiotic" was first used by Lilly and Stillwell in 1965 to address substances secreted by microorganisms that can stimulate the growth of other microorganisms (Fuller, 1992). In 1971, Sperti described probiotics as extracts that can stimulate microbial growth, as opposed to antibiotics that can prevent microbial growth. In 1974, Parker defined probiotics as organisms and substances which contribute to intestinal microbial balance (Fuller, 1992), and the addition of 'substances' in the definition included 'antibiotics.' Therefore, Fuller in 1989 modified the definition of probiotics as live microbial feed supplements which beneficially affect the host animal by improving intestinal microbial balance (Fuller, 1992). After several modifications, the definition of probiotics has been defined currently as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Rijkers et al., 2010; Lew and Liong, 2013).

1.5.2. Mechanistic benefits of probiotics in human health and disease

The gastrointestinal tract of an animal is inhabited by a highly diverse group of

microorganisms, and for humans, more than 500 different types of microorganisms are localized mainly in the colon (Tuohy et al., 2003; Guarner et al., 2003). The major functions of these gut microbiota are to prevent pathogen colonization (Tuohy et al., 2003), secrete bactericidal or bacteriostatic substances (Collado et al., 2009), utilize energy and nutrients (Guarner et al., 2003), and modulate the host immune response (Stamatova et al., 2009). Probiotics are effective against chronic inflammation in Crohn's disease (Fujimori et al., 2006; Stamatova et al., 2009), remission of ulcerative colitis (Zocco et al., 2006; Kruis et al., 2004), reduction of inflammatory bowel disease (IBD) (Baroja et al., 2007), reduction of bowel movements, and pain (Andriulli et al., 2008), nonspecific diarrhea (Henker et al., 2008), improvement in lactose intolerance (Reid, 1999), reduction of urinary tract infections (Reid, 1999), prevalence of eczema in young ones (Wickens et al., 2012), and against cancer (Reid, 1999). In addition, probiotics along with glutamine supplementation reduced infection in brain injured patients (de Arruda and Jose, 2004).

1.5.3. Probiotics in poultry

Nowadays consumers are concerned about antibiotic resistant bacteria, and antibiotic residues in the meat products and eggs. Therefore, probiotics have emerged as an alternative to antibiotics in poultry industry globally eliminating the use of chemicals, pesticides, and herbicides (Reid and Robert 2002; Apata et al., 2009). Previously, poultry farmers were using antibiotics as feed additives in sub-therapeutic doses, as these

compounds could eradicate common enteric pathogens in poultry such as *Salmonella*, *Campylobacter*, and *Escherichia*, thereby promoting optimal growth. Adversely, antibiotic supplementation in feed has resulted in the emergence of antibiotic-resistant bacteria that contaminated the human food chain, resulting in infections difficult to treat with existing antibiotics (Khachatourians, 1998; Marshall and Levy, 2011; Timothy et al., 2012). The first indication about bacteria acquiring antibiotic resistance was reported by the UK Swan Committee in 1969. The Committee recommended that antibiotics used in human chemotherapy or those that promote cross-resistance should not be used as growth promoters in animals (Witte, 1998). Many countries banned incorporation of antibiotics in poultry feed, including Denmark in the early 90s. The FDA banned the use of fluoroquinolones in poultry feed since 2005. Ever since the FDA has been instrumental in persuading the industry to eliminate/reduce the use of medically important antibiotics from production agriculture, and that veterinary supervision has become mandatory for antibiotic use in livestock, including poultry (FDA, 2015).

After Nurmi and Rantala (1974), there has been a steady progress in evaluating beneficial microorganisms for pathogen reduction in poultry (Snoeyenbos et al., 1978). Following Nurmi's concept of competitive exclusion cultures, in the early 90s, Nisbet et al. (1994) used a defined composition of anaerobic bacteria, namely probiotics, to competitively exclude *Salmonella* from poultry. This composition consisted of anaerobic bacteria that could ferment lactic acid to produce organic acids such as acetic, propionic, and butyric acids, and a second culture, an anaerobic bacterium capable of producing

lactic acid from the fermentable carbohydrates. The two bacterial cultures were added into poultry feed that eventually prevented *Salmonella* colonization in poultry gut.

Higgins et al. (2005) studied the efficacy of probiotics in preventing diarrhea, and stunted growth in turkey poults. The study was conducted in three independent experiments. Results revealed a significant weight gain in the probiotic-treated group when compared to antibiotic treated group in the first experiment. In the second experiment, however, no significant difference was observed between the two groups. In the third experiment, when there was *Salmonella enterica* serovar Senftenberg infection, poults that received antibiotic treatment after probiotic administration gained significant weight when compared to antibiotic -, and probiotic – treated groups. The research indicates inconsistency in the beneficial effects of probiotics in turkeys.

Rodriguez et al. (2007) investigated the effect of a *Lactobacillus* probiotic, FM-B11 (IVS/Wynco, LLC, Springdale, AR) on turkey body weight, performance, and health. The study was conducted in 118 commercial turkey hen lot containing 1,542 to 30,390 hens per lot either belonging to Nicholas or Hybrid genetic lines. Sixty lots were selected as treatment groups whereas 58 were set as control groups. The probiotic was administered orally through drinking water at the level 10^6 CFU/ml for 3 consecutive days at placement (day of age) and move out (6 weeks of age). The study found a significant increase in market body weight, and average daily weight gain after probiotic administration ($P < 0.05$), and genetic line ($P < 0.01$), whereas, no significant difference

was observed in feed conversion efficiency between the treatment groups ($P > 0.05$). The cost of production was found to be less in probiotic fed group when compared to the control group.

Rahimi et al. (2008) determined the effect of a direct fed microbial (DFM), Primalac (Star- Labs Inc., Clarksdale, MO) supplemented through mash or crumbled feed and determined its effect on the structure and ultrastructure of the small intestine. There were 8 treatment groups with six replicates carrying 7 hens in each replication. The DFM group was supplemented with Primalac from day 1 to day 21. Fifty percent of the poults were challenged with 1ml of 10^10 CFU/ml *Salmonella enterica* serovar Typhimurium, *Salmonella enterica* serovar Heidelberg, and *Salmonella enterica* serovar Kentucky as oral gavage, and housed separately. At day 21, one poult from each treatment group was selected randomly, euthanized and performed a necropsy. Intestinal samples such as duodenum, jejunum, and ileum were collected to perform light and electron microscopy. The DFM-fed birds were found to possess increased goblet cell numbers, total goblet cell area, goblet cell mean size, and mucosal thickness when compared to treatment, and the intestinal lumen of these birds were found to be possessing more filamentous bacteria.

Zulkifli et al. (2000) studied the effect of probiotic cultures on growth performance and immune response in two commercial broiler strains, Hubbard × Hubbard (HH) and Shaver × Shaver (SS). The broiler strains were supplemented with either 50mg/kg oxytetracyline (OTC) or 1g/Kg *Lactobacillus* culture (LC) from day 1 onwards and were

exposed to a daily temperature of 36 ± 1°C for 3 h/day from day 21 to 42. Chicks supplemented with *Lactobacillus* culture showed higher weight gain followed by OTC group and control group during day 1 to day 21. Among the chicks, HH showed greater body weight and weight gain when compared to the SS strain. After heat exposure from day 21 to 42, it was found that strains provided with *Lactobacillus* cultures gained significant weight, showed high feed intake, and low food efficiency when compared to OTC and control groups.

Vahjen et al. (2010) studied the effect of *Enterococcus faecium* strain as probiotic against the bacterial population in the small intestine of growing turkey poults. The poults were fed with a diet containing 10^10 viable probiotic cells/kg feed for 42 days. Lactate production along with colony forming units of different bacteria such as anaerobic bacteria, lactic acid bacteria, enterobacteria, and enterococci were determined. The study found increased lactate concentration in probiotic fed birds throughout 42 days of study. In addition, a 10-fold increase in the enterococci colony forming units in treatment groups when compared to control group was observed. The study also found that the probiotic has stimulated other lactic acid bacteria in intestine such as *Lactobacillus*.

Menconi et al. (2010) determined the effect of commercial lactic acid bacteria cultures FloraMax (IVS-Wynco LLC, Springdale, AR) against *Salmonella enterica* serovar Heidelberg in broiler chicks and turkey poults. The study found a significant reduction in *Salmonella enterica* serovar Heidelberg in ceca of both species

supplemented with probiotics. In addition, the study concluded that the pathogen was more colonized in turkey ceca than broiler ceca.

Higgins et al. (2010) investigated the effect of 3 ATCC Lactobacilli (LAB3) combinations and a commercially available probiotic (PROB) in reducing *Salmonella enterica* serovar Enteritidis colonization in neonatal broiler chicks. In the first three set of experiments, chicks were challenged with *Salmonella enterica* serovar Enteritidis by oral gavage. After 1-hour challenge, chicks were treated separately with probiotics, LAB3 or PROB, and cecal tonsils were collected after 24 hours for *Salmonella* recovery. In the next set of four experiments, day-old chicks were supplemented with PROB by oral gavage, and then challenged with *Salmonella enterica* serovar Enteritidis as oral gavage after 24 hours. Cecal tonsils were also collected for *Salmonella* recovery after another 24 hours. In the last set of three experiments, day-old chicks were challenged with *Salmonella enterica* serovar Enteritidis, and after 24 hours, the chicks were provided with probiotic culture, PROB by oral gavage. Cecal tonsils were collected for enrichment. In the first set of experiments, the probiotic, PROB considerably reduced cecal *Salmonella* populations when compared to LAB3 and the control ($P<0.05$). Similarly, in the second set of experiments, PROB significantly reduced cecal recovery of *Salmonella* when administered 24 hours before challenge. On the contrary, when PROB was administered 24 hours after *Salmonella* challenge, no significant reduction of *Salmonella* was detected in the cecal tonsils. The study revealed that PROB is better than LAB3 in reducing *Salmonella*, but the reduction will be less if administered 24 hours after pathogen challenge.

Wolfenden et al. (2011) evaluated the effect of DFM cultures on live performance and pathogen reduction in turkeys for 23 days. A *Bacillus lateroporus* isolate named PHL-MM65, and a *Bacillus subtilis* isolate, PHL-NP122 were fed at 10^6 spores/g of feed to 7-day old turkey poults grouped as two treatment groups. In addition to the above two treatments, two more groups were included as a negative control, and 0.019% nitarsone (feed additive), respectively. On day 23, the average body weights of birds in groups treated with probiotic isolate, PHL-NP122 (853g) and nitarsone (852g) were found to be significantly different ($P<0.05$) compared to the control group (784g). In addition, both isolates significantly reduced *Salmonella* when compared with the control ($P<0.05$). The study concluded that the isolate PHL-NP122 supplemented in poult diet can significantly improve weight gain and can reduce *Salmonella* in the cecum.

Agboola et al. (2014) determined the effect of probiotics and synbiotics on gut microbiota and gut histomorphological characteristics in turkey poults. Day-old turkey poults were brooded for 7 days, and were divided into four different treatment groups such as control, probiotics, antibiotics, and synbiotics. Necropsy was conducted on day 56 and ileal digesta samples were collected for microbial enumeration and pH determination. Probiotic and synbiotic administration significantly reduced intestinal coliform count and total bacterial count ($P<0.05$), whereas pH was found to be acidic in treatment groups (6.45-6.83). The villus height and crypt depth of treatment groups were improved significantly ($P<0.05$) when compared to control groups, but less significant

from antibiotic treated groups. The lactic acid producing bacteria increased in probiotic and synbiotic groups.

1.5.4. Suggested probiotic mechanisms in poultry

Various mechanisms are suggested for the efficacy of probiotics in poultry. For example, Edens et al. (2003) suggested that probiotics could be alternatives to antibiotics because they can potentially modulate metabolism, conserve energy utilization, stimulate host immunity, competitively exclude and kill pathogens, enhance nutrient uptake from the intestinal tract, improve host performance, and ultimately resulting in reduced contamination of meat with enteric pathogens. In addition, use of probiotics in poultry may potentially stimulate immunity and boost performance (Dhama et al., 2011; Kabir et al., 2009). Moreover, probiotics can create a physical barrier in the intestinal tract against pathogen attachment, increase host digestive enzymes activity, decrease activity of digestive enzymes activity in pathogens, decrease ammonia production, neutralize enterotoxins produced by pathogens, improve nutrient uptake and host performance (Kral, 2012), maintain integrity of bone, and antagonistic to intestinal parasites in poultry (Khan et al., 2013).

1.6. Conclusions and objectives

Non-typhoidal salmonellosis is a serious public health concern in the United States of America. We are observing the emergence of certain *Salmonella* serotypes in poultry,

including the MDR *Salmonella enterica* serovar Heidelberg that has surfaced causing human infections difficult to treat with conventional antibiotics. With antibiotic resistance emerging as a major threat of the century, the FDA has persuaded the livestock industries to comply with the veterinary feed directive that aims at the elimination of the use of medically important antibiotics from production agriculture and facilitating veterinary supervision for antibiotic use on production farms. Probiotics are beneficial bacteria that can produce health benefits to the host. Probiotics, when administered in adequate quantities, could competitively exclude pathogenic bacteria from colonizing the gut. In addition, probiotics can stimulate host immune system and can maintain a healthy microbial balance inside the gut. Since MDR *Salmonella enterica* serovar Heidelberg could harbor inside the turkey gut, it is a serious public health concern, and so far, no studies have determined the potential of host-specific *Lactobacillus* strains against this MDR pathogen. Therefore, we hypothesized that (turkey gut-derived) *Lactobacillus salivarius* and *Lactobacillus ingluviei* in combination would reduce cecal colonization and organ invasion in turkey poults. Our specific objectives were:

1. To determine the effect of supplementation of turkey gut-derived *Lactobacillus salivarius* and *Lactobacillus ingluviei* in combination against MDR *Salmonella enterica* serovar Heidelberg colonization in turkey poults, and

2. To determine the probiotic qualities of turkey gut-derived *Lactobacillus salivarius* and *Lactobacillus ingluivei* isolates *in vitro.*

Chapter 2

Determining the effect of supplementation of turkey gut-derived *Lactobacillus salivarius* and *Lactobacillus ingluviei* in combination against multidrug-resistant *Salmonella enterica* serovar Heidelberg colonization in turkey poults

Synopsis

In this study, we determined the efficacy of *Lactobacillus salivarius* and *Lactobacillus ingluviei*, *Lactobacillus* of turkey-gut origin, against MDR *Salmonella enterica* Heidelberg colonization in commercial straight-run Hybrid Converter turkey poults. Three independent experiments were conducted. The treatment groups were negative control (-*S. enterica* ser. Heidelberg, -*Lactobacillus*), *S. enterica* ser. Heidelberg control (+*S. enterica* ser. Heidelberg, -*Lactobacillus*), and a treatment group (+*S. enterica* ser. Heidelberg, +*Lactobacillus*), with at least nine birds in each treatment group per experiment. *Lactobacillus* (8 log10 CFU/gallon of water) was supplemented through drinking water continuously for 14 days. Poults were challenged with the 2011 turkey outbreak strain of *S. enterica* ser. Heidelberg (5.0 log10 CFU/ml) on day 7, and *S. enterica* ser. Heidelberg counts were determined on days 2 and 7 post-inoculation. Results indicated that the treatments significantly reduced *S. enterica* ser. Heidelberg in the cecum, liver, and spleen of turkey poults compared to the *S. enterica* ser. Heidelberg control in two of the three experiments conducted ($P<0.05$). The combination of *Lactobacillus* isolates could be an alternative to antibiotics strategy in turkey production.

2.1. Introduction

Probiotics are used in the poultry industry as a substitute for antibiotics, as a growth promoter, and for eliminating pathogenic bacteria. On the other side of the coin, increased use of antibiotics in livestock, and poultry has led to the emergence of antibiotic-resistant pathogenic bacteria in the animal gastrointestinal tracts (GIT) (Khachatourians 1998). This has become a great concern for the consumers who prefer foods that are not treated with antibiotics, for that matter, any chemicals of human health concern (Donoghue 2003). A few countries have banned the use of antibiotics in poultry feed, and the FDA has invested a great effort to persuade industry to reduce the use of medically important antibiotics from animal agriculture (FDA, 2015). In this context, probiotics have become an area of great promise in the animal industry, including poultry production as several probiotics can deliver multiple benefits to the host.

Research of Nurmi and Rantala (1973) on competitive exclusion opened new horizons in the alternative antimicrobial discovery in the early 1970s. They reported that oral administration of cultured gut flora of an adult chicken could prevent *Salmonella enterica* serovar Infantis colonization in young chicks. It became evident that host-specific cultures could be protective against pathogenic organisms. However, standardizing defined cultures – cultures of which the identity of the included organisms is known to the strain, dose, stability, and concentration levels, is still a difficult task for the industry and researchers. In this regard, lactic acid bacteria have been tremendously researched

since they are the predominant inhabitants among the gut microbes of humans and animals (Chou and Weimer, 1999). They have tremendous potential to be considered as host-specific probiotics (Tortuero et al., 1973; Pascual et al., 1999; Ghareeb et al., 2012; Bielke et al., 2003; Torrez- Rodriguez et al., 2007; Vicente et al., 2007; Higgins et al., 2008). *Lactobacillus* strains have the ability to produce bacteriocins, lactic acid, and peroxide that can inhibit pathogenic bacteria (Harimuthu and Widodo, 2015).

Previous studies used either commercially available *Lactobacillus* strains or cecal microflora from another bird (non-defined cultures). No studies are available to date regarding the use of turkey-gut derived defined *Lactobacillus* cultures against MDR *Salmonella enterica* serovar Heidelberg colonization in turkeys. To meet this goal, the objective of this study was to determine the use of turkey-gut derived *Lactobacillus salivarius* and *Lactobacillus ingluviei* on cecal colonization, and organ invasion of *Salmonella enterica* serovar Heidelberg in turkey poult challenge-intervention-response model.

2.2. Materials and Methods

All experiments were approved by the Institutional Animal Care and Use Committee at the University of Minnesota and carried out according to the protocol.

2.2.1. Bacteria

2.2.1.1. MDR *Salmonella* isolate, culture conditions, inoculum preparation

An MDR (resistant to ampicillin, tetracycline, gentamicin, and streptomycin; CLSI standards) isolate of *Salmonella enterica* serovar Heidelberg obtained from the 2011 ground turkey outbreak were used for challenging poults in the experiment (Source – University of Tennessee, Knoxville, TN). For selective enumeration, and to avoid any inherent MDR *Salmonella*, the input *Salmonella enterica* serovar Heidelberg isolate was made resistant against nalidixic acid (NA; Amresco; 50µg/ml) by gradually growing the bacteria in tryptic soy broth (TSB; Criterion, Hardy Diagnostics, CA) from 5µg/ml to 50µg/ml. The isolates were confirmed for resistance induction by streaking on XLD containing 50µg/mL of NA (XLD-NA). For determining the bacterial count, NA resistant isolate was grown overnight aerobically in 500 mL of TSB supplemented with 50µg/ml NA at 37° C. The overnight bacterial culture was serially diluted 10-fold in phosphate buffered saline (PBS; pH 7.2), and 0.1 mL of the appropriate dilutions were plated on XLD-NA, and incubated at 37° C for 24 h. For preparing inoculum, the overnight bacterial culture was grown in 500 mL of TSB-NA and was sedimented by centrifugation (4000 x g, 15 min at 4° C; Allegra 15X Beckman Coulter), and the pellet was resuspended in 100 mL of PBS to get a bacterial concentration of 5 x10^8 CFU/mL. Appropriate serial 10-fold dilution was carried out to obtain a final 5 x 10^5 CFU/ml, and was used a bacterial inoculum (Kollanoor Johny et al., 2009; 2010a, b; 2012a, b; 2013; Nair and Kollanoor Johny, 2016; Nair et al., 2016).

2.2.1.2. Probiotic isolates, and inoculum preparation

Probiotic (*Lactobacillus*) isolates were provided by Dr. Timothy Johnson, Veterinary and Biomedical Sciences, University of Minnesota. The two isolates, *Lactobacillus salivarius,* and *Lactobacillus ingluviei*, were lab adapted by continuously sub-culturing aerobically at 37°C, and bacterial enumeration on deMan Rogosa Sharpe (MRS; Criterion, Hardy Diagnostics, CA) agar after aerobic incubation at 37° C for 48 h. Both cultures were grown overnight separately in 500mL MRS broth at 37° C, aerobically. Bacterial enumeration of overnight cultures was done by serially diluting (1:10) in PBS, and plating 0.1 mL of corresponding dilutions on MRS plates, and allowing to incubate at 37° C for 48h. Bacterial cultures were then subjected to centrifugation at 4000g, 15m, and 4° C. The button formed for each probiotic was reconstituted in 25mL PBS (pH 7.2) and were mixed to get a final volume of 50 mL. The final volume (50 mL) was dispensed in 1-gallon drinking water to attain a final concentration of 10^6 CFU/ml and fed for 14 days to birds belonging to the probiotic, and treatment groups (Kollanoor Johny et al., 2013; Nair and Kollanoor Johny, 2016; Nair et al., 2016).

2.2.2. Experimental birds

Day-of-hatch, commercial, turkey poults (Hybrid converter), male and female in

equal, obtained from the Willmar Poultry Company, Willmar, MN, were weighed and allocated to isolators located at the Research Animal Resources biocontainment (isolation) units at the University of Minnesota. Poults were provided with non-medicated, *Salmonella*-free feed (Famo feeds Inc.), and water *ad libitum*. Testing confirmed the feed was negative. Arrangements were made for providing age-appropriate temperatures, and bedding.

2.2.3. Experimental Design

Three separate experiments were conducted to determine the effect of *Lactobacillus salivarius* and *Lactobacillus ingluviei* in combination in reducing MDR *Salmonella enterica* serovar Heidelberg in turkey poults. Weighing of each bird was performed on day 0, and random screening was performed in the incoming flock to determine the presence of any inherent *Salmonella* (n=6/experiment). Briefly, two birds from each group were euthanized with CO_2, cecal contents were collected, and enriched in 10mL selenite cystine broth (SCB; Criterion, Hardy Diagnostics, CA) for 6h. The enriched cecal contents were streaked on xylose lysine desoxycholate agar (XLD; Criterion, Hardy Diagnostics, CA), and incubated at 37°C for 24h. There were 3 treatments in each experiment: a negative control (no *S. enterica* ser. Heidelberg challenge and no probiotic), a positive control (*S. enterica* ser. Heidelberg challenge and no probiotic), and a treatment group (*S. enterica* ser. Heidelberg challenge and probiotic).

Birds in the negative control were not provided probiotic. Fifty ml of the probiotics made from equal volumes of *Lactobacillus salivarius* (10^9cfu/mL) and *Lactobacillus ingluviei* (10^9 CFU/mL) were added to 1 gallon of drinking water daily to attain a final concentration of ~10^6 CFU/ml for 14 days to the probiotic group. Birds in the positive control group and the probiotic group were challenged with *Salmonella enterica* serovar Heidelberg at ~10^6 CFU/ml on day 7. After 48 h of challenge, two birds were randomly selected from each group to ensure *Salmonella* colonization in the ceca. At the end of each experiment, birds were weighed and euthanized by CO_2 asphyxiation, and ceca were collected aseptically for *Salmonella* colonization in the ceca and liver and spleen for checking *Salmonella* invasion.

2.2.4. Determination of cecal colonization of MDR *Salmonella enterica* serovar Heidelberg

Ceca with their contents from each bird were collected aseptically in separate 50mL tubes containing 10mL PBS (pH 7.2), weighed, and homogenized thoroughly. The cecal contents were serially diluted (1:10) in PBS, and corresponding dilutions were plated on XLD-NA. The plates were then incubated aerobically at 37° C for 48 h. Those samples which were not enumerated by serial dilution and plating were enriched with 10mL SCB, aerobically, and incubated at 37° C for 6h, and streaked on XLD-NA plates. The streaked XLD-NA was then aerobically incubated at 37° for 24 h (Kollanoor Johny et al., 2009; 2010a, b; 2012a, b; 2013; Nair and Kollanoor Johny, 2016; Nair et al., 2016).

2.2.5. Determination of invasion of MDR *Salmonella enterica* serovar Heidelberg to liver and spleen

Liver and spleen were collected aseptically from each bird into 50mL tubes containing 10mL PBS and mixed homogeneously. The homogenate was then enriched with 10mL SCB, and allowed to incubate aerobically at 37° C for 6h. The homogenate was then streaked on XLD-NA plates and was aerobically incubated at 37° for 24 h (Kollanoor Johny et al., 2009; 2010a, b; 2012a, b; Nair and Kollanoor Johny, 2016; Nair et al., 2016).

2.2.6. Statistical analysis

A completely randomized design was used to analyze the effect of probiotics on *Salmonella enterica* serovar Heidelberg in all experiments. The treatment structure included 3 treatment groups (Negative Control, *Salmonella* Control, and Probiotic + *Salmonella*) and 3 organ samples (cecum, liver, and spleen), and the experimental unit was a pen (isolator). The number of *Salmonella* colonies was logarithmically transformed ($\log_{10}$ CFU/g) before analysis to achieve homogeneity of variance (Byrd et al., 2003). The data were analyzed using the PROC-MIXED procedure of the statistical analysis software (version 9.4, SAS Institute Inc., Cary, NC). Differences among the least squares means were detected using Fisher's least significance difference test. A P-value <0.05 was considered statistically significant. The liver and spleen data were analyzed with a

binary approach to determine the effect of probiotic treatment on the presence (positive after either direct plating or enrichment) or absence (negative after both direct plating and enrichment) of *Salmonella* in different organ samples. An isolator was the experimental unit, and the analysis was done for each organ separately. The corresponding body weights in different groups from three experiments were combined for analysis.

2.3. Results

No significant difference was observed for day 1 body weights in the experiments ($P>0.05$). Also, no significant difference was found in day 14 body weight among the three groups ($P>0.05$) (Figure 1).

After 48h post-inoculation, *Salmonella enterica* serovar Heidelberg colonized the poult ceca in the range of 5- and 6- log_{10}CFU/g of cecal contents in all experiments. After 7 days' post inoculation, *Salmonella enterica* serovar Heidelberg continued colonizing the cecum of *Salmonella* controls in the range of 4.7 to 6.2 log10 CFU/g of cecal contents (Figure 2). However, the probiotic supplementation reduced *Salmonella enterica* serovar Heidelberg colonization in the poultry ceca significantly, except in the second experiment (Figure 2). In the first and third experiments, probiotic supplementation significantly reduced cecal colonization of *Salmonella enterica* serovar Heidelberg by 1.79- and 3.9 logs, respectively, compared to the *Salmonella* controls.

Similar to the cecal colonization, liver invasion by *Salmonella enterica* serovar Heidelberg was also significantly affected by the probiotic supplementation in experiments 1 and 3. In the first experiment, 71% of the liver samples in the *Salmonella* control were invaded by *Salmonella enterica* serovar Heidelberg. The supplementation of probiotic reduced liver invasion by the pathogen to 21% (P<0.05; Figure 3). Similarly, in the third experiment, the probiotic supplementation reduced liver invasion of the pathogen from 83% in control to 12.5% (Figure 3; P<0.05). No significant difference between the treatments was observed in the second experiment.

Invasion of spleen by *Salmonella enterica* serovar Heidelberg varied significantly across the experiments (Figure 4). In the first experiment, the percentage of splenic invasion in the probiotic supplemented group (7%) was substantially lower than in the control group (33%; P<0.05; Figure 4). In the second experiment, no significant difference between the treatments was observed (P>0.05). A 100% invasion of the spleen was noticed in both control and treatment groups. However, in the third experiment, probiotic supplementation significantly decreased *Salmonella enterica* serovar Heidelberg splenic invasion by >30% (P<0.05; Figure 4).

2.4. Discussion

Following Nurmi and Rantala's research on competition exclusion cultures against *Salmonella enterica* serovar Infantis in chickens (Snoeyenbos et al., 1978), several researchers investigated the potential of non-defined and defined probiotic cultures for competitive exclusion of pathogenic bacteria (Pascual et al., 1999; Higgins et al., 2007; Rodriguez et al., 2007; Ghareeb et al., 2012). However, there are various functions for which probiotics are being used now-a-days, including modulation of the immune system, balancing the enteric flora, and maintenance of cellular integrity in the GIT, among several others. There has been a renewed interest in using host-specific bacteria as probiotics in livestock production. One important aspect is the colonization potential of the bacteria used, which most of the non-host-specific species lack. In addition, one of the challenges that the probiotic industry face is the difficulty in delivering high, and stable populations of probiotics into the gut from external sources, and keeping them alive for longer duration in the GIT.

Lactobacillus species is the most common, and a major role player in animal gut microbiome balance. Since they are the major species that colonize in the chicken gut (Fuller and Brooker 1974), the use of host-specific *Lactobacillus* could be a viable strategy for controlling pathogens in poultry; studies that investigated host-specific *Lactobacillus* is scarce in turkeys. The current study explores the potential of such a strategy – use of *Lactobacillus* of turkey gut origin – to control MDR *Salmonella enterica*

serovar Heidelberg in turkeys. If found effective, this strategy could be easily implemented in the turkey farms for pathogen control.

The probiotic exerted significant reduction against *Salmonella enterica* serovar Heidelberg in two of the three experiments (Figure 2). It could be inferred that a 2-3 log10 CFU/g reduction of *Salmonella enterica* serovar Heidelberg could be obtained if the *Lactobacillus* combination is applied at 10^6 CFU/ml through water and the *Salmonella enterica* serovar Heidelberg colonization is at 10^5 log10 CFU/bird (Figure 2). This is important with regards to field situations because *Salmonella* if present as few as 100 cells can infect day old chicks, and make them consistent pathogen shedders (Van Immerseel et al., 2004; Gast and Holt, 1998). However, as they are a week old, it may take a million cells to consistently infect the chicks (Van Immerseel et al., 2004; Trampel et al., 2014). Moreover, the infectious dose of *Salmonella* in humans is 50-100 cells (Waterman et al., 1998). The reduction observed in the current study indicates that preharvest food safety associated with bird-level colonization of the pathogen, and the potential of cross-infection of fresh incoming flock could be improved with turkey-derived *Lactobacillus* based probiotics. Contrary to the first and third experiment, the birds in the second experiment may have encountered transportation stress that resulted in a higher rate of pathogen colonization (10^6 log10 CFU/g vs. <10^5 CFU/g) although the initial inoculation was the same (5X10^5 log10 CFU/bird) (Figure 2).

The reduction in *Salmonella* number could be due to competitive exclusion by the

probiotic as *Salmonella* was unable to adhere to the cecal/intestinal epithelium (Jin et al., 1996a). Also, *Salmonella* could have encountered competition from probiotics for nutrients (Patterson and Burkholder 2003) and the high cell surface hydrophobicity of *Lactobacillus* - a measure of greater ability to adhere to the intestinal cells, when compared to *Salmonella* (Gusils et al., 1999). In addition, bacteriocins produced by *Lactobacillus* could have exerted potent antimicrobial activity (Bogovič-Matijašić et al., 1998). In addition, Vicente et al. (2007) have observed that when probiotics are administered orally, the reduction of *Salmonella* in the cecum could be achieved due to an increased concentration of short-chain fatty acids such as propionic acid, acetic acid, lactic acid, and butyric acid.

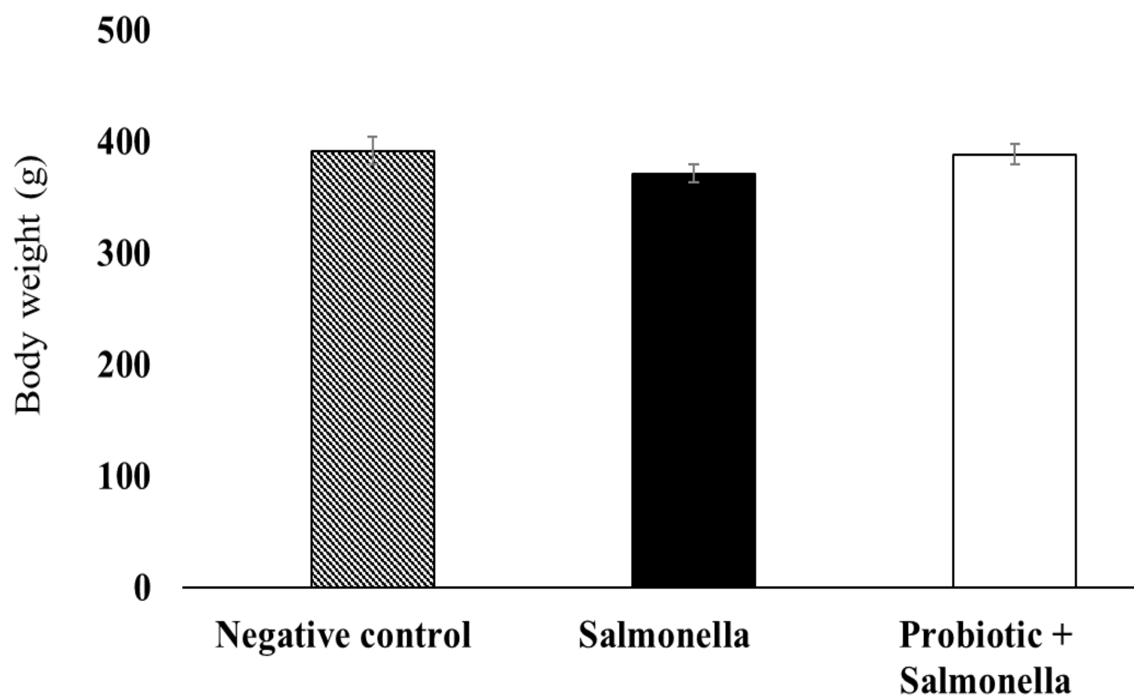
We also found that host-specific *Lactobacillus* could reduce invasion of the liver by *Salmonella enterica* serovar Heidelberg, evidence obtained from 2 of the 3 experiments. The liver has stellate macrophages – the Kupffer cells – that could help in the clearance of the bacteria much faster than any other organ (Llorente and Bernd, 2016). However, reduction of invasion to the spleen is hard to obtain. Splenic polymorphonuclear cells have been identified as a ‘safe-site’ for *Salmonella enterica* serovar Typhimurium survival (Dunlap, 1992). The decrease in liver invasion may be due to a significant reduction in pathogen load in the gut due to competitive exclusion by *Lactobacillus*. The other protective mechanisms that might have played vital roles in preventing organ invasion of *Salmonella* could be immune modulation (Hatcher and Lambrecht, 1993), and bacteriocin production (Bogovič-Matijašić et al., 1998).

Although not host-specific, *Lactobacillus* species have high potential as a general probiotic in chickens. For example, Higgins et al. (2007) found that administration of *Lactobacilli* 1h after challenge with *Salmonella enterica* serovar Enteritidis, and *Salmonella enterica* serovar Typhimurium significantly reduced pathogens in the cecal tonsils, and ceca after 24h. The potential of *Lactobacillus salivarius* CTC2197 to inhibit *Salmonella enterica* serovar Enteritidis for 21 days was explored by Pascual et al. (1999). Similarly, Ghareeb et al. (2012) found that *Campylobacter jejuni*, a pathogenic bacterium colonizing the ceca of chickens can be competitively excluded when administered with a multispecies probiotic that contain *Lactobacillus salivarius* and *Lactobacillus reuteri*.

In conclusion, the oral supplementation of combined probiotic cultures of *Lactobacillus Salivarius* and *Lactobacillus ingluviei* at 10^6 CFU/ml significantly reduced cecal colonization and invasion of the liver and spleen by MDR *Salmonella enterica* serovar Heidelberg, reflecting its potential to be studied in larger turkeys.

Figure 1

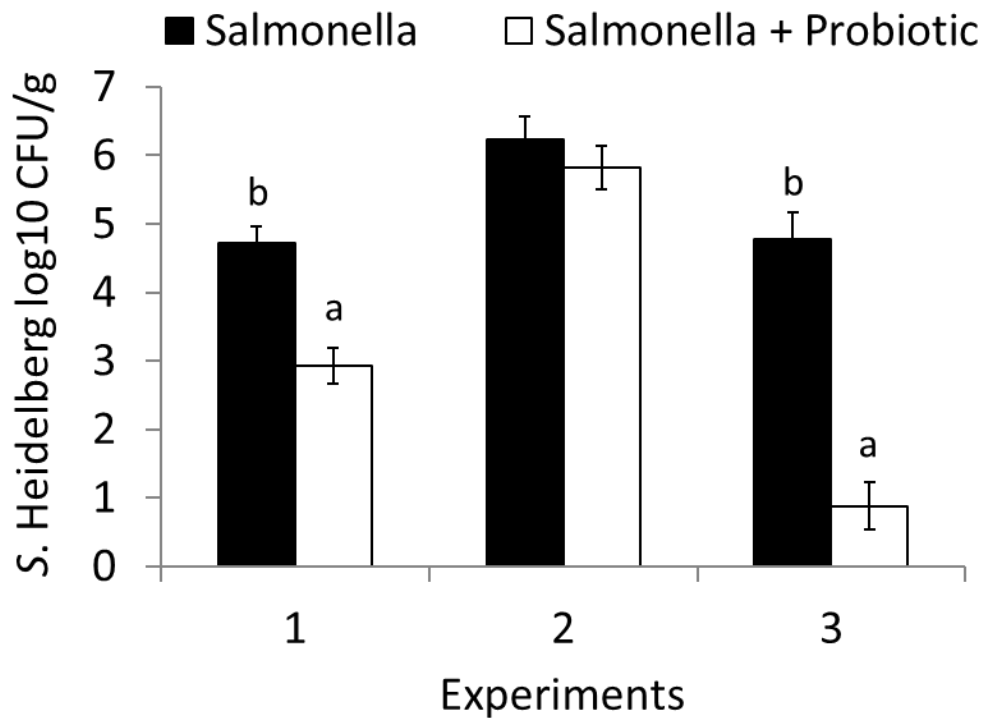
Effect of *Lactobacillus* probiotic on body weights of turkey poults with and without *S. enterica* ser. Heidelberg challenge.



* No significant difference between the treatments (P>0.05) on day 14. Each group had 40 birds in the beginning, from which 8 birds per group were euthanized on day 2 post-inoculation to ensure *S. enterica* ser. Heidelberg colonization. Final body weights were taken from a minimum of 29 birds per group.

Figure 2

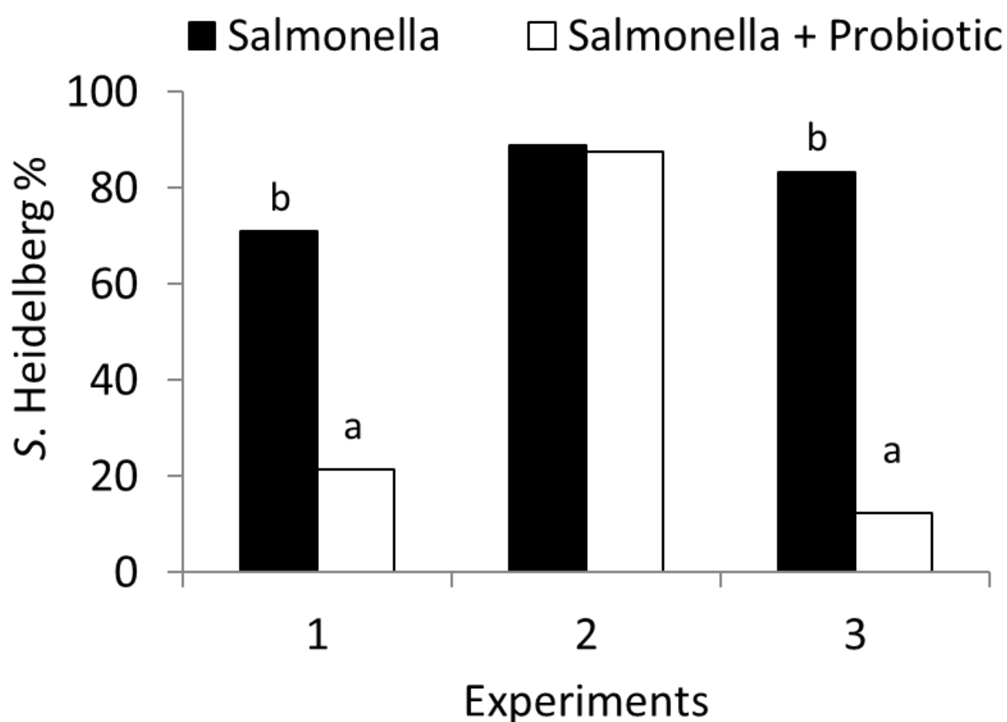
Effect of *Lactobacillus* probiotic on cecal colonization with *S. enterica* ser. Heidelberg challenge in turkey poults on day 14*



* Different superscripts indicate that the treatment groups were significantly different at P<0.05 on day 14. Treatment groups were: Negative Control, *Salmonella* Control, and *Salmonella* + Probiotic. Study 1 had 14 birds in each treatment group, Study 2 had 9 birds in each treatment group, and Study 3 had 9 birds in the Negative control, 6 birds in the *Salmonella* Control, and 8 birds in the *Salmonella* + Probiotic group.

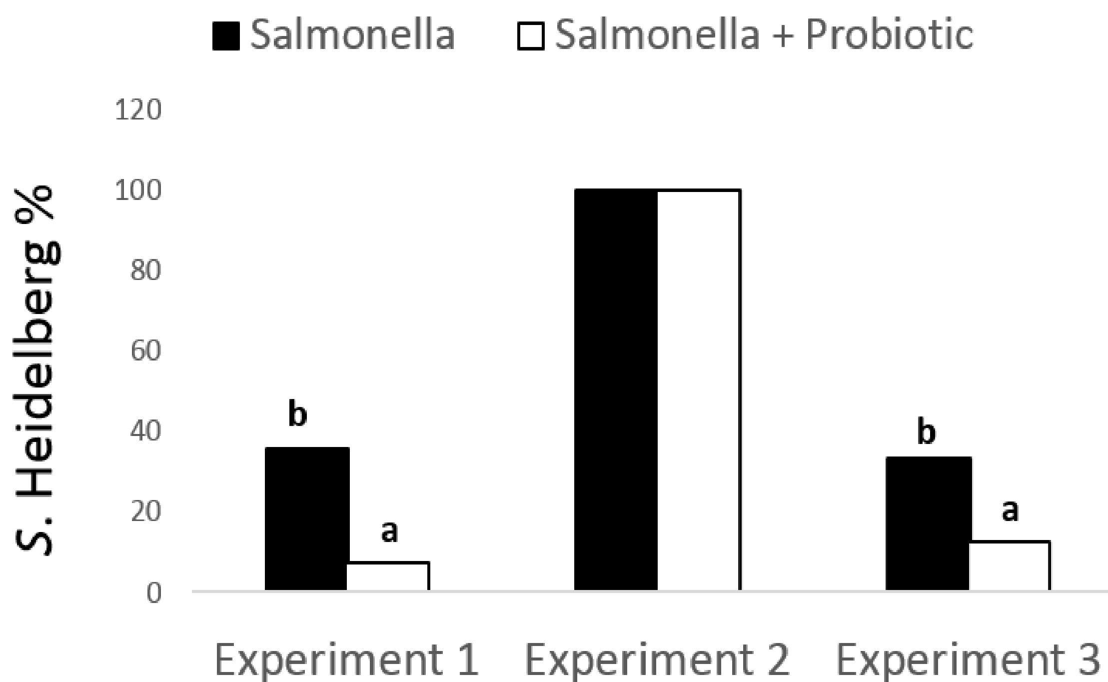
Figure 3

Effect of *Lactobacillus* probiotic on the liver invasion with *S. enterica* ser. Heidelberg in turkey poults on day 14*



* Different superscripts indicate that the treatment groups within an experiment were significantly different at P<0.05 on day 14. Treatment groups were: Negative Control *Salmonella* Control, and *Salmonella* + Probiotic. Study 1 had 14 birds in each treatment group, Study 2 had 9 birds in each treatment group, and Study 3 had 9 birds in the Negative control, 6 birds in the *Salmonella* Control, and 8 birds in the *Salmonella* + Probiotic group.

Figure 4
Effect of *Lactobacillus* probiotic on the spleen invasion with *S. enterica* ser. Heidelberg in turkey poults on day 14*



* Different superscripts indicate that the treatment groups were significantly different at $P<0.05$ on day 14. Treatment groups were: Negative Control, *Salmonella* Control, and *Salmonella* + Probiotic. Study 1 had 14 birds in each treatment group, Study 2 had 9 birds in each treatment group, and Study 3 had 9 birds in the Negative control, 6 birds in the *Salmonella* Control, and 8 birds in the *Salmonella* + Probiotic group.

Chapter 3

To determine the probiotic qualities of turkey gut-derived *Lactobacillus salivarius* and *Lactobacillus ingluviei* isolates *in vitro*.

Synopsis

In this study, we determined the qualities of *Lactobacillus ingluviei* and *Lactobacillus salivarius* to be considered as potential probiotics, *in vitro*. Although turkey gut-derived, any potential probiotic strain will face a series of physiological challenges until efficient colonization in the cecum occurs, once administered orally. These obstacles include the low acid environment in the proventriculus, and the detergent action of bile and bile salts, before its colonization in the lower part of the intestine. Once these obstacles are traversed, the probiotic strain should have the ability to colonize strongly to the intestinal epithelium for performing colonization resistance against invading pathogens. In this process, potential probiotic strains will induce strong antimicrobial property. Our studies indicated that both *Lactobacillus* exerted significant resistance to low pH and bile salts ($P<0.05$). Our cell culture studies indicated that the tested *Lactobacillus* isolates had high adhesion to model avian intestinal epithelial cells, validating the *in vivo* studies. Moreover, the cell-free extracts of *Lactobacillus salivarius* and *Lactobacillus ingluviei* showed high antimicrobial activity separately against three major serotypes of *Salmonella*, namely, *Salmonella enterica* serovar Enteritidis, *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Heidelberg ($P<0.05$). We also tested the antibiotic susceptibility of the potential *Lactobacillus* isolates. *Lactobacillus salivarius* and *Lactobacillus ingluviei* were sensitive to a variety of common antibiotics used in human therapy.

3.1. Introduction

Probiotics are beneficial microbes that can contribute to the gut health (Pagnini et al., 2009; Gartz et al., 2010). The word "probiotic" was first used by Lilly and Stillwell in 1965 to address substances secreted by microorganisms that can stimulate the growth of other microorganisms (Fuller, 1992). In 1971, Sperti described probiotics as extracts that can stimulate microbial growth, as opposed to antibiotics that can prevent microbial growth. In 1974, Parker defined probiotics as organisms and substances which contribute to intestinal microbial balance (Fuller, 1992), and the addition of 'substances' in the definition included 'antibiotics.' Therefore, Fuller in 1989 modified the definition of probiotics as live microbial feed supplements which beneficially affect the host animal by improving intestinal microbial balance (Fuller, 1992). After several modifications, the definition of probiotics has been defined currently as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Rijkers et al., 2010; Lew and Liong, 2013).

Nowadays consumers are concerned about the presence of antibiotic resistant bacteria, and residues in the meat products and eggs. Therefore, probiotics have emerged as an alternative to antibiotics in poultry industry globally eliminating the use of chemicals, pesticides, and herbicides (Reid and Robert 2002; Apata et al., 2009). Various mechanisms are suggested for the efficacy of probiotics in poultry. For example, Edens et al. (2003) suggested that probiotics can potentially modulate metabolism, conserve

energy utilization, stimulate host immunity, competitively exclude and kill pathogens, enhance nutrient uptake from the intestinal tract, improve host performance, and ultimately resulting in reduced contamination of meat with enteric pathogens. In addition, use of probiotics in poultry may potentially stimulate immunity and boost performance (Dhama et al., 2011; Kabir et al., 2009). Moreover, probiotics can create a physical barrier in the intestinal tract against pathogen attachment, increase host digestive enzyme activity, decrease digestive enzyme activity in pathogens, reduce ammonia production, neutralize enterotoxins produced by pathogens, improve nutrient uptake and host performance, maintain integrity of bone, and effective against intestinal parasites (Kral, 2012; Khan et al., 2013).

Ideal probiotics will be good colonizers in the host intestines. The organisms have to go through different segments of the gastrointestinal tract to result in efficient colonization. During this transit, they will have to encounter the low pH prevailing in the gastric environment, detergent properties of bile in the small intestine, possess bile salt hydrolase activity, and finally, adhere to the intestinal epithelium (Schillinger et al., 2005). Moreover, the probiotic bacteria should possess antibacterial activity against harmful pathogens and do not invade the host epithelial cells (Owusu-Kwarteng et al., 2015). Furthermore, these organisms should be susceptible to conventional antibiotics used for human therapy.

We observed that turkey-derived *Lactobacillus* isolates could significantly reduce the

colonization and invasion of MDR *Salmonella enterica* serovar Heidelberg in turkey poults (Chapter 2). Therefore, in this chapter, our objective was to determine the probiotic qualities of the *Lactobacillus* isolates to encounter the stressors in the gut environment and ultimately perform antimicrobial activity against *Salmonella enterica* serovar Heidelberg *in vitro*.

3.2. Materials and methods

3.2.1. Probiotic isolates, growth conditions, and inoculum preparation

Probiotic (*Lactobacillus*) isolates were kindly provided by Dr. Timothy Johnson, Veterinary and Biomedical Sciences, University of Minnesota. The two isolates, *Lactobacillus salivarius,* and *Lactobacillus ingluviei* were lab adapted by continuously sub-culturing aerobically at 37 °C, and bacterial enumeration on deMan Rogosa Sharpe (MRS; Criterion, Hardy Diagnostics, CA) agar after aerobic incubation at 37° C for 48 h. *Lactobacillus salivarius* and *Lactobacillus ingluviei* cultures were grown overnight separately in 500mL MRS broth at 37 °C, aerobically. Bacterial enumeration of overnight cultures was done by serially diluting (1:10) in PBS, and plating 0.1 mL of corresponding dilutions on MRS plates, and allowing to incubate at 37°C for 48h. Bacterial cultures were then subjected to centrifugation at 4000g, 15m, and 4° C. The button formed for each probiotic was reconstituted in PBS (pH 7.2) and used as inoculum (Kollanoor Johny et al., 2013).

3.2.2. Determination of resistance to low pH

A fresh overnight culture (18 h) of either *Lactobacillus salivarius* or *Lactobacillus ingluviei* grown in 10 mL MRS broth (n=12) was centrifuged at 4000 x g, 15 min, 4° C (Allegra 15X Beckmann Coulter). The button formed was washed twice using PBS buffer (pH 7.2), and was resuspended (n=6) in 10 mL PBS buffer having a pH=2.5 (adjusted using 0.1N HCl) to simulate acidic conditions in the stomach (Farner 1942), and also in PBS buffer having a pH=7.2 (n=6), as control. The resuspended *Lactobacillus* were then allowed to incubate anaerobically (5% CO_2) at 41°C for 3.5h, the approximate time the feed spends in the poultry alimentary tract (Mateos et al., 1982). Viable bacterial colonies were enumerated at 0 h and after 3.5 h, after serial dilution (1:10), and plating 0.1 mL of corresponding dilutions on MRS agar plates, and incubation at 37°C aerobically for 48h (Owusu-Kwarteng et al., 2015).

3.2.3. Determination of resistance to bile salt

A fresh overnight culture (18 h) of either *Lactobacillus salivarius* or *Lactobacillus ingluviei* grown in 10 mL MRS broth (n=12) was centrifuged at 4000 x g, 15 min, 4 °C. The button formed was washed twice using PBS buffer (pH 7.2), and was resuspended in 2 mL PBS. From this, 0.5 mL of resuspended bacteria was taken, and inoculated into 5 mL of MRS containing 0.3% bile salt (n=6). The pH was adjusted to 8 using 1N NaOH.

MRS tubes without added bile salt pH 7.2 (n=6) were kept as negative controls. The resuspended bacteria were then allowed to grow anaerobically (5% CO_2) at 41° C for 3.5h, the time for the transit of feed through the gut (Mateos et al., 1982). Viable bacterial colonies were enumerated at 0 h and after 3.5 h, after serial dilution (1:10) and plating 0.1 mL of corresponding dilutions on MRS agar plates, and incubated at 37°C aerobically for 48h (Owusu-Kwarteng et al., 2015).

3.2.4. Determination of bile salt hydrolase activity

Overnight *Lactobacillus* (18 h) cultures were streaked on MRS agar plates (n=6) that contained 0.5% (w/v) taurodeoxycholic acid. Normal MRS agar plates were set as controls (n=6). The plates were then allowed to incubate anaerobically (5% CO_2) at 41°C for 48h. Bile salt hydrolase activity was identified by different colony morphology in treatment plates when compared to the control plates. *Listeria monocytogenes* having proven bile salt hydrolase activity, streaked on 0.5% (w/v) tauro deoxycholic acid was kept as a positive control (Owusu-Kwarteng et al., 2015).

3.2.5. Determination of hemolytic activity

Overnight *Lactobacillus* (18 h) cultures were streaked on blood agar plates (n=6) made from Columbia CNA (Colistin + NA, C-CNA, Criterion, Hardy Diagnostics, CA) agar mixed with 5% (w/v) defibrinated turkey blood (Rockland Immunological, PA).

Normal blood agar plates (n=6) streaked with MRS broth were set as control. The plates were then allowed to incubate anaerobically (5% CO_2) at 41 °C for 48h, and examined for β-hemolysis (clear zones around colonies), α-hemolysis (greenish discoloration) and γ-hemolysis (no zones around colonies). *Streptococcus pyogenes* that possess β-hemolytic activity when streaked on blood agar were kept as positive controls (Owusu-Kwarteng et al., 2015).

3.2.6. Determination of antimicrobial activity

Lactobacillus cultures grown for 72 hours aerobically at 37° C were centrifuged at 4000 x g, 15 min, 4°C, and filter sterilized using a 0.22-micrometer filter (Corning Incorporated, Corning, NY) to separate supernatants. The cell- free culture supernatants (CFCS) were screened for inhibitory activity against different pathogenic *Salmonella* serotypes such as *Salmonella enterica* serovar Heidelberg, *Salmonella enterica* serovar Typhimurium, and *Salmonella enterica* serovar Enteritidis. Briefly, CFCS at various concentrations, 5%, 10%, 15%, and 20%, were added to 2mL tryptic soy broth (n=6) taken in 6-well cell culture plates and the pH was recorded. Controls were set for each concentration of treatments with 2mL tryptic soy broth (n=6) after adjusting the pH to the corresponding treatment groups with 1N HCl. An initial inoculum of 10^5 CFU/ml of each *Salmonella* serotype was added into the treatment wells. Optical density reading was taken at 610nm at 0h and 24h after incubating anaerobically in 5% CO_2 at 41° C (Owusu-Kwarteng et al., 2015).

3.2.7. Determination of antibiotic susceptibility

The antibiotic susceptibility of the *Lactobacillus* isolates was tested against some of the common antibiotics such as Amoxicillin, Ceftiofur, Clindamycin, Enrofloxacin, Erythromycin, Florfenicol, Gentamicin, Neomycin, Novobiocin, Oxytetracycline, Penicillin, Spectinomycin, Streptomycin, Sulfadimethoxine, Sulphathiazole, Tetracycline, Trimethoprim/Sulphamethoxazole, and Tylosin by using Sensititre avian plates according to the Clinical Laboratory Standard Institute's (CLSI) guidelines. This experiment was carried out at the Veterinary Diagnostic Laboratory, University of Minnesota.

3.2.8. Cell culture

3.2.8.1. Avian epithelial cell line

A permanent avian epithelial cell line obtained from the abdominal tumor cells known as Budgerigar abdominal tumor cells (BATC) was used for cell culture studies. BATCs were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FBS). The cells were propagated three times, and were seeded into wells of 24-well tissue culture plates added with 1 mL whole medium (DMEM + 10% FBS) so that a final concentration of 1x 10^5 cells/ well was attained. The tissue culture plates were incubated at 37 °C with 5% CO_2 for 48h to reach

a confluence > 95%. The cell viability was checked using trypan vital dye exclusion assay. In short, before each experiment, 50μL of the diluted BATC suspension was mixed with 50μL of trypan blue vital dye and 10uL was loaded in counting chambers of a hemocytometer. The nonviable cells are indicated by blue cells and counted under the low- power objective of a light microscope after one minute (Kollanoor Johny et al., 2012a, b; Nair and Kollanoor-Johny, 2016; Nair et al., 2016).

3.2.8.2. Epithelial association assay

The study was conducted to determine the adherence ability of *Lactobacillus* to the avian intestinal epithelium. *Lactobacillus ingluviei* and *Lactobacillus salivarius* were grown separately for 2 to 3 generations in MRS broth at 37 °C in 5% CO_2 with shaking (100rpm) to obtain a final concentration of 10^9 CFU/mL and used to inoculate the BATC. Briefly, the BATC cell line grown in 1 mL DMEM were inoculated with 10^9 CFU/mL of each *Lactobacillus* separately and incubated for 1 h at 37 °C under 5% CO_2 for attachment. The control and treatment wells were added with 0.1% Triton-X and allowed to incubate at 37◦C for 15 minutes. The total number of *Lactobacillus* adhered to the BATC were enumerated after plating cell homogenates on MRS agar, and incubating at 37◦C for 48h (Kollanoor Johny et al., 2012a, b; Nair and Kollanoor-Johny, 2016; Nair et al., 2016).

3.2.9. Statistical analysis

Differences between groups were analyzed using one-way ANOVA, and $P < 0.05$ was considered statistically significant.

3.3. Results

3.3.1 Resistance to low pH

Both *Lactobacillus salivarius* and *Lactobacillus ingluviei* showed resistance to pH = 2.5 for 3.5h. For *Lactobacillus salivarius*, the survival rate obtained after 3.5h of exposure was 54.96% (Figure 1), whereas for *Lactobacillus ingluviei*, the survival rate obtained was 75.62% (Figure 2), compared to their respective growth at pH = 7.2 which were 86.26%, and 96.56%, respectively.

3.3.2. Resistance to bile salt

An ideal probiotic must pass through the small intestine with an alkaline pH and the secreted bile before reaching the cecum. The detergent properties of bile salt can damage the bacterial cell membrane. Therefore, resistance to bile salt is considered as a probiotic quality. In our study, the resistance against a bile salt concentration of 0.3% in MRS at pH of 8 was determined for the 3.5 h duration. *Lactobacillus salivarius* showed a survival

rate of 86.79% (Figure 3), and *Lactobacillus ingluviei* showed 88.20% survival rate (Figure 4), compared to their respective survival rates of 105.90% and 106.06%, respectively (Figures 3 & 4).

3.3.3. Bile salt hydrolase activity

Both isolates did not show the zone of white precipitation indicative of the absence of bile salt hydrolase activity. In the positive control plates, *Listeria monocytogenes* showed white opaque colonies indicative of bile salt hydrolase activity (Figure 12).

3.3.4. Hemolytic activity

The isolates did not show β-hemolytic activity.

3.3.5. Antibiotic susceptibility

Minimum inhibitory concentration (MIC) of *Lactobacillus salivarius* and *Lactobacillus ingluviei* against common antibiotics was determined using Sensititre plates (Thermo Scientific) as per CLSI guidelines, and the results are shown in Table 1. *Lactobacillus salivarius* was found to be susceptible to common antibiotics used in poultry such as enrofloxacin, erythromycin, florfenicol, gentamicin, neomycin, novobiocin, and Trimethoprim / Sulphamethoxazole combination. Similarly,

Lactobacillus ingluviei was susceptible to florfenicol, gentamicin, neomycin, and Trimethoprim / Sulphamethoxazole combination (Table 1).

3.3.6. Antimicrobial property

The CFCS of *Lactobacillus salivarius* and *Lactobacillus ingluviei* were tested against *Salmonella enterica* serovar Heidelberg, *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis at different concentrations such as 5%, 10%, 15% and 20%. The CFCS of *Lactobacillus salivarius* inhibited *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Heidelberg starting at 5% (Figures 5 & 6) whereas, 10% CFCS inhibited *Salmonella enterica* serovar Typhimurium (Figure 7). Similar results were obtained with *Lactobacillus ingluviei* CFCS against the tested pathogens (Figures 8, 9 & 10).

3.3.7. Epithelial association

The results indicated that both *Lactobacillus* isolates adhered to the BATCs. *Lactobacillus salivarius* attached to the epithelium at 52.3% (initial inoculum at 8.69 log10 CFU/ml and recovery at 4.55 log10 CFU/ml), whereas, *Lactobacillus ingluviei* adhered at 69.9% (initial inoculum at 8.69 log10 CFU/ml and recovery at 6.07 log10 CFU/ml) (Figure 11).

3.4. Discussion

In the previous chapter, we discussed that the combination of *Lactobacillus ingluviei* and *Lactobacillus salivarius* reduced MDR *Salmonella enterica* serovar Heidelberg colonization in the turkey poult cecum in two of the three experiments conducted. This reduction indicated that the isolates have excellent potential to colonize the cecum resulting in the competitive exclusion of the incoming pathogen. Although turkey gut-derived, determination of the probiotic potential of the two *Lactobacillus* isolates in simulated gut environments *in vitro* would support the hypothesis that these isolates could be developed as probiotics in turkeys. However, any potential probiotic strain will face a series of physiological challenges until efficient colonization in the cecum occurs, once administered orally. These obstacles include the low acid environment in the gizzard, and the detergent action of bile and bile salts (Ehrmann et al., 2002), before its colonization in the lower part of the intestine. The pH of turkey gastrointestinal tract ranges from 6.07 in the crop to 5.86 in the ceca with the highest pH of 6.39 reported in the ileum (Farner 1942). The lowest pH of 2.19 had been reported in the gizzard (Farner 1942). The average passage rate of ingesta in poultry digestive tract is 3 to 3.5h (Mateos et al., 1982). In this study, we found that *Lactobacillus salivarius* and *Lactobacillus ingluviei*, when exposed to a low pH of 2.5 for 3.5h, could retain the survivability of 54.96% and 75.62%, respectively (Figures 1 & 2).

The bile acids synthesized in the liver are released into the small intestine (Myant and

Mitropoulos 1977) and possess detergent properties detrimental to the survival of microorganisms as their membrane is made up of lipids and fatty acids (Erkkila and Petaja 2000). Resistance towards a concentration of 0.3% bile salt is critical to identify the tolerant and potential probiotic Lactobacilli (Erkkila and Petaja 2000). Both isolates used in the current study showed survival rates of 86.79% and 88.20% (Figures 3 & 4), respectively, at pH=8, indicating their potential to resist the detergent action of bile. However, the isolates did not show bile salt hydrolase activity, a desirable quality of a potential probiotic isolate (Figure 12). Bile salt hydrolase activity of the isolates on the nutrient (MRS) agar is dependent mainly on two factors such as possession of enzymes responsible for bile salt hydrolysis and the capability of acidifying the medium to add hydrogen ion to the liberated bile acids (Dashkevicz and Scott 1989). It has been previously described that some strains of the same *Lactobacillus* may not show bile salt hydrolase activity (Dashkevicz and Scott 1989) and does not rule out the isolates under the current study to select for other probiotic qualities.

Once these proximal gut obstacles are successfully encountered, the strain should have the ability to colonize strongly to the intestinal epithelium for performing colonization resistance against invading pathogens. In this process, potential probiotic strains will induce strong antimicrobial property. Our cell culture studies indicated that the tested *Lactobacillus* isolates had high adhesion to model avian intestinal epithelial cells (Figure 11), validating the *in vivo* studies. Moreover, the CFCSs of *Lactobacillus salivarius* and *Lactobacillus ingluviei* showed high antimicrobial activity separately against three major

serotypes of *Salmonella*, namely, *Salmonella enterica* serovar Enteritidis, *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Heidelberg. A similar observation was made by Stern et al. (2006) that a bacteriocin isolated from the cell-free extract of *Lactobacillus salivarius* strain NRRL B-30514 inhibited the growth of *Campylobacter jejuni* in the chicken GIT. One of the characteristics of *Lactobacillus* is the reduction of colonization of pathogenic bacteria in the gut. The reduction is achieved by immune stimulation, an alteration in the cecal microbial environment, and production of inhibitory metabolites (Neal-McKinney et al., 2012). The great diversity of metabolites secreted by the probiotic *Lactobacillus* such as peroxides, lactic acid, diacetyl, carbon dioxide, biogenic amines, slime, and bacteriocins can act as preservatives, sensory enhancers, and inhibit the growth of pathogenic bacteria (Harimurti and Widodo 2015, Jin et al., 1997, Holzapfel et al., 1995). The *Lactobacillus* has the capability of producing significant amounts of lactic acid that can reduce pH and are therefore detrimental to other bacteria (Jernigan et al., 1985). Also, macrophage augmentation activity was also detected by CFCS's of *Lactobacillus* (Hatcher and Randall 1993), indicating potential recruitment of the body's immune system in pathogen inhibition.

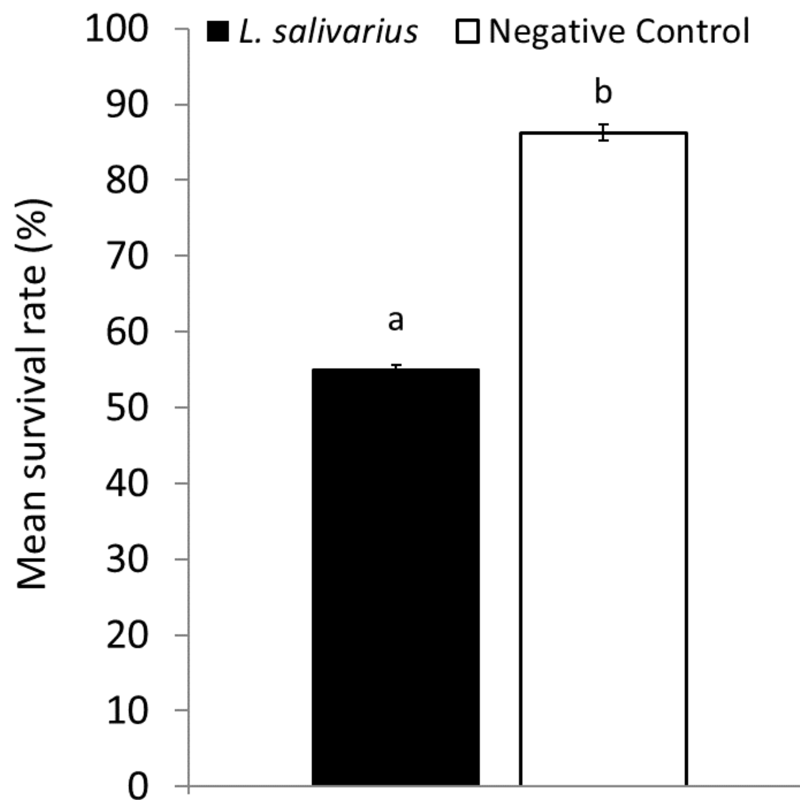
We also tested the antibiotic susceptibility of the potential *Lactobacillus* isolates (Table 1). *Lactobacillus salivarius* was sensitive to a variety of antibiotics. Primarily, susceptibility to antibiotics depends upon the *Lactobacillus* spp. (Danielsen and Wind, 2003). Also, several studies (William et al., 2001; Katla et al., 2001) suggest natural intrinsic resistance present in *Lactobacillus.* Since the resistance genes are

chromosomally integrated, they cannot be horizontally transferred to other organisms. For example, some Lactobacilli were found to possess high intrinsic resistance against bacitracin, cefoxitin, ciprofloxacin, fusidic acid, gentamicin kanamycin, metronidazole, norfloxacin, nitrofurantoin, streptomycin, teicoplanin, sulphadiazine, vancomycin and trimethoprim/sulphamethoxazole (Danielsen and Wind 2003; Mathur and Rameshwar 2005). This property of *Lactobacillus* strains could be beneficial that their population won't be affected even when antibiotics are used to treat pathogens in the gut during disease situations.

In conclusion, in this study, we observed that the tested turkey gut-derived *Lactobacillus* isolates have good qualities to be developed as probiotics in turkey production. These isolates were resistant to low pH, and bile salts, adhered very well to the avian intestinal epithelial cells, and exhibited high antimicrobial activity against major *Salmonella* serotypes encountered in turkey production. Moreover, these observations validate our finding in the previous study that a combination of *Lactobacillus salivarius* and *Lactobacillus ingluviei* reduced MDR *Salmonella enterica* serovar Heidelberg colonization and organ invasion in turkey poults.

Figure. 1

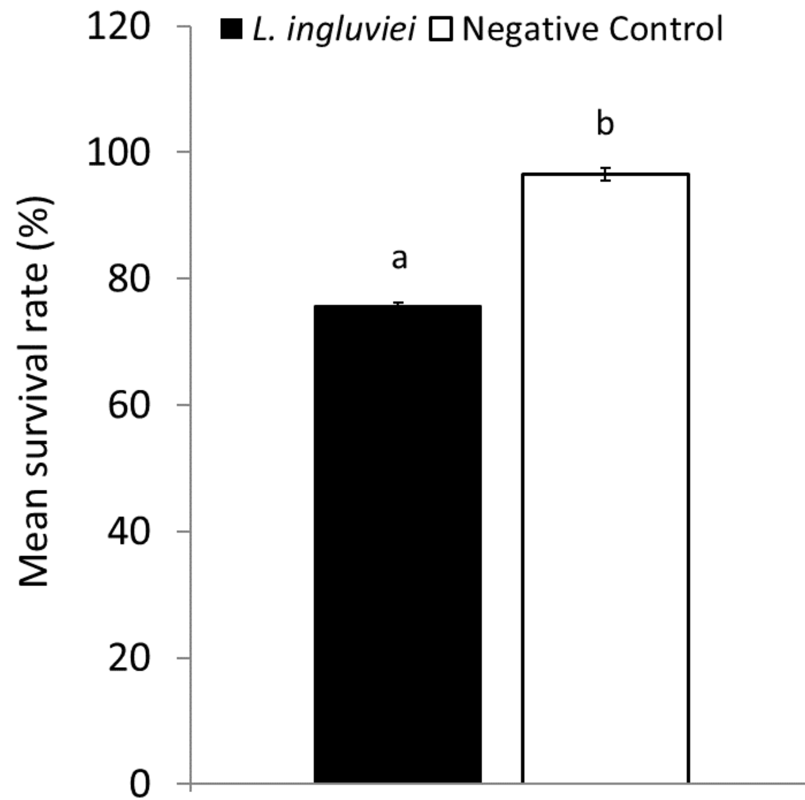
Effect of low pH on the survival of *Lactobacillus salivarius* *



*Different superscripts indicate that the treatments were significantly different from each other at P<0.05 (n=6).

Figure. 2

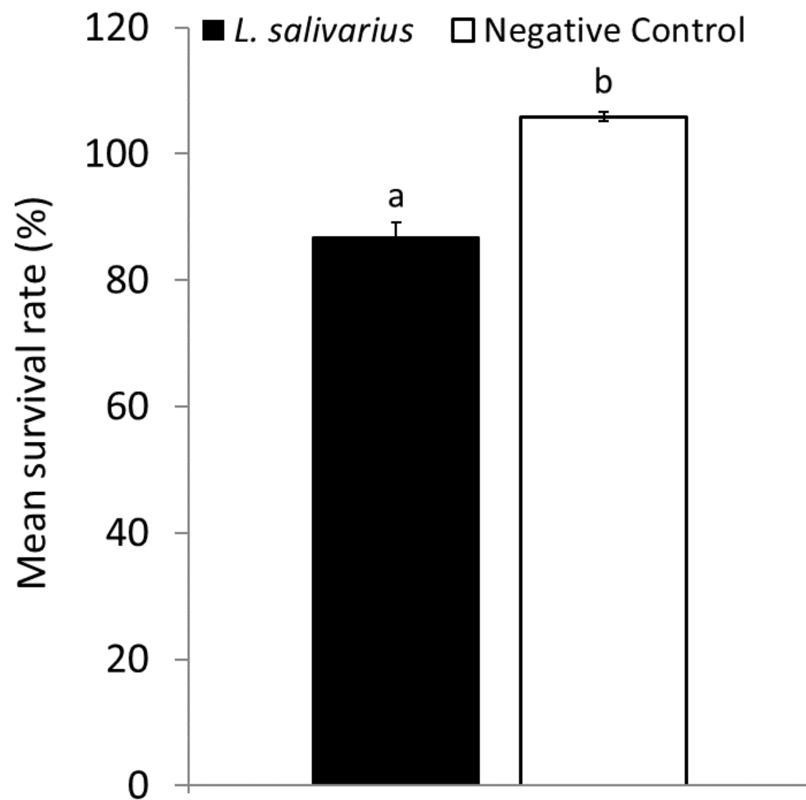
Effect of low pH on the survival of *Lactobacillus ingluviei* *



*Different superscripts indicate that the treatments were significantly different from each other at P<0.05 (n=6).

Figure. 3

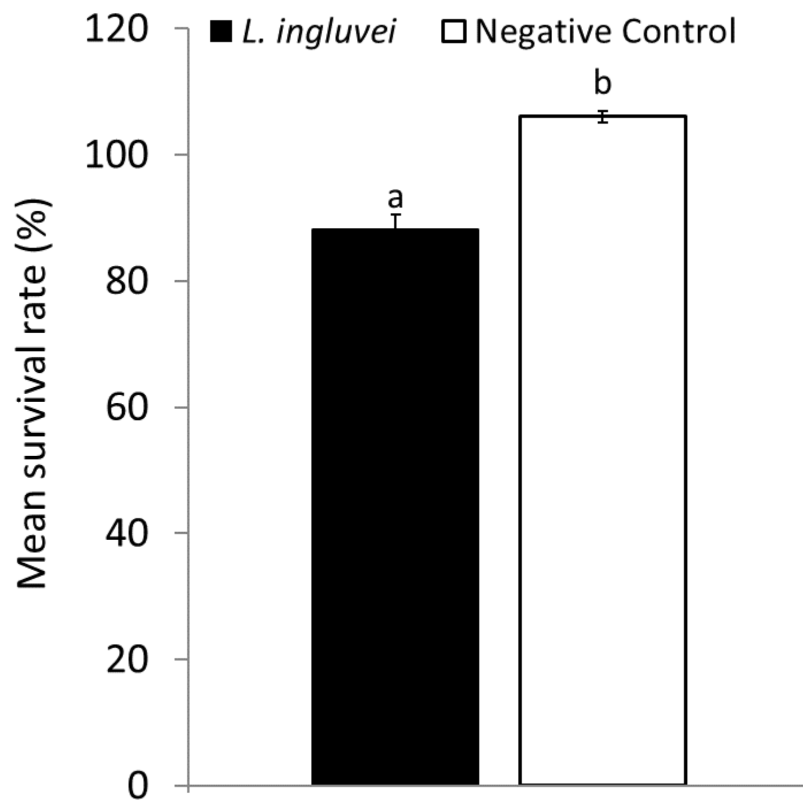
Effect of 0.3% bile salt on the survival of *Lactobacillus salivarius* *



*Different superscripts indicate that the treatments were significantly different from each other at P<0.05 (n=6).

Figure. 4

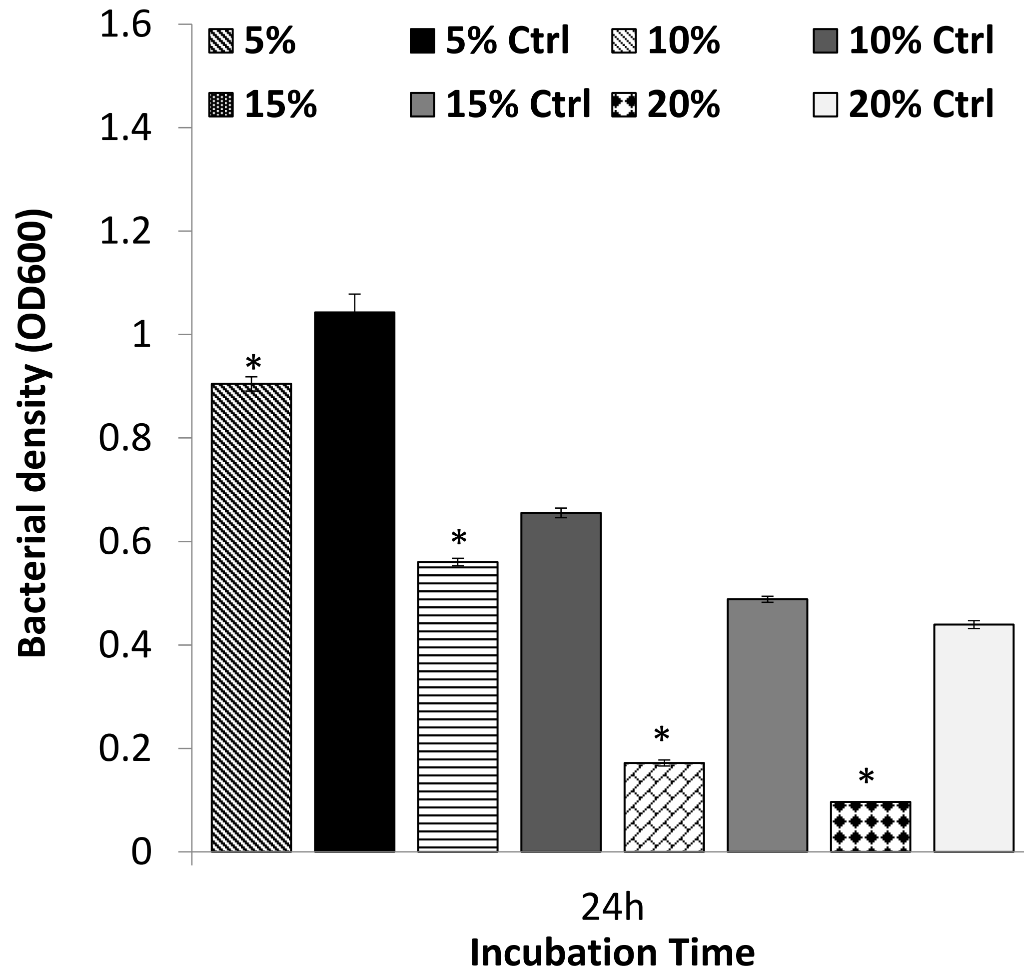
Effect of 0.3% bile salt on the survival of *Lactobacillus ingluviei* *



*Different superscripts indicate that the treatments were significantly different from each other at $P<0.05$ (n=6).

Figure. 5

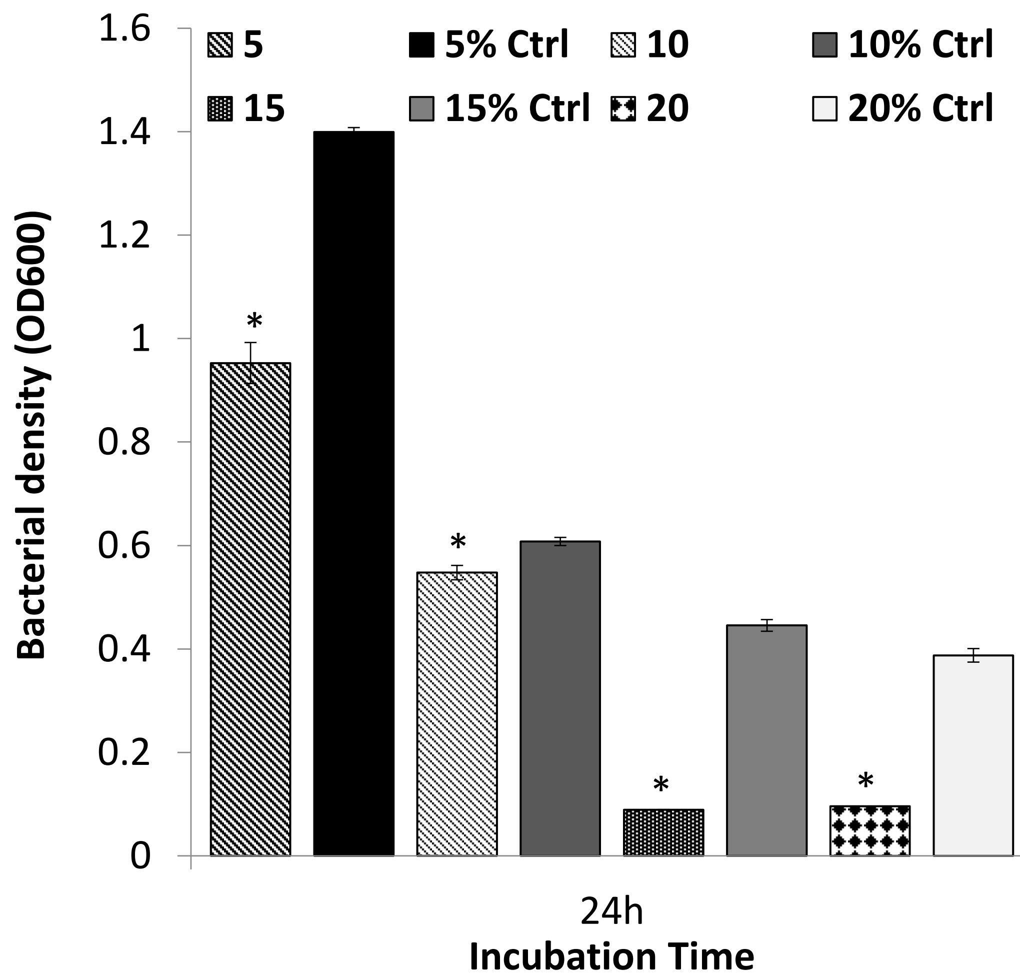
Effect of *Lactobacillus salivarius* cell-free extracts on *Salmonella enterica* serovar Enteritidis at 41ºC



*Treatment significantly different from the respective control neutralized for pH at P<0.05 (n=6)

Figure. 6

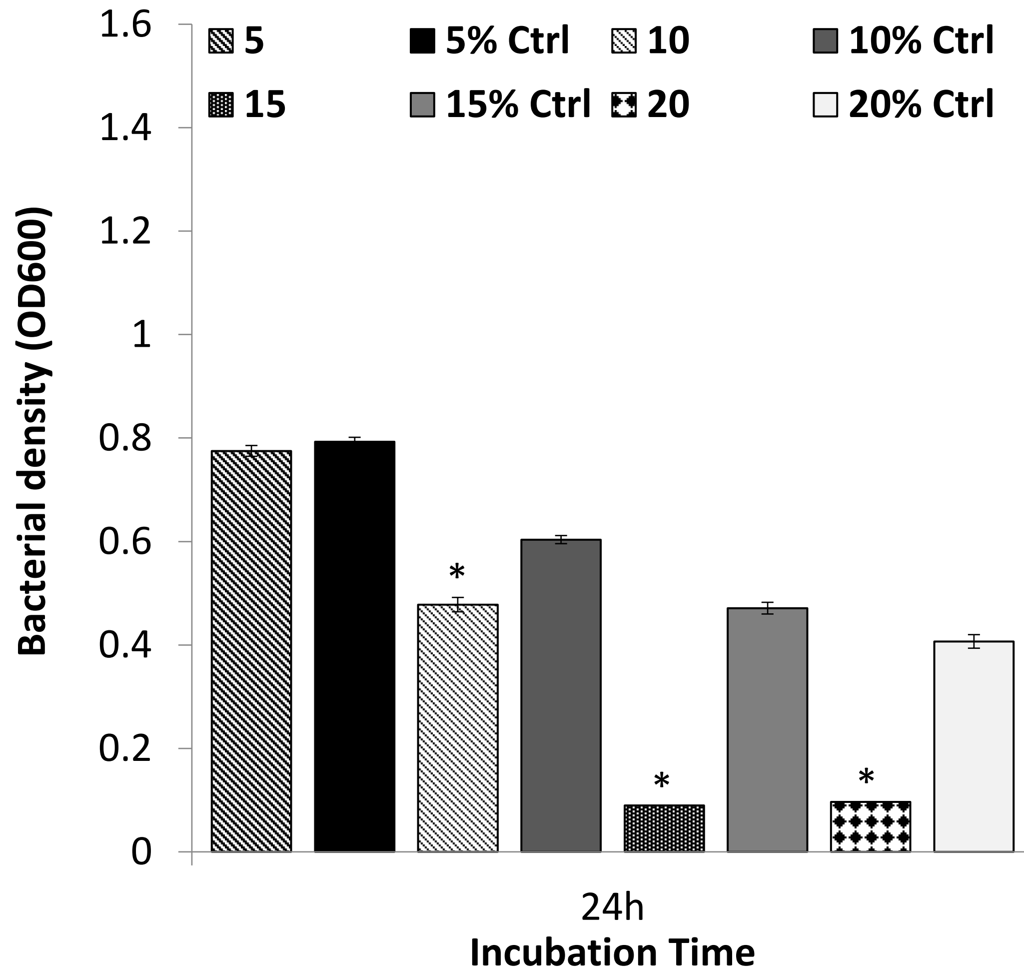
Effect of *Lactobacillus salivarius* cell-free extracts on *Salmonella enterica* serovar Heidelberg at 41ºC



*Treatment significantly different from the respective control neutralized for pH at $P<0.05$ (n=6)

Figure. 7

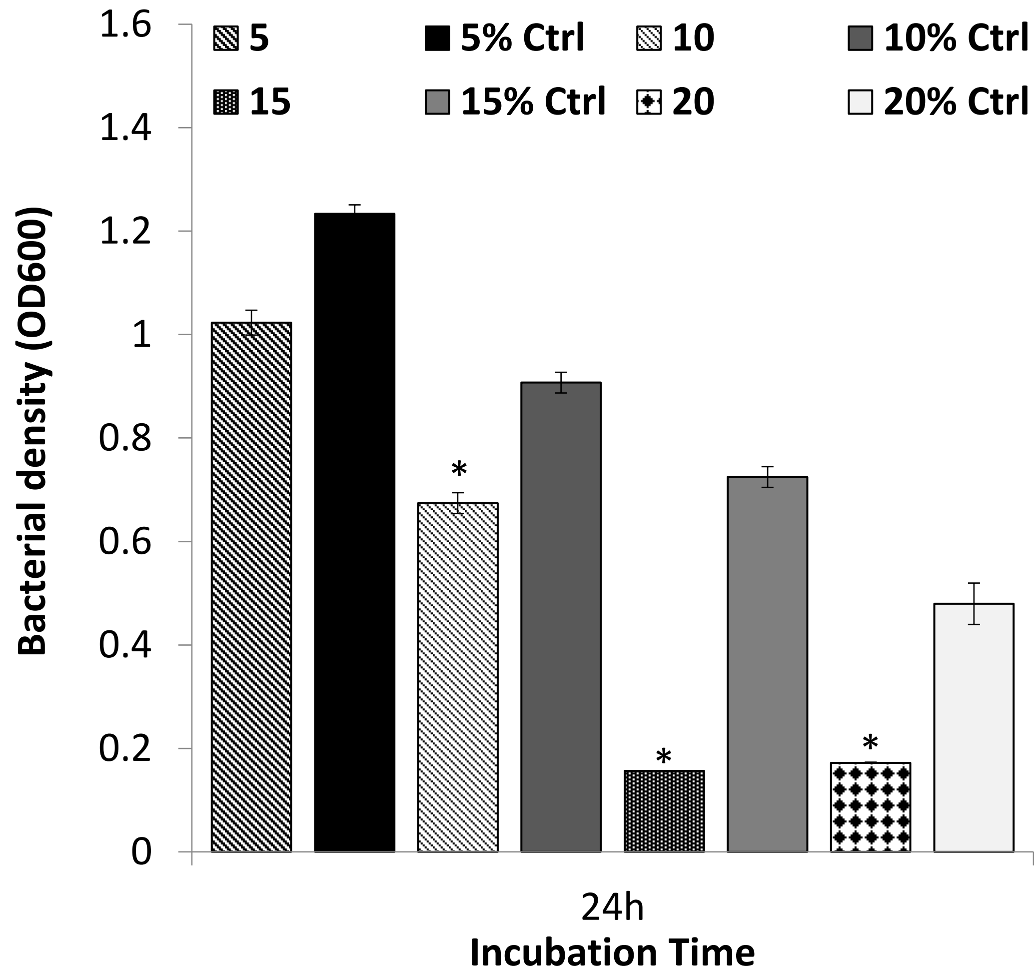
Effect of *Lactobacillus salivarius* cell-free extracts on *Salmonella enterica* serovar Typhimurium at 41ºC



*Treatment significantly different from the respective control neutralized for pH at P<0.05 (n=6)

Figure. 8

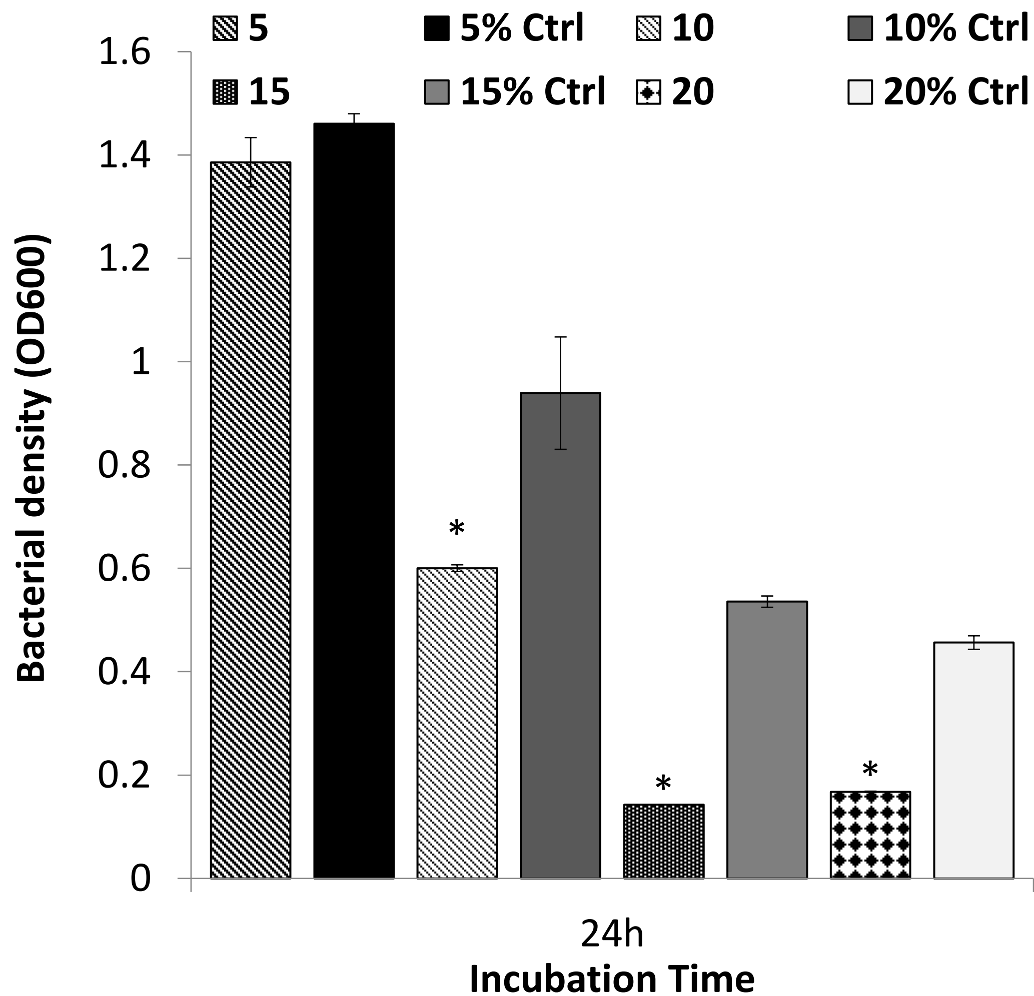
Effect of *Lactobacillus ingluviei* cell-free extracts on *Salmonella enterica* serovar Enteritidis at 41ºC



*Treatment significantly different from the respective control neutralized for pH at P<0.05 (n=6)

Figure. 9

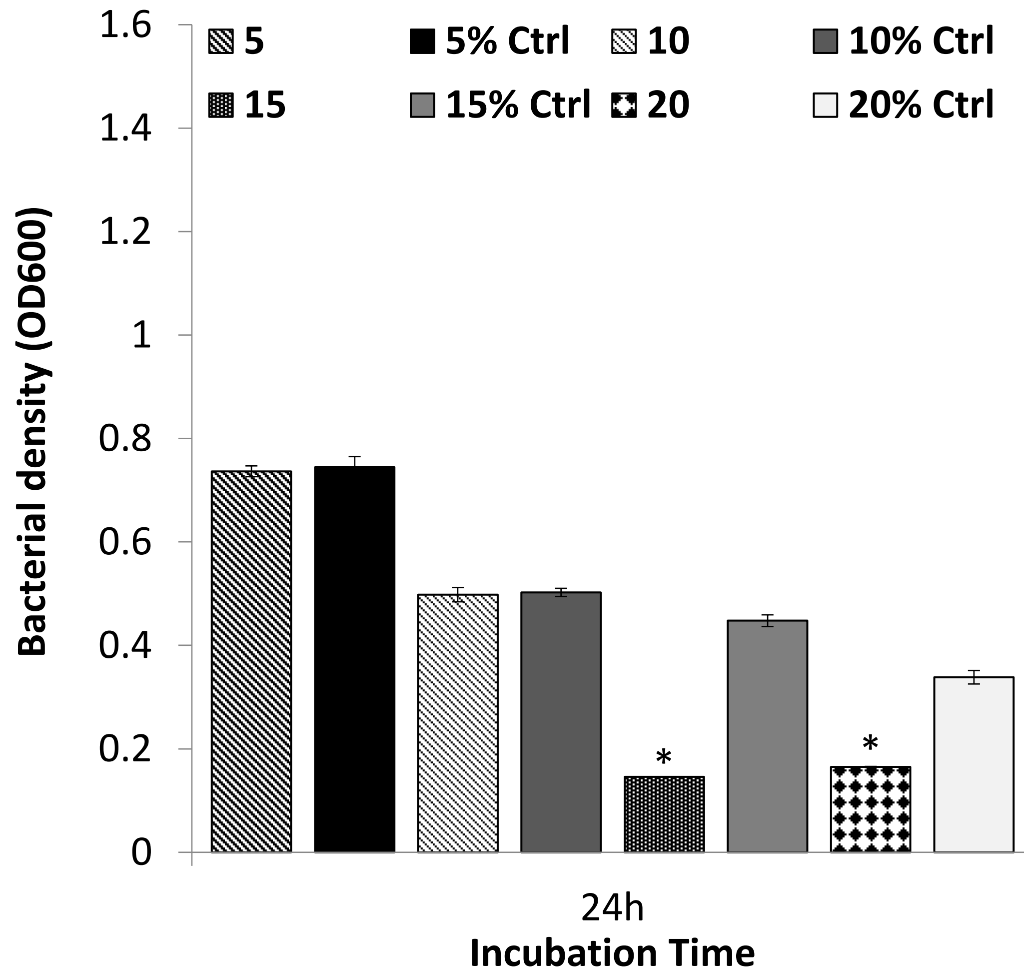
Effect of *Lactobacillus ingluviei* cell-free extracts on *Salmonella enterica* serovar Heidelberg at 41°C



*Treatment significantly different from the respective control neutralized for pH at $P<0.05$ (n=6)

Figure. 10

Effect of *Lactobacillus ingluviei* cell-free extracts on *Salmonella enterica* serovar Typhimurium at 41ºC



*Treatment significantly different from the respective control neutralized for pH at P<0.05 (n=6)

Figure. 11

Relative association of *Lactobacillus salivarius* and *Lactobacillus ingluviei* on BATCs

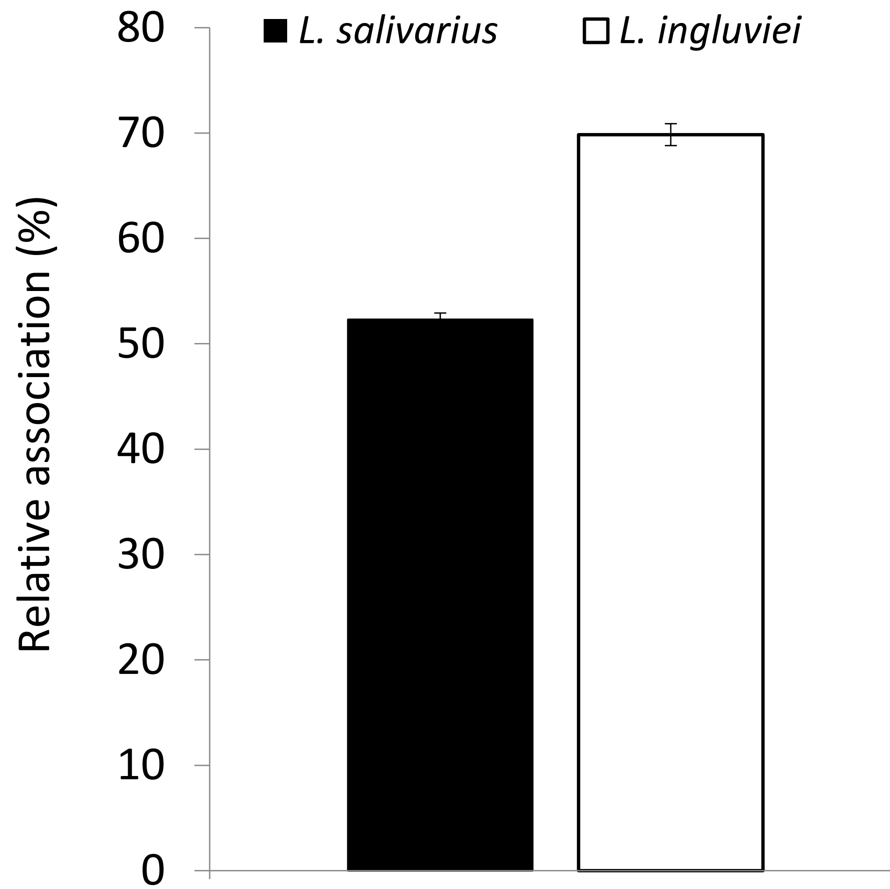
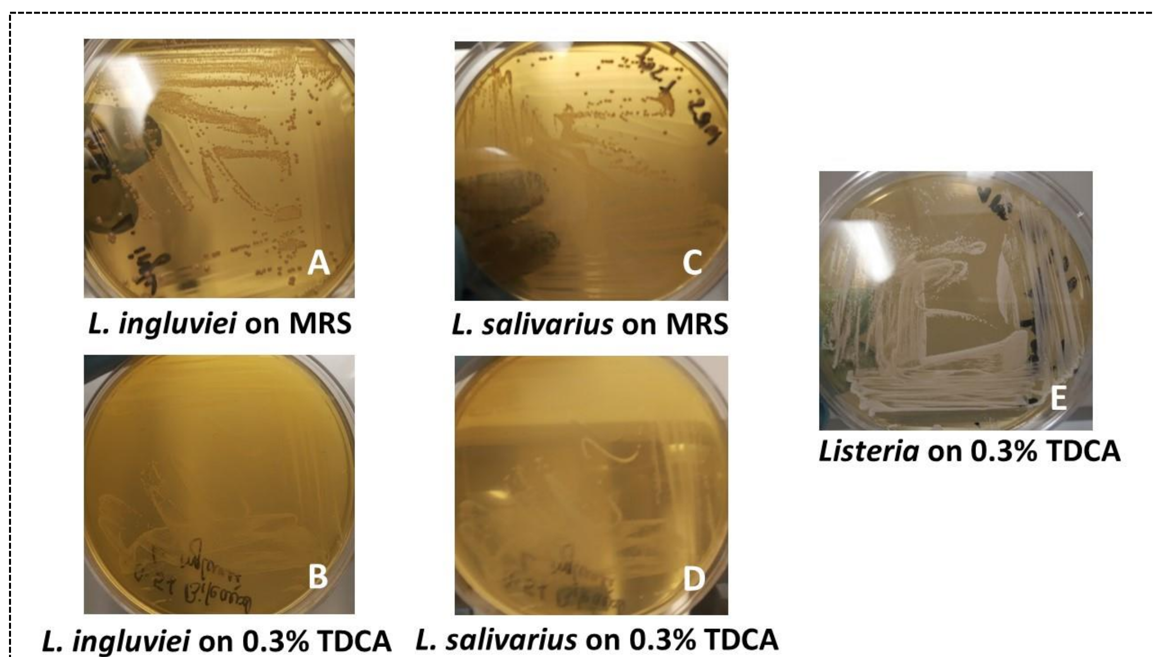


Figure. 12

Demonstration of bile salt hydrolase activity of the *Lactobacillus* isolates



A. *Lactobacillus ingluviei* on MRS agar showing normal growth with opaque colonies

B. *Lactobacillus ingluviei* on MRS + 0.3% taurodeoxycholic acid agar with no bile salt hydrolysis

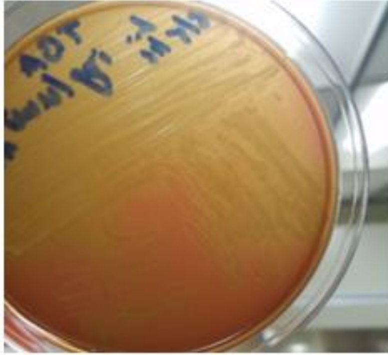
C. *Lactobacillus salivarius* on MRS agar showing normal growth with opaque colonies

D. *Lactobacillus salivarius* on MRS + 0.3% taurodeoxycholic acid agar with no bile salt hydrolysis

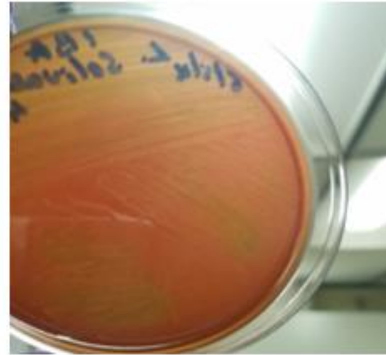
E. *Listeria monocytogenes* on MRS + 0.3% taurodeoxycholic acid agar with bile salt hydrolysis

Figure. 13

Demonstration of absence of β- hemolytic activity of the *Lactobacillus* isolates in Turkey blood agar



A. *L. Ingluviei* on Turkey blood agar



B. *L. Salivarius* on Turkey blood agar

C. Turkey blood agar

D. *S. pyogenes* on Turkey blood agar

A. *Lactobacillus ingluviei* on Turkey blood agar not showing β- hemolysis

B. *Lactobacillus salivarius* on Turkey blood agar not showing β- hemolysis

C. Normal Turkey blood agar

D. *Streptococcus pyogenes* on turkey blood agar showing β- hemolysis

Table. 1.

Minimum inhibitory concentration of 18 antibiotics against *Lactobacillus ingluviei and Lactobacillus salivarius* (The Clinical and Laboratory Standards Institute)

Antibiotic	***Lactobacillus salivarius*** (MIC- µg/ml)	***Lactobacillus ingluviei*** (MIC- µg/ml)
Amoxicillin	4.00	2.00
Clindamycin	2.00	4.00
Enrofloxacin	0.50	1.00
Erythromycin	0.12	1.00
Florfenicol	1.00	1.00
Gentamicin	0.50	0.50
Neomycin	2.00	2.00
Novobiocin	0.50	0.50
Oxytetracycline	8.00	8.00
Penicillin	8.00	8.00
Streptomycin	32.00	32.00
Sulphadimethoxine	256.00	256.00
Sulphathiazole	256.00	256.00
Tetracycline	8.00	8.00
Trimethoprim/Sulphamethoxazole	1.00	1.00

Literature cited

Agboola AF, Aroniyo I, Suberu SA, Adeyemi WT. 2014. Dietary supplementation of probiotics and synbiotics on intestinal microbial populations and gut morphology of turkey poults. Afr J of Liv Ext. 14(1):13-20. http://s3.amazonaws.com/academia.edu.documents/43200738/Agboola_gut_morphology-ANJOL.pdf?AWSAccessKeyId=AKIAIWOWYYGZ2Y53UL3A&Expires=1496302705&Signature=OdUoiJ1DsRuo%2B4O1crSvUaqmJvk%3D&response-content-disposition=inline%3B%20filename%3DDIETARY_SUPPLEMENTATION_OF_PROBIOTICS_AN.pdf

Agunos A, Ibuki M, Yokomizo F, Mine Y. 2007. Effect of dietary β 1–4 mannobiose in the prevention of *Salmonella enterica* serovar Enteritidis infection in broilers. Br Poult Sci. 48(3):331-41. http://www.tandfonline.com/doi/abs/10.1080/00071660701370442

Altekruse SF, Cohen ML, Swerdlow DL. Emerging foodborne diseases. 1997. Emerg Infect Diseases. 3(3):285. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2627632/

Andriulli A, Neri M, Loguercio C, Terreni N, Merla A, Cardarella MP, Federico A, Chilovi F, Milandri GL, De Bona M, Cavenati S. 2008. Clinical trial on the efficacy of a new symbiotic formulation, Flortec, in patients with irritable bowel syndrome: a multicenter, randomized study. J Clin Gastroenterol. 42:S218-23. http://journals.lww.com/jcge/Abstract/2008/09002/Clinical_Trial_on_the_Efficacy_of_a_New_Symbiotic.18.aspx

Antunes P, Réu C, Sousa JC, Peixe L, Pestana N. 2003. Incidence of *Salmonella* from poultry products and their susceptibility to antimicrobial agents. Int J Food Microbiol. 82(2):97-103. http://www.sciencedirect.com/science/article/pii/S0168160502002519

Apata, D. F. 2009. Antibiotic resistance in poultry. Int J Poult Sci 8. 404-408. http://scialert.net/abstract/?doi=ijps.2009.404.408

Awad WA, Ghareeb K, Abdel-Raheem S, Böhm J. 2009. Effects of dietary inclusion of probiotic and synbiotic on growth performance, organ weights, and intestinal histomorphology of broiler chickens. Poult Sci. 88(1):49-56. https://academic.oup.com/ps/article/88/1/49/1487480/Effects-of-dietary-inclusion-of-probiotic-and

Barnhart HM, Dreesen DW, Bastien R, Pancorbo OC. 1991. Prevalence of *Salmonella enterica* serovar Enteritidis and other serovars in ovaries of layer hens at time of slaughter. J Food Prot. 54 (7):488-91. http://www.jfoodprotection.org/doi/abs/10.4315/0362-028X-54.7.488?code=fopr-site

Barrow PA, Simpson JM, Lovell MA. 1998. Intestinal colonization in the chicken by food-poisoning *Salmonella* serotypes; Microbial characteristics associated with fecal

excretion. Avian Pathol. 17(3):571-88. http://www.tandfonline.com/doi/abs/10.1080/03079458808436478

Barton MD. 2000. Antibiotic use in animal feed and its impact on human health. Nutr Res Rev. 13(02):279-99. https://www.cambridge.org/core/services/aop-cambridge-core/content/view/S0954422400000767

Bogovič-Matijašić B, Rogelj I. 1998. Bacteriocin complex of *Lactobacillus acidophilus* LF221—production studies in MRS media at different pH values and effect against *Lactobacillus helveticus* ATCC 15009. Process Biochem. 33(3):345-52. http://www.sciencedirect.com/science/article/pii/S0032959297000733

Bohaychuk VM, Gensler GE, King RK, Manninen KI, Sorensen O, Wu JT, Stiles ME, McMullen LM. 2006. Occurrence of pathogens in raw and ready-to-eat meat and poultry products collected from the retail marketplace in Edmonton, Alberta, Canada. J Food Prot. 69(9):2176-82. http://jfoodprotection.org/doi/pdf/10.4315/0362-028X-69.9.2176

Bokanyi RP, Stephens JF, Foster DN. 1990. Isolation and characterization of *Salmonella* from broiler carcasses or parts. Poult Sci. 1990. 69(4):592-8. https://academic.oup.com/ps/article/69/4/592/1514401/Isolation-and-Characterization-of-Salmonella*Salmonella*-from

Bryan FL. 1980. Foodborne diseases in the United States associated with meat and poultry. J Food Prot. 43(2):140-50. http://jfoodprotection.org/doi/pdf/10.4315/0362-028X-43.2.140

Buenrostro JL, Kratzer FH. 1983. Effect of *Lactobacillus* inoculation and antibiotic feeding of chickens on availability of dietary biotin. Poult Sci. 62(10):2022-9. https://academic.oup.com/ps/article/62/10/2022/1591328/Effect-of-Lactobacillus*Lactobacillus*-Inoculation-and-Antibiotic

Byrd JA, Anderson RC, Callaway TR, Moore RW, Knape KD, Kubena LF, Ziprin RL, Nisbet DJ. 2003. Effect of experimental chlorate product administration in the drinking water on *Salmonella enterica* serovar Typhimurium contamination of broilers. Poult Sci. 82(9):1403-6. https://academic.oup.com/ps/article/82/9/1403/1537480/Effect-of-experimental-chlorate-product

Byrd JA, Hargis BM, Caldwell DJ, Bailey RH, Herron KL, McReynolds JL, Brewer RL, Anderson RC, Bischoff KM, Callaway TR, Kubena LF. 2001. Effect of lactic acid administration in the drinking water during pre-slaughter feed withdrawal on *Salmonella* and *Campylobacter* contamination of broilers. Poult Sci. 80(3):278-83. https://academic.oup.com/ps/article/80/3/278/1501686/Effect-of-Lactic-Acid-Administration-in-the

Bywater RJ. 2005. Identification and surveillance of antimicrobial resistance dissemination in animal production. Poult Sci. 84 (4):644-8. https://academic.oup.com/ps/article/84/4/644/1562303/Identification-and-surveillance-of-antimicrobial

Centers for Disease Control (CDC. 1986. *Salmonella enterica* serovar Heidelberg outbreak at a convention--New Mexico. MMWR. Morb Mortal Wkly Rep. 35 (6):91. https://www.cdc.gov/mmwr/preview/mmwrhtml/00000683.htm

Centers for Disease Control and Prevention (CDC). 2011. National *Salmonella* Surveillance Annual Summary, 2007. Atlanta, Georgia: US Department of Health and Human Services, CDC. https://www.cdc.gov/ncezid/dfwed/pdfs/salmonella*Salmonella*-annual-summary-2007-508c.pdf

Centers for Disease Control and Prevention (CDC). 2013. National *Salmonella* Surveillance Annual Report — Appendices, 2011. Atlanta, Georgia: US Department of Health and Human Services, CDC. https://www.cdc.gov/ncezid/dfwed/pdfs/salmonella*Salmonella*-annual-report-2011-508c.pdf

Centers for Disease Control and Prevention (CDC. 2013. Outbreak of *Salmonella enterica* serovar Heidelberg infections linked to a single poultry producer--13 states, 2012-2013.MMWR. Morb Mortal Wkly Rep. 62.27: 553. https://www.cdc.gov/mmwr/preview/mmwrhtml/mm6227a3.htm

Centers for Disease Control and Prevention. (CDC). 2015. Multistate outbreak of multidrug-resistant *Salmonella enterica* serovar Heidelberg infections linked to Foster Farms brand chicken (Final Update). https://www.cdc.gov/salmonella*Salmonella*/heidelberg-10-13/

Chadfield, M. S, and M. H. Hinton. 2003. Evaluation of treatment and prophylaxis with nitro furans and comparison with alternative antimicrobial agents in experimental *Salmonella enterica* serovar Enteritidis infection in chicks. Vet Res Commun. 27.4: 257-273. https://link.springer.com/article/10.1023%2FA%3A1024039506986?LI=true

Charteris WP, Kelly PM, Morelli L, Collins JK. 2001. Gradient diffusion antibiotic susceptibility testing of potentially probiotic lactobacilli. J Food Prot. 64(12):2007-14. http://www.jfoodprotection.org/doi/pdf/10.4315/0362-028X-64.12.2007

Chittick P, Sulka A, Tauxe RV, Fry AM. 2006. A summary of national reports of foodborne outbreaks of *Salmonella enterica* serovar Heidelberg infections in the United States: clues for disease prevention. J Food Prot. 69(5):1150-3. http://dx.doi.org/10.4315/0362-028X-69.5.1150

Chou LS, Weimer B. 1999. Isolation and Characterization of Acid-and Bile-Tolerant Isolates from Strains of *Lactobacillus acidophilus*1. J Dairy Sci. 82(1):23-31. http://www.sciencedirect.com/science/article/pii/S0022030299752045

Collado MC, Isolauri E, Salminen S, Sanz Y. 2009. The impact of probiotic on gut health. Curr Drug Metab. 10(1):68-78. http://www.ingentaconnect.com/content/ben/cdm/2009/00000010/00000001/art00008

Crump JA, Medalla FM, Joyce KW, Krueger AL, Hoekstra RM, Whichard JM, Barzilay EJ. 2011. Emerging Infections Program NARMS Working Group. Antimicrobial resistance among invasive non-typhoidal *Salmonella enterica* in the United States, national antimicrobial resistance monitoring system, 1996-2007. Antimicrob Agents and Chemother. http://aac.asm.org/content/early/2011/01/03/AAC.01333-10.short

Cui S, Ge B, Zheng J, Meng J. 2005. Prevalence and antimicrobial resistance of *Campylobacte*r spp. and *Salmonella* serovars in organic chickens from Maryland retail stores. Appl Environ Microbiol. 71(7):4108-11. http://aem.asm.org/content/71/7/4108.full

Currie A, MacDougall L, Aramini J, Gaulin C, Ahmed R, Isaacs S. 2005. Frozen chicken nuggets and strips and eggs are leading risk factors for *Salmonella enterica* serovar Heidelberg infections in Canada. Epidemiol Infect. 133(05):809-16. https://doi.org/10.1017/S0950268805004383

Danielsen M, Wind A. 2003. Susceptibility of *Lactobacillus* spp. to antimicrobial agents. Int J Food Microbiol. 82(1):1-1. http://www.sciencedirect.com/science/article/pii/S0168160502002544

Dashkevicz MP, Feighner SD. 1989. Development of a differential medium for bile salt hydrolase-active *Lactobacillus* spp. Appl Environ Microbiol. 55(1):11-6. http://aem.asm.org/content/55/1/11.full.pdf+html

de Arruda ISF, de Aguilar-Nascimento JE. 2004. Benefits of early enteral nutrition with glutamine and probiotics in brain injury patients. Clin Sci. 106(3):287-92. http://www.clinsci.org/content/106/3/287

Dhama K, Verma V, Sawant PM, Tiwari R, Vaid RK, Chauhan RS. 2011. Applications of probiotics in poultry: Enhancing immunity and beneficial effects on production performances and health: A review. J Immunol Immunopathol. 13(1):1-9. https://www.researchgate.net/profile/Kuldeep_Dhama/publication/229596530_Applications_of_Probiotics_in_Poultry_Enhancing_Immunity_and_Beneficial_Effects_on_Production_Performances_and_Health_-_A_Review/links/00b7d52e7439cd14d4000000.pdf

Dobson A, Cotter PD, Ross RP, Hill C. 2012. Bacteriocin production: a probiotic trait? Appl Environ Microbiol. 78(1):1-6. http://aem.asm.org/content/78/1/1.full

Domınguez C, Gomez I, Zumalacarregui J. 2002. Prevalence of *Salmonella* and *Campylobacter* in retail chicken meat in Spain. Int J Food Microbiol. 72(1):165-8. http://www.sciencedirect.com/science/article/pii/S0168160501006389

Donoghue DJ. 2003. Antibiotic residues in poultry tissues and eggs: human health concerns? Poult Sci. 82(4):618-21. https://academic.oup.com/ps/article/82/4/618/1502622/Antibiotic-residues-in-poultry-tissues-and-eggs

Dueger EL, House JK, Heithoff DM, Mahan MJ. 2003. *Salmonella* DNA adenine methylase mutants prevent colonization of newly hatched chickens by homologous and heterologous serovars. Int J Food Microbiol. 80(2):153-9. http://www.sciencedirect.com/science/article/pii/S0168160502001526

Dunlap NE, Benjamin WH, Berry AK, Eldridge JH, Briles DE. 1992. A ‘safe-site’ for *Salmonella enterica* serovar Typhimurium is within splenic polymorphonuclear cells. Microb Pathog. 13(3):181-90. http://www.sciencedirect.com/science/article/pii/088240109290019K

Dutil L, Irwin R, Finley R, Ng LK, Avery B, Boerlin P, Bourgault AM, Cole L, Daignault D, Desruisseau A, Demczuk W. 2010. Ceftiofur resistance in *Salmonella enterica* serovar Heidelberg from chicken meat and humans, Canada. Emerg Infect Diseases. 16(1):48. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2874360/

Edens FW. 2003. An alternative for antibiotic se in poultry: probiotics. Rev Bras de Ciênc Avíc. (2):75-97. http://www.scielo.br/scielo.php?pid=s1516-635x2003000200001&script=sci_arttext

Ehrmann MA, Kurzak P, Bauer J, Vogel RF. 2002. Characterization of lactobacilli towards their use as probiotic adjuncts in poultry. J Appl Microbiol. 92(5):966-75. http://onlinelibrary.wiley.com/doi/10.1046/j.1365-2672.2002.01608.x/full

Erkkilä S, Petäjä E. 2000. Screening of commercial meat starter cultures at low pH and in the presence of bile salts for potential probiotic use. Meat Sci. 55(3):297-300. http://www.sciencedirect.com/science/article/pii/S0309174099001564

Farner DS. 1942. The hydrogen ion concentration in avian digestive tracts. Poult Sci. 21(5):445-50. https://academic.oup.com/ps/article/21/5/445/1517785/The-Hydrogen-Ion-Concentration-in-Avian-Digestive

Fernandez F, Hinton M, Gils BV. 2002. Dietary mannan-oligosaccharides and their effect on chicken caecal microflora in relation to *Salmonella enterica* serovar Enteritidis colonization. Avian Pathol. 31(1):49-58. http://www.tandfonline.com/doi/abs/10.1080/03079450120106000

Fernandez F, Hinton M, Van Gils B. 2000. Evaluation of the effect of mannan-oligosaccharides on the competitive exclusion of *Salmonella enterica* serovar

Enteritidis colonization in broiler chicks. Avian Pathol. 29(6):575-81. http://www.tandfonline.com/doi/abs/10.1080/03079450020016823

Fiorentin L, Vieira ND, Barioni Jr W. 2005. Oral treatment with bacteriophages reduces the concentration of *Salmonella enterica* serovar Enteritidis PT4 in caecal contents of broilers. Avian Pathol. 34 (3):258-63. http://www.tandfonline.com/doi/abs/10.1080/01445340500112157

Foley SL, Lynne AM, Nayak R. 2008. Challenges: Prevalence in swine and poultry and potential pathogenicity of such isolates. J Anim Sci 86(14_suppl): E149-62. https://dl.sciencesocieties.org/publications/jas/abstracts/86/14_suppl/E149

Foley SL, Nayak R, Hanning IB, Johnson TJ, Han J, Ricke SC. 2011. Population dynamics of *Salmonella enterica* serotypes in commercial egg and poultry production. Appl Environ Microbiol. 77(13):4273-9. http://aem.asm.org/content/77/13/4273.short

Folster JP, Pecic G, Rickert R, Taylor J, Zhao S, Fedorka-Cray PJ, Whichard J, Mcdermott P. 2012. Characterization of multidrug-resistant *Salmonella enterica* serovar Heidelberg from a ground turkey-associated outbreak in the United States in 2011. Antimicrob Agents Chemother. 56(6):3465-6. http://aac.asm.org/content/56/6/3465.short

Food and Drug Administration Federal Register 2015. Veterinary Feed Directive. Accessed online at https://www.federalregister.gov/documents/2015/06/03/2015-13393/veterinary-feed-directive

Francis C, Janky DM, Arafa AS, Harms RH. 1978. Interrelationship of *Lactobacillus* and zinc bacitracin in the diets of turkey poults. Poult Sci. 57(6):1687-9. https://academic.oup.com/ps/article/57/6/1687/1569256/Interrelationship-of-Lactobacillus*Lactobacillus*-and-Zinc

Fujimori S, Tatsuguchi A, Gudis K, Kishida T, Mitsui K, Ehara A, Kobayashi T, Sekita Y, Seo T, Sakamoto C. 2007. High dose probiotic and prebiotic cotherapy for remission induction of active Crohn's disease. J Gastroenterol Hepatol. 22(8):1199-204. http://onlinelibrary.wiley.com/doi/10.1111/j.1440-1746.2006.04535.x/full

Fuller R, Brooker BE. 1974. Lactobacilli which attach to the crop epithelium of the fowl. Am J Clin Nutr. 27(11):1305-12. http://ajcn.nutrition.org/content/27/11/1305.short

Fuller R, Gibson GR. 1997. Modification of the intestinal microflora using probiotics and prebiotics. Scand J Gastroenterol. 32(sup222):28-31. http://www.tandfonline.com/doi/abs/10.1080/00365521.1997.11720714

Fuller R. History and development of probiotics. *In* Probiotics 1992 (pp. 1-8). Springer Netherlands.

Gaggìa F, Mattarelli P, Biavati B. 2010. Probiotics and prebiotics in animal feeding for safe food production. Int J Food Microbiol. 141:S15-28. http://www.sciencedirect.com/science/article/pii/S0168160510001121

Gantois I, Eeckhaut V, Pasmans F, Haesebrouck F, Ducatelle R, Van Immerseel F. 2008. A comparative study on the pathogenesis of egg contamination by different serotypes of *Salmonella*. Avian Pathol. 37(4):399-406.

Gast RK, Guard-Bouldin J, Holt PS. 2004. Colonization of reproductive organs and internal contamination of eggs after experimental infection of laying hens with *Salmonella enterica* serovar Heidelberg and *Salmonella enterica* serovar Enteritidis. Avian Dis. 48 (4):863-9. http://www.bioone.org/doi/abs/10.1637/7204-05050R

Gast RK, Guraya R, Guard-Bouldin J, Holt PS, Moore RW. 2007. Colonization of specific regions of the reproductive tract and deposition at different locations inside eggs laid by hens infected with *Salmonella enterica* serovar Enteritidis or *Salmonella enterica* serovar Heidelberg. Avian Dis. 51(1):40-4. http://www.bioone.org/doi/abs/10.1637/0005-2086(2007)051%5B0040:COSROT%5D2.0.CO%3B2

Gast RK, Guraya R, Guard-Bouldin J, Holt PS. 2007. In vitro penetration of egg yolks by *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Heidelberg strains during thirty-six-hour ambient temperature storage. Poult Sci. 86(7):1431-5. https://academic.oup.com/ps/article/86/7/1431/1623427/In-Vitro-Penetration-of-Egg-Yolks-by-*Salmonella*

Gast RK, Holt PS, Murase T. 2005. Penetration of *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Heidelberg into egg yolks in an in vitro contamination model. Poult Sci. 84 (4):621-5. https://academic.oup.com/ps/article/84/4/621/1562293/Penetration-of-Salmonella*Salmonella*-enteritidis-and

Gast RK, Holt PS. 1998. Persistence of *Salmonella enterica* serovar Enteritidis from one day of age until maturity in experimentally infected layer chickens. Poult Sci. 77(12):1759-62. https://academic.oup.com/ps/article/77/12/1759/1567650/Persistence-of-Salmonella-enteritidis-from-one-day

Gibson GR, Roberfroid MB. 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J Nutr. 125(6):1401. http://search.proquest.com/docview/197433031?pq-origsite=gscholar

Gibson GR. 1999. Dietary modulation of the human gut microflora using the prebiotics oligo fructose and inulin. J Nutr. 129(7):1438S-41s. http://jn.nutrition.org/content/129/7/1438S.full

Gratz SW, Mykkanen H, ElNezami HS. 2010. Probiotics and gut health: a special focus on liver diseases. World Journal of Gastroenterology. http://dx.doi.org/10.3748%2Fwjg.v16.i4.403

Guarner F, Malagelada JR. 2003. Gut flora in health and disease. The Lancet. 361 (9356):512-9. http://www.sciencedirect.com/science/article/pii/S0140673603124890

Gusils C, Chaia AP, Gonzalez S, Oliver G. 1999. Lactobacilli isolated from chicken intestines: potential use as probiotics. J Food Prot. 62(3):252-6. http://jfoodprotection.org/doi/abs/10.4315/0362-028X-62.3.252

Hajati H, Rezaei M. 2010. The application of prebiotics in poultry production. International Journal of Poult Sci. 9(3):298-304. http://docsdrive.com/pdfs/ansinet/ijps/2010/298-304.pdf

Harimurti S, Hadisaputro W. Probiotics in Poultry. 2015. *In* Beneficial Microorganisms in Agriculture, Aquaculture and Other Areas. (pp. 1-19). Springer International Publishing. https://link.springer.com/chapter/10.1007/978-3-319-23183-9_1#page-1

Hatcher GE, Lambrecht RS. 1993. Augmentation of macrophage phagocytic activity by cell-free extracts of selected lactic acid-producing bacteria. J Dairy Sci. 76(9):2485-92. http://www.sciencedirect.com/science/article/pii/S0022030293775839

Henker J, Laass MW, Blokhin BM, Maydannik VG, Bolbot YK, Elze M, Wolff C, Schreiner A, Schulze J. 2008. Probiotic *Escherichia coli* Nissle 1917 versus placebo for treating diarrhea of greater than 4 days duration in infants and toddlers. Pediatr Infect Disease J. 27(6):494-9. http://journals.lww.com/pidj/Abstract/2008/06000/Probiotic_Escherichia_coli_Nissle_1917_Versus.3.aspx

Hennessy TW, Cheng LH, Kassenborg H, Ahuja SD, Mohle-Boetani J, Marcus R, Shiferaw B, Angulo FJ. 2004. Emerging Infections Program FoodNet Working Group. Egg consumption is the principal risk factor for sporadic *Salmonella enterica* serotype Heidelberg infections: a case-control study in FoodNet sites. Clin Infect Dis. 2004. 38(Supplement 3):S237-43. https://academic.oup.com/cid/article/38/Supplement_3/S237/355332/Egg-Consumption-is-the-Principal-Risk-Factor-for

Heres L, Engel B, Urlings HA, Wagenaar JA, Van Knapen F. 2004. Effect of acidified feed on susceptibility of broiler chickens to intestinal infection by *Campylobacter* and *Salmonella*. Vet Microbiol. 99(3):259-67. http://www.sciencedirect.com/science/article/pii/S0378113504000136

Hidaka H, Eida T, Hamaya T, inventors; Meiji Seika Kaisha, Ltd., assignee. Livestock feed containing inulo-oligosaccharides and breeding of livestock by using the same. United States patent US 4,778,680. 1988 Oct 18. https://www.google.com/patents/US4778680

Higgins JP, Higgins SE, Vicente JL, Wolfenden AD, Tellez G, Hargis B. 2007. Temporal effects of lactic acid bacteria probiotic culture on *Salmonella* in neonatal broilers. Poult Sci. 86(8):1662-6. https://academic.oup.com/ps/article/86/8/1662/1521579/Temporal-Effects-of-Lactic-Acid-Bacteria-Probiotic

Higgins JP, Higgins SE, Wolfenden AD, Henderson SN, Torres-Rodriguez A, Vicente JL, Hargis BM, Tellez G. 2010. Effect of lactic acid bacteria probiotic culture treatment timing on *Salmonella enterica* serovar Enteritidis in neonatal broilers. Poult Sci. 89(2):243-7. https://academic.oup.com/ps/article/89/2/243/1547738/Effect-of-lactic-acid-bacteria-probiotic-culture

Higgins SE, Torres-Rodriguez A, Vicente JL, Sartor CD, Pixley CM, Nava GM, Tellez G, Barton JT, Hargis BM. 2005. Evaluation of intervention strategies for idiopathic diarrhea in commercial turkey brooding houses. J Appl Poult Res. 14(2):345-8. https://academic.oup.com/japr/article/14/2/345/696626/Evaluation-of-Intervention-Strategies-for

Holzapfel WH, Geisen R, Schillinger U. 1995. Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. Int J Food Microbiol. 24(3):343-62. http://www.sciencedirect.com/science/article/pii/0168160594000366

Humphrey T, Jørgensen F. 2006. Pathogens on meat and infection in animals–Establishing a relationship using *Campylobacter* and *Salmonella* as examples. Meat Sci. 74 (1):89-97. http://www.sciencedirect.com/science/article/pii/S0309174006001410

Iji PA, Saki AA, Tivey DR. 2001. Intestinal structure and function of broiler chickens on diets supplemented with a mannan oligosaccharide. J Sci Food Agr. 81 (12):1186-92. http://onlinelibrary.wiley.com/doi/10.1002/jsfa.925/full

Inoue AY, Berchieri Jr A, Bernardino A, Paiva JB, Sterzo EV. 2008. Passive immunity of progeny from broiler breeders vaccinated with oil-emulsion bacterin against *Salmonella enterica* serovar Enteritidis. Avian Dis. 52 (4):567-71. http://www.bioone.org/doi/abs/10.1637/8096-082707-Reg.1

Jackson BR, Griffin PM, Cole D, Walsh KA, Chai SJ. 2013. Outbreak-associated *Salmonella enterica* Serotypes and Food Commodities, United States, 1998–2008. Emerg Infect Dis. 19(8):1239-1244. https://dx.doi.org/10.3201/eid1908.121511

Jernigan MA, Miles RD, Arafa AS. 1985. Probiotics in poultry nutrition—a review. Worlds Poult Sci J. 41(02):99-107. https://www.cambridge.org/core/services/aop-cambridge-core/content/view/S0043933985000125

Jin LZ, Ho YW, Abdullah N, Ali MA, Jalaludin S. 1996. Antagonistic effects of intestinal *Lactobacillus* isolates on pathogens of chicken. Lett Appl Microbiol.

23(2):67-71. http://onlinelibrary.wiley.com/doi/10.1111/j.1472-765X.1996.tb00032.x/epdf

Jin LZ, Ho YW, Abdullah N, Jalaludin S. 1997. Probiotics in poultry: modes of action. Worlds Poult Sci J. 53(04):351-68. https://www.cambridge.org/core/services/aop-cambridge-core/content/view/S0043933997000366

Jin LZ, Ho YW, Ali MA, Abdullah N, Jalaludin S. 1996. Effect of adherent *Lactobacillus* spp. on in vitro adherence of *Salmonella* to the intestinal epithelial cells of chicken. J Appl Bacteriol. 81(2):201-6. http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2672.1996.tb04501.x/epdf

Johny AK, Baskaran SA, Charles AS, Amalaradjou MA, Darre MJ, Khan MI, Hoagland TA, Schreiber DT, Donoghue AM, Donoghue DJ. Venkitanarayanan K. 2009. Prophylactic supplementation of caprylic acid in feed reduces *Salmonella enterica* serovar Enteritidis colonization in commercial broiler chicks. J Food Prot. 72(4):722-7. http://jfoodprotection.org/doi/pdf/10.4315/0362-028X-72.4.722

Johny AK, Darre MJ, Donoghue AM, Donoghue DJ, Venkitanarayanan K. 2010b. Antibacterial effect of trans-cinnamaldehyde, eugenol, carvacrol, and thymol on *Salmonella enterica* serovar Enteritidis and *Campylobacter jejuni* in chicken cecal contents in vitro. J Appl Poult Res. 19(3):237-44. https://academic.oup.com/japr/article/19/3/237/708306/Antibacterial-effect-of-trans-cinnamaldehyde

Johny AK, Hoagland T, Venkitanarayanan K. 2010a. Effect of sub inhibitory concentrations of plant-derived molecules in increasing the sensitivity of multidrug-resistant *Salmonella enterica* serovar Typhimurium DT104 to antibiotics. Foodborne Pathog and Dis. 7(10):1165-70. http://online.liebertpub.com/doi/pdf/10.1089/fpd.2009.0527

Juven BJ, Cox NA, Bailey JS, Thomson JE, Charles OW, Shutze JV. 1984. Survival of *Salmonella* in dry food and feed. J Food Prot. 47(6):445-8. http://jfoodprotection.org/doi/pdf/10.4315/0362-028X-47.6.445

Katla AK, Kruse H, Johnsen G, Herikstad H. 2001. Antimicrobial susceptibility of starter culture bacteria used in Norwegian dairy products. Int J Food Microbiol. 67(1):147-52. http://www.sciencedirect.com/science/article/pii/S0168160500005225

Kermanshahi H, Rostami H. 2006. Influence of supplemental dried whey on broiler performance and cecal flora. Int. J. Poult. Sci. 5(6):538-43. http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.493.5923&rep=rep1&type=pdf

Khachatourians GG. 1998. Agricultural use of antibiotics and the evolution and transfer of antibiotic-resistant bacteria. CMAJ. 159(9):1129-36. http://www.cmaj.ca/content/159/9/1129.full.pdf+html

Khan RU, Naz S. 2013. The applications of probiotics in poultry production. Worlds Poult Sci J. 69 (03):621-32. https://www.cambridge.org/core/services/aop-cambridge-core/content/view/S0043933913000627

Khan SA, Stratford R, Wu T, Mckelvie N, Bellaby T, Hindle Z, Sinha KA, Eltze S, Mastroeni P, Pickard D, Dougan G. 2003. *Salmonella enterica* serovar Typhi and *Salmonella enterica* serovar Typhimurium derivatives harboring deletions in aromatic biosynthesis and *Salmonella* Pathogenicity Island-2 (SPI-2) genes as vaccines and vectors. Vaccine. 21(5):538-48. http://www.sciencedirect.com/science/article/pii/S0264410X02004103

Kim GB, Seo YM, Kim CH, Paik IK. 2011. Effect of dietary prebiotic supplementation on the performance, intestinal microflora, and immune response of broilers. Poult Sci. 90(1):75-82. https://academic.oup.com/ps/article/90/1/75/1512172/Effect-of-dietary-prebiotic-supplementation-on-the

Klaenhammer TR. 1998. Functional activities of *Lactobacillus* probiotics: genetic mandate. Int Dairy J. 8(5-6):497-505. http://www.sciencedirect.com/science/article/pii/S0958694698000764

Kollanoor-Johny A, Mattson T, Baskaran SA, Amalaradjou MA, Babapoor S, March B, Valipe S, Darre M, Hoagland T, Schreiber D, Khan MI. 2012b. Reduction of *Salmonella enterica* serovar Enteritidis colonization in 20-day-old broiler chickens by the plant-derived compounds trans-cinnamaldehyde and eugenol. Appl Environ Microbiol. 78(8):2981-7. http://aem.asm.org/content/78/8/2981.short

Kollanoor-Johny A, Mattson T, Baskaran SA, Amalaradjou MA, Hoagland TA, Darre MJ, Khan MI, Schreiber DT, Donoghue AM, Donoghue DJ, Venkitanarayanan K. 2012a. Caprylic acid reduces *Salmonella enterica* serovar Enteritidis populations in various segments of digestive tract and internal organs of 3-and 6-week-old broiler chickens, therapeutically. Poult Sci. 91(7):1686-94. https://academic.oup.com/ps/article/91/7/1686/1523352/Caprylic-acid-reduces-*Salmonella*-Enteritidis

Kollanoor-Johny A, Nair M, Venkitanarayanan K. 2013. Effect of probiotic supernatants on adhesion to and invasion of *Salmonella enterica* serovar Heidelberg isolates on avian abdominal epithelial cells, and virulence and antibiotic resistance genes. IFT Annual Mtg.

Král M, Angelovičová M, Mrázová Ľ. 2012. Application of probiotics in poultry production. SPASB. 45(1):55-7. http://spasb.ro/index.php/spasb/article/view/13

Kruis W, Frič P, Pokrotnieks J, Lukáš M, Fixa B, Kaščák M, Kamm MA, Weismueller J, Beglinger C, Stolte M, Wolff C. 2004. Maintaining remission of ulcerative colitis with the probiotic *Escherichia coli* Nissle 1917 is as effective as with standard mesalazine. Gut. 53(11):1617-23. http://gut.bmj.com/content/53/11/1617.short

Lammerding AM, Garcia MM, Mann ED, Robinson Y, Dorward WJ, Truscott RB, Tittiger F. 1988. Prevalence of *Salmonella* and thermophilic *Campylobacter* in fresh pork, beef, veal and poultry in Canada. J Food Prot. 51(1):47-52. http://jfoodprotection.org/doi/pdf/10.4315/0362-028X-51.1.47

Landers TF, Cohen B, Wittum TE, Larson EL. 2012. A review of antibiotic use in food animals: perspective, policy, and potential. Public health reports. 127(1):4-22. http://journals.sagepub.com/doi/pdf/10.1177/003335491212700103

Layton MC, Calliste SG, Gomez TM, Patton C, Brooks S. 1997. A mixed foodborne outbreak with *Salmonella enterica* serovar Heidelberg and *Campylobacter jejuni* in a nursing home. Infect Control Hosp Epidemiol. 18(02):115-21. http://www.jstor.org/stable/30142400#fndtn-references_tab_contents

Lew LC, Liong MT. 2013. Bioactives from probiotics for dermal health: functions and benefits. J Appl Microbiol. 114(5):1241-53. http://onlinelibrary.wiley.com/doi/10.1111/jam.12137/full

Licciardi PV, Wong SS, Tang ML, Karagiannis TC. 2010. Epigenome targeting by probiotic metabolites. Gut Pathog. 2(1):24. https://gutpathogens.biomedcentral.com/articles/10.1186/1757-4749-2-24

Llorente C, Schnabl B. 2016. Fast-Track Clearance of Bacteria from the Liver. Cell Host Microbe. 20(1):1-2. http://www.sciencedirect.com/science/article/pii/S1931312816302670

Logue CM, Sherwood JS, Olah PA, Elijah LM, Dockter MR. 2003. The incidence of antimicrobial-resistant *Salmonella* spp. on freshly processed poultry from US Midwestern processing plants. J Appl Microbiol. 94(1):16-24. http://onlinelibrary.wiley.com/doi/10.1046/j.1365-2672.2003.01815.x/full

Lorea Baroja M, Kirjavainen PV, Hekmat S, Reid G. 2007. Anti-inflammatory effects of probiotic yogurt in inflammatory bowel disease patients. Clin Exp Immunol. 149(3):470-9. http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2249.2007.03434.x/full

Marcus R, Varma JK, Medus C, Boothe EJ, Anderson BJ, Crume T, Fullerton KE, Moore MR, White PL, Lyszkowicz E, Voetsch AC. 2007. Re-assessment of risk factors for sporadic *Salmonella enterica* serotype Enteritidis infections: a case-control study in five FoodNet Sites, 2002–2003. Epidemiol Infect. 135(01):84-92. https://www.cambridge.org/core/services/aop-cambridge-core/content/view/S0950268806006558

Marshall BM, Levy SB. 2011. Food animals and antimicrobials: impacts on human health. Clin Microbiol Rev. 24(4):718-33. http://cmr.asm.org/content/24/4/718.full

Mateos GG, Sell JL, Eastwood JA. 1982. Rate of food passage (transit time) as influenced by level of supplemental fat. Poult Sci. 61(1):94-100.

https://academic.oup.com/ps/article/61/1/94/1505407/Rate-of-Food-Passage-Transit-Time-as-Influenced-by

Mathur S, Singh R. 2005. Antibiotic resistance in food lactic acid bacteria—a review. Int J Food Microbiol. 105(3):281-95. http://www.sciencedirect.com/science/article/pii/S0168160505002618

Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, Griffin PM, Tauxe RV. 1999. Food-related illness and death in the United States. Emerg Infect Diseases. 5(5):607. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2627714/pdf/10511517.pdf

Menconi A, Wolfenden AD, Shivaramaiah S, Terraes JC, Urbano T, Kuttel J, Kremer C, Hargis BM, Tellez G. 2011. Effect of lactic acid bacteria probiotic culture for the treatment of *Salmonella enterica* serovar Heidelberg in neonatal broiler chickens and turkey poults. Poult Sci. 90(3):561-5. https://academic.oup.com/ps/article/90/3/561/1588733/Effect-of-lactic-acid-bacteria-probiotic-culture

Methner U, Barrow PA, Martin G, Meyer H. 1997. Comparative study of the protective effect against *Salmonella* colonization in newly hatched SPF chickens using live, attenuated *Salmonella* vaccine strains, wild-type *Salmonella* strains or a competitive exclusion product. Int J Food Microbiol. 35(3):223-30. http://www.sciencedirect.com/science/article/pii/S0168160596012366

Myant NB, Mitropoulos KA. 1977. Cholesterol 7a-hydroxylase. J. Lipid Res. 18: 135-153. http://www.jlr.org/content/18/2/135.full.pdf

Nair DV, Kollanoor-Johny A. 2016. Effect of *Propionibacterium freudenreichii* on *Salmonella* multiplication, motility, and association with avian epithelial cells. Poult Sci. pew367. https://academic.oup.com/ps/article/96/5/1376/2282772/Effect-of-Propionibacterium-freudenreichii-on

Nair DV, Kollanoor-Johny A. Effect of *Propionibacterium freudenreichii* on *Salmonella* multiplication, motility, and association with avian epithelial cells. Poult Sci. 2016 Oct 12:pew367. https://academic.oup.com/ps/article/96/5/1376/2282772/Effect-of-Propionibacterium-freudenreichii-on

Nair DVT, Thomas JV, Johny AK. 2016. *Propionibacterium freudenreichii* reduces cecal colonization of multidrug-resistant *Salmonella enterica* serovar Heidelberg in turkey poults. Abstr Poult Sci. Abstr 64 E-Suppl. 1. P 21. http://www.poultryscience.org/psa16/abstracts/20.pdf

Nava GM, Bielke LR, Callaway TR, Castaneda MP. 2005. Probiotic alternatives to reduce gastrointestinal infections: the poultry experience. Anim Health Res Rev. 6(01):105-18. https://www.cambridge.org/core/services/aop-cambridge-core/content/view/S1466252305000071

Neal-McKinney JM, Lu X, Duong T, Larson CL, Call DR, Shah DH, Konkel ME. 2012. Production of organic acids by probiotic lactobacilli can be used to reduce pathogen load in poultry. PLoS One. 7(9):e43928. http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0043928

Nisbet DJ, Corrier DE, Scanlan CM, Hollister AG, Beier RC, DeLoach JR. 1993. Effect of a defined continuous-flow derived bacterial culture and dietary lactose on *Salmonella enterica* serovar Typhimurium colonization in broiler chickens. Avian Dis. 1017-25. http://www.jstor.org/stable/1591908?seq=2#page_scan_tab_contents

Olaimat AN, Holley RA. 2015. Control of *Salmonella* on fresh chicken breasts by κ-carrageenan/chitosan-based coatings containing allyl isothiocyanate or deodorized Oriental mustard extract plus EDTA. Food Microbiol. 48:83-8. http://www.sciencedirect.com/science/article/pii/S0740002014003086

Olsen SJ, Bishop R, Brenner FW, Roels TH, Bean N, Tauxe RV, Slutsker L. 2001. The changing epidemiology of *Salmonella*: trends in serotypes isolated from humans in the United States, 1987–1997. J Infect Dis. 183(5):753-61. https://academic.oup.com/jid/article/183/5/753/891160/The-Changing-Epidemiology-of *Salmonella*-Trends-in

O'mahony M, Cowden J, Smyth B, Lynch D, Hall M, Rowe B, Teare EL, Tettmar RE, Rampling AM, Coles M, Gilbert RJ. 1990. An outbreak of *Salmonella enterica* serovar Saintpaul infection associated with beansprouts. Epidemiol Infect. 104(02):229-35. https://www.cambridge.org/core/services/aop-cambridge-core/content/view/S0950268800059392

Owusu-Kwarteng J, Tano-Debrah K, Akabanda F, Jespersen L. 2015. Technological properties and probiotic potential of *Lactobacillus fermentum* strains isolated from West African fermented millet dough. BMC Microbiol. 15(1):261. https://bmcmicrobiol.biomedcentral.com/articles/10.1186/s12866-015-0602-6

Pagnini C, Saeed R, Bamias G, Arseneau KO, Pizarro TT, Cominelli F. 2010. Probiotics promote gut health through stimulation of epithelial innate immunity. Proceedings of the national academy of sciences. 107(1):454-9. http://www.pnas.org/content/107/1/454.full

Parvez S, Malik KA, Ah Kang S, Kim HY. 2006. Probiotics and their fermented food products are beneficial for health. J Appl Microbiol. 100(6):1171-85. http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2672.2006.02963.x/full

Pascual M, Hugas M, Badiola JI, Monfort JM, Garriga M. 1999. *Lactobacillus salivarius* CTC2197 prevents *Salmonella enterica* serovar Enteritidis colonization in chickens. Appl Environ Microbiol. 65(11):4981-6. http://aem.asm.org/content/65/11/4981.full

Patterson JA, Burkholder KM. 2003. Application of prebiotics and probiotics in poultry production. Poult Sci. 82(4):627-31.

https://academic.oup.com/ps/article/82/4/627/1502639/Application-of-prebiotics-and-probiotics-in

Rabsch W, Altier C, Tschäpe H, Baumler AJ. 2008. Microbial Food Safety in Animal Agriculture: Current Topics. *In* Blackwell Publishing. 97-108. http://onlinelibrary.wiley.com/doi/10.1002/9780470752616.ch12/pdf

Rahimi S, Grimes JL, Fletcher O, Oviedo E, Sheldon BW. 2009. Effect of a direct-fed microbial (Primalac) on structure and ultrastructure of small intestine in turkey poults. Poult Sci. 88(3):491-503. https://academic.oup.com/ps/article/88/3/491/1511785/Effect-of-a-direct-fed-microbial-Primalac-on

Rantala M, Nurmi E. 1973 Prevention of the growth of *Salmonella enterica* serovar Infantis in chicks by the flora of the alimentary tract of chickens. British Poult Sci. 14(6):627-630. http://www.tandfonline.com/doi/abs/10.1080/00071667308416073

Revolledo L, Ferreira CS, Ferreira AJ. 2009. Prevention of *Salmonella enterica* serovar Typhimurium colonization and organ invasion by combination treatment in broiler chicks. Poult Sci. 88(4):734-43. https://academic.oup.com/ps/article/88/4/734/1557478/Prevention-of-*Salmonella*-Typhimurium-colonization

Rijkers GT, Bengmark S, Enck P, Haller D, Herz U, Kalliomaki M, Kudo S, Lenoir-Wijnkoop I, Mercenier A, Myllyluoma E, Rabot S. 2010. Guidance for substantiating the evidence for beneficial effects of probiotics: current status and recommendations for future research. J Nutr. 140(3):671S-6S. http://jn.nutrition.org/content/140/3/671S.short

Roberts T. 1998. Salmonellosis control: estimated economic costs. Poult Sci. 67(6):936-43. https://academic.oup.com/ps/article/67/6/936/1509227/Salmonellosis-Control-Estimated-Economic-Costs1

Roy P, Dhillon AS, Lauerman LH, Schaberg DM, Bandli D, Johnson S. 2002. Results of*Salmonella* isolation from poultry products, poultry, poultry environment, and other characteristics. Avian Dis. 46(1):17-24. http://www.bioone.org/doi/abs/10.1637/0005-2086(2002)046%5B0017:ROSIFP%5D2.0.CO%3B2

Salminen S, Ouwehand A, Benno Y, Lee YK. 1999. Probiotics: how should they be defined? Trends Food Science Technol. 10(3):107-10. http://www.sciencedirect.com/science/article/pii/S0924224499000278

Scharff RL. 2012. Economic burden from health losses due to foodborne illness in the United States. J Food Prot. 75(1):123-31. http://jfoodprotection.org/doi/abs/10.4315/0362-028X.JFP-11-058?code=fopr-site

Schoeni JL, Glass KA, McDermott JL, Wong AC. 1995. Growth and penetration of *Salmonella enterica* serovar Enteritidis, *Salmonella enterica* serovar Heidelberg and

Salmonella enterica serovar Typhimurium in eggs. Int J Food Microbiol. 24(3):385-96. http://www.sciencedirect.com/science/article/pii/0168160594000425

Shea KM. 2003. Antibiotic resistance: What is the impact of agricultural uses of antibiotics on children's health? Pediatrics. 112(Supplement 1):253-8. http://pediatrics.aappublications.org/content/112/Supplement_1/253.full

Sinovec Z, Marković R. 2005. Use of prebiotics in poultry nutrition. Biotech Anim Husbandry. 21(5-6):235-9. http://www.doiserbia.nb.rs/img/doi/1450-9156/2005/1450-91560506235S.pdf

Slavin J. 2013. Fiber and prebiotics: mechanisms and health benefits. Nutrients. 5(4):1417-35. http://www.mdpi.com/2072-6643/5/4/1417/html

Snoeyenbos GH, Carlson VL, Smyser CF, Olesiuk OM. 1969. Dynamics of *Salmonella* infection in chicks reared on litter. Avian Dis. 72-83. http://www.jstor.org/stable/1588416?seq=1#page_scan_tab_contents

Snoeyenbos GH, Weinack OM, Smyser CF. 1978. Protecting chicks and poults from *Salmonella* by oral administration of normal gut microflora. Avian Dis. 273-87. http://www.jstor.org/stable/1589539?seq=1#page_scan_tab_contents

Spring P, Wenk C, Dawson KA, Newman KE. 2000. The effects of dietary mannanoligosaccharides on cecal parameters and the concentrations of enteric bacteria in the ceca of *Salmonella*-challenged broiler chicks. Poult Sci. 79(2):205-11. https://academic.oup.com/ps/article/79/2/205/1536315/The-effects-of-dietary-mannaoligosaccharides-on

St. Amand JA, Otto SJ, Cassis R, Annett Christianson CB. 2013. Antimicrobial resistance of *Salmonella enterica* serovar Heidelberg isolated from poultry in Alberta. Avian Pathol. 42(4):379-86. http://www.tandfonline.com/doi/abs/10.1080/03079457.2013.811465

Stamatova I, Meurman JH. 2009. Probiotics: health benefits in the mouth. Am J Dent. 22(6):329-8. https://www.researchgate.net/profile/Jukka_Meurman/publication/41547287_Probiotics_Health_benefits_in_the_mouth/links/55cde60d08ae6a881380957a.pdf

Stern NJ, Cox NA, Bailey JS, Berrang ME, Musgrove MT. 2001. Comparison of mucosal competitive exclusion and competitive exclusion treatment to reduce *Salmonella* and *Campylobacter* spp. colonization in broiler chickens. Poult Sci. 80(2):156-60. https://academic.oup.com/ps/article/80/2/156/1602843/Comparison-of-Mucosal-Competitive-Exclusion-and

Stern NJ, Svetoch EA, Eruslanov BV, Perelygin VV, Mitsevich EV, Mitsevich IP, Pokhilenko VD, Levchuk VP, Svetoch OE, Seal BS. 2006. Isolation of a *Lactobacillus salivarius* strain and purification of its bacteriocin, which is inhibitory

to *Campylobacter jejuni* in the chicken gastrointestinal system. Antimicrob Agents Chemother. 50(9):3111-6. http://aac.asm.org/content/50/9/3111.full

Stock K, Stolle A. 2001. Incidence of *Salmonella* in minced meat produced in a European Union–approved cutting plant. J Food Prot. 64(9):1435-8. http://jfoodprotection.org/doi/pdf/10.4315/0362-028X-64.9.1435

Swanson KS, Grieshop CM, Flickinger EA, Bauer LL, Healy HP, Dawson KA, Merchen NR, Fahey GC. 2002. Supplemental fructooligosaccharides and mannanoligosaccharides influence immune function, ileal and total tract nutrient digestibilities, microbial populations and concentrations of protein catabolites in the large bowel of dogs. J Nutr. 132(5):980-9. http://jn.nutrition.org/content/132/5/980.short

Tanaka H, Doesburg K, Iwasaki T, Mierau I. 1999. Screening of lactic acid bacteria for bile salt hydrolase activity. J Dairy Sci. 82(12):2530-5. http://www.sciencedirect.com/science/article/pii/S0022030299755062

Tauxe RV. 1991. *Salmonella*: A Postmodern Pathogen 1. J Food Prot. 54(7):563-8. http://www.jfoodprotection.org/doi/pdf/10.4315/0362-028X-54.7.563

Tellez G, Petrone VM, Escorcia M, Morishita TY, Cobb CW, Villaseñor L, Promsopone B. 2001. "Evaluation of avian-specific probiotic and *Salmonella enterica* serovar Enteritidis-, *Salmonella enterica* serovar Typhimurium-, and *Salmonella enterica* serovar Heidelberg-specific antibodies on cecal colonization and organ invasion of *Salmonella enterica* serovar Enteritidis in broilers." J Food Prot. 64(3) 287-291. https://www.ncbi.nlm.nih.gov/pubmed/11252468

Tellez G, Pixley C, Wolfenden RE, Layton SL, Hargis BM. 2012. Probiotics/direct fed microbials for *Salmonella* control in poultry. Food Res Int. 45(2):628-33. http://www.sciencedirect.com/science/article/pii/S0963996911002067

Torres-Rodriguez A, Donoghue AM, Donoghue DJ, Barton JT, Tellez G, Hargis BM. 2007. Performance and condemnation rate analysis of commercial turkey flocks treated with a *Lactobacillus* spp.-based probiotic. Poult Sci. 86(3):444-6. https://academic.oup.com/ps/article/86/3/444/1569722/Performance-and-Condemnation-Rate-Analysis-of

Tortuero F. 1973. Influence of the implantation of *Lactobacillus acidophilus* in chicks on the growth, feed conversion, malabsorption of fats syndrome and intestinal flora. Poult Sci. 52(1):197-203. https://academic.oup.com/ps/article/52/1/197/1560403/Influence-of-the-Implantation-of-*Lactobacillus*

Trampel DW, Holder TG, Gast RK. 2014. Integrated farm management to prevent *Salmonella enterica* serovar Enteritidis contamination of eggs. J Appl Poult Res.

japr944. https://academic.oup.com/japr/article/23/2/353/762531/Integrated-farm-management-to-prevent-Salmonella

Tuohy KM, Probert HM, Smejkal CW, Gibson GR. 2003. Using probiotics and prebiotics to improve gut health. Drug Discov Today. 8(15):692-700. http://www.sciencedirect.com/science/article/pii/S1359644603027466

Upadhyaya I. 2015. PhD thesis. University of Connecticut, Connecticut, CT. "Investigating the Efficacy of Natural Antimicrobial Molecules in Reducing Egg-borne Transmission of *Salmonella enterica* serovar Enteritidis in Layer Hens." http://digitalcommons.uconn.edu/cgi/viewcontent.cgi?article=6869&context=dissertations

Uyttendaele MR, Debevere JM, Lips RM, Neyts KD. 1998. Prevalence of *Salmonella* in poultry carcasses and their products in Belgium. Int J Food Microbiol. 40(1):1-8. http://www.sciencedirect.com/science/article/pii/S0168160598000129

Vahjen W, Jadamus A, Simon O. 2002. Influence of a probiotic *Enterococcus faecium* strain on selected bacterial groups in the small intestine of growing turkey poults. Arch Anim Nutr. 56(6):419-29. http://www.tandfonline.com/doi/abs/10.1080/00039420215634

Van Immerseel F, Cauwerts K, Devriese LA, Haesebrouck F, Ducatelle R. 2002. Feed additives to control *Salmonella* in poultry. Worlds Poult Sci Journal. 58(04):501-13. https://www.cambridge.org/core/services/aop-cambridge-core/content/view/S0043933902000363

Van Immerseel F, De Buck J, Pasmans F, Bohez L, Boyen F, Haesebrouck F, Ducatelle R. 2004. Intermittent long-term shedding and induction of carrier birds after infection of chicken early post hatch with a low or high dose of *Salmonella enterica* serovar Enteritidis. Poult Sci. 83(11):1911-6. https://academic.oup.com/ps/article/83/11/1911/1502509/Intermittent-long-term-shedding-and-induction-of

Verschuere L, Rombaut G, Sorgeloos P, Verstraete W. 2000. Probiotic bacteria as biological control agents in aquaculture. Microbiol Mol Biol Reviews. 64(4):655-71. http://mmbr.asm.org/content/64/4/655.full

Vicente JL, Torres-Rodriguez A, Higgins SE, Pixley C, Tellez G, Donoghue AM, Hargis BM. 2008. Effect of a Selected *Lactobacillus* spp.–Based Probiotic on *Salmonella enterica* Serovar Enteritidis–Infected Broiler Chicks. Avian Dis. 52(1):143-6. http://www.bioone.org/doi/abs/10.1637/7847-011107-ResNote

Waterman SR, Small PL. 1998. Acid-sensitive enteric pathogens are protected from killing under extremely acidic conditions of pH 2.5 when they are inoculated onto certain solid food sources. Appl Environ Microbiol. 64(10):3882-6. http://aem.asm.org/content/64/10/3882.full

Watkins BA, Miller BF, Neil DH. 1982. *In vivo* inhibitory effects of *Lactobacillus acidophilus* against pathogenic *Escherichia coli* in gnotobiotic chicks. Poult Sci. 61(7):1298-308. https://academic.oup.com/ps/article/61/7/1298/1518232/In-Vivo-Inhibitory-Effects-of-*Lactobacillus*

White DG, Zhao S, Sudler R, Ayers S, Friedman S, Chen S, McDermott PF, McDermott S, Wagner DD, Meng J. 2001. The isolation of antibiotic-resistant *Salmonella* from retail ground meats. N Engl J Med. 345(16):1147-54. http://www.nejm.org/doi/full/10.1056/nejmoa010315#t=article

Wickens K, Black P, Stanley TV, Mitchell E, Barthow C, Fitzharris P, Purdie G, Crane J. 2012. A protective effect of *Lactobacillus rhamnosus* HN001 against eczema in the first 2 years of life persists to age 4 years. Clin Exp Allergy. 42(7):1071-9. http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2222.2012.03975.x/full

Witte W. 1998. Medical consequences of antibiotic use in agriculture. Science. 279(5353):996-7. http://science.sciencemag.org/content/279/5353/996.full

Young VB. 2012. The Intestinal Microbiota in Health and Disease. Curr Opin Gastroenterol. 28(1):63-69. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3707308/

Zhao C, Ge B, De Villena J, Sudler R, Yeh E, Zhao S, White DG, Wagner D, Meng J. 2001. Prevalence of *Campylobacter* spp., *Escherichia coli*, and *Salmonella* serovars in retail chicken, turkey, pork, and beef from the Greater Washington, DC, area. Appl Environ Microbiol. 67(12):5431-6. http://aem.asm.org/content/67/12/5431.full

Zhao S, White DG, Friedman SL, Glenn A, Blickenstaff K, Ayers SL, Abbott JW, Hall-Robinson E, McDermott PF. 2008. Antimicrobial resistance in *Salmonella enterica* serovar Heidelberg isolates from retail meats, including poultry, from 2002 to 2006. Appl Environ Microbiol. 74(21):6656-62. http://aem.asm.org/content/74/21/6656.full

Zocco MA, Dal Verme LZ, Cremonini F, Piscaglia AC, Nista EC, Candelli M, Novi M, Rigante D, Cazzato IA, Ojetti V, Armuzzi A. 2006. Efficacy of *Lactobacillus* GG in maintaining remission of ulcerative colitis. Aliment Pharmacol Ther. 23(11):1567-74. http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2036.2006.02927.x/full

Zulkifli I, Abdullah N, Azrin NM, Ho YW. 2000. Growth performance and immune response of two commercial broiler strains fed diets containing *Lactobacillus* cultures and oxytetracyline under heat stress conditions. British Poult Sci. 41(5):593-7. http://www.tandfonline.com/doi/pdf/10.1080/713654979?needAccess=true

Appendix A

Table. 2. Famo feeds game bird starter guaranteed analysis

CONTENT	PERCENTAGE
Crude Protein (Min)	30.0%
Lysine (Min)	1.7%
Methionine (Min)	0.7%
Crude Fat (Min)	3.5%
Crude Fiber (Max)	5.0%
Calcium (Ca) (Min)	1.3%
Calcium (Ca) (Max)	1.8%
Phosphorus (P) (Min)	0.9%
Salt (NaCl) (Max)	0.1%
Salt (NaCl) (Max)	0.6%

Appendix B

Table. 3. Famo feeds game bird starter ingredient statement

INGREDIENTS
Dehulled Soybean Meal
Ground Corn
Animal Protein Products
Wheat Middlings
Corn Distillers Dried Grains with Solubles
Dehydrated Alfalfa Meal
Fish Meal
Animal Fat
Choline Chloride
Calcium Carbonate
Manganese sulfate
Magnesium oxide
Zinc sulfate
Niacin Supplement
Vitamin E supplement
Ferrous sulfate
Biotin
Vitamin A supplement
Calcium Pantothenate
Copper sulfate
Riboflavin Supplement
Folic Acid
Vitamin D_3 Supplement
Menadione Sodium Bisulfite Complex
Pyridoxine Hydrochloride
Thiamine Mononitrate
Vitamin B12 Supplement
Ethylene diamine Dihydroiodide
Cobalt Carbonate
Salt
DL-Methionine Propionic Acid
Ammonium Hydroxide
Zinc Oxide
Sodium Selenite
Ferrous Carbonate
Cobalt Sulfate
Calcium Iodate