ISOLATION OF HOST-ASSOCIATED, POULTRY-DERIVED LACTOBACILLUS SPECIES AND ASSESSMENT OF THEIR PROBIOTIC POTENTIAL THROUGH EXAMINATION OF PREFERABLE STRAIN CHARACTERISTICS

by

ALYXANDRA E. REED

(Under the Direction of Hendrik den Bakker)

ABSTRACT

This study served to isolate host-associated, poultry-derived *Lactobacillus* spp. from production broilers for use as probiotics within the poultry industry. 100 isolates from this study identified 60 *Enterococcus* spp. and 40 *Lactobacillus* spp. using 16S rRNA sequencing, while WGS subsequently identified 8 *L. salivarius*, 2 *L. johnsonii*, 4 *E. faecium*, 3 *E. faecium*, and 1 *E. hirae*. *Lactobacillus* spp. isolated in this study were assessed for putative bacteriocin-producing genes, antimicrobial resistance (AMR) genes, and plasmids. *Lactobacillus* strains contained a variety of bacteriocin-producing genes (bacteriocins, salivaricins, enterolysins, nisin, pediocins, and the MR10B gene) and AMR genes (Lincosamide, Streptogramin B, and Tetracycline resistance). Strains contained no plasmids. Five *Lactobacillus* strains were challenged with six poultry-associated pathogens, with *L. salivarius* showing the greatest results. Overall, *Lactobacillus* strains isolated in this study showed promising characteristics for use as host-associated, poultry-

derived probiotics to increase food safety of final products (i.e., poultry products) in the future.

INDEX WORDS: Poultry, Probiotics, *Lactobacillus*, Poultry-Derived, Host-Associated, Antimicrobial resistance (AMR), Bacteriocins, Plasmids

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DEDICATION

This project is dedicated to everyone who has supported me along the way, including my family, friends, and colleagues. To my Mother and Father who supported me throughout my personal endeavors, internships, moving states multiple times, and my entire school experience. My Mom, Lorraine Reed, would have made sure I owned half of the UGA merchandise in the state, as well as quadruple the amount of cat toys. To my Dad, Charlie Reed, who always asked how my research was going, even if he still has no idea what I am talking about. Well Dad, you keep asking me when I will be done with school, well this is my largest (and last) assignment to date! To my brother, Ashley Reed, who kept me up to date on everything that was going on in Texas while I am in Georgia and made sure Dad didn't get bored in his retirement, thank you. Also, to James Bennett, who listened to me stress about every aspect of this project and tried to help in the best way he could by telling me to go eat cake, squid rings, or something sweet and unhealthy. To the rest of my family and friends, thank you.

This is whole-heartedly dedicated to everyone who has a passion for agriculture, have pursued their goals despite their personal or educational background, and have continually strived to follow their interests, no matter how crazy.

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ABBREVIATIONS

AMR	Antimicrobial Resistance
NAE	No-Antibiotics-Ever
NA	No-Antibiotics (Sample Labels)
CV	Conventional (Sample Labels)
C	Ceca (Sample Labels)
IL	Ilea (Sample Labels)
ATM / A	Atmospheric CO_2 Level (Sample Labels)
CO_2 / C	Elevated CO_2 (5%) Level (Sample Labels)
MRS	De Man, Rogosa, and Sharpe Agar (Commercial)
mMRS	De Man, Rogosa, and Sharpe Agar (Modified)
CO_2	Carbon Dioxide Gas
TSB	Tryptic Soy Broth
TSA	Tryptic Soy Agar
PCR	Polymerase Chain Reaction
16S rRNA	16S Ribosomal RNA
WGS	Whole Genome Sequencing
GIT	Gastrointestinal Tract
sp.	Species (Singular)
spp.	Species (Plural)

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CHAPTER 1

INTRODUCTION

Poultry products contribute to many cases of food-borne illness every year. With a recent push towards less antibiotic use during flock production, alternative measures of pathogen control have been employed such as probiotics and direct-fed microbials, to alleviate the food safety concerns surrounding poultry products. Probiotics have been shown to greatly increase food safety and flock production characteristics, and are usually comprised of a variety of bacterial strains such as *Bacillus* spp., *Lactobacillus* spp., and *Bifidobacterium* spp. (Garriga et al., 1998). Lactobacilli-based probiotics have rapidly gained interest within food safety due to their ability to control pathogens within poultry that are commonly associated with food-borne outbreaks, such as *Salmonella*, *Listeria*, *Campylobacter*, and *Clostridium perfringens* (Garriga et al., 1998; Pascual et al., 1999). Within food-production animals (poultry, cattle, swine, goats, sheep, fish) *Lactobacillus* spp. are commonly used as a food safety and production aid (Garriga et al., 1998).

Identification and evaluation of novel probiotic strains for use within the industry should meet certain genetic criteria in order to work effectively within the host, as well as confer the greatest benefits. Strains used as probiotics should be 1) of host-origin, 2) non-pathogenic, 3) persist in the intestinal tract, and 4) modulate the immune system to be more effective within the GIT (Patterson & Burkholder, 2003; Simmering & Blaut, 2001). Probiotics are thought to work through a variety of methods, such as competitive

exclusion, however the exact mechanism is not understood. Competitive exclusion mechanisms are thought to work more effectively when the above mentioned criteria are met within probiotic strains (Clavijo & Flórez, 2018; Collado et al., 2005). While many food-production systems employ lactobacilli species and strains as probiotics, the emergence of host-associated lactobacilli probiotics are becoming highly sought after in the scientific community and the industry (Chaucheyras-Durand & Durand, 2010; Clavijo & Flórez, 2018; Collado et al., 2005).

The current study was performed to evaluate *Lactobacillus* spp. isolated from the ceca and ilea of both conventionally grown and no-antibiotics-ever flocks for their probiotic potential as an agent to increase food safety of poultry products in the future. *Lactobacillus* spp. were cultured and isolated, and these isolates were 16S rRNA and Whole Genome sequenced to further examine and assess their genetic probiotic characteristics. Isolates were taxonomically identified, examined for genetic diversity, host-association, putative bacteriocin-producing genes, antimicrobial resistance (AMR) genes, and plasmids. *Lactobacillus* strains isolated in this study were then subjected to a challenge study against six poultry-associated pathogens using an overlay technique. This study was expected to yield *Lactobacillus* spp. strains that contain positive genomic characteristics, such as host-association and bacteriocin-producing genes, which would be ideal in probiotic strains used within the industry in the future.

CHAPTER 2

LITERATURE REVIEW

Lactobacillus spp. Information

Lactobacillus spp. are a genus of bacteria within the family *Lactobacillaceae*, which are gram-positive, non-spore-forming, rod or coccoid-shaped cells that vary in their metabolic end-products depending on species (full taxonomy shown at right in **Figure 1**) (Schleifer, 2015). To date about 200 species and 25 subspecies of *Lactobacillus* spp. have been categorized from a wide variety of sources (https://bacdive.dsmz.de/; http://www.bacterio.net/lactobacillus.html) (Parte, 2018; Reimer et al., 2019; Schleifer, 2015). *Lactobacillus* spp. are fastidious organisms adapted to growing into a wide variety of environments, such as temperatures ranging from 2 to 53°C and show limited growth at pH ranges <5.0, but instead prefer pH values between 5.5 and 6.3 (Schleifer, 2015). *Lactobacillus* spp. have a genome size between 1.2 and 4.9 Mb (megabases) with the number of protein-coding genes ranging from 1,200 to 4,750 in number, depending on

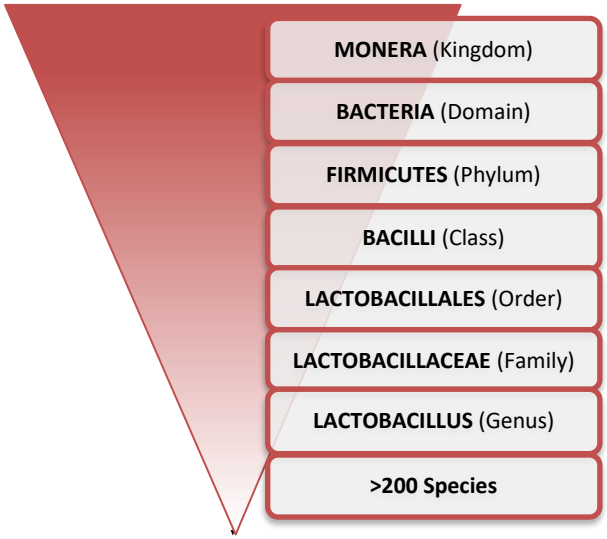

Figure 1: Taxonomic Classification of *Lactobacillus* spp. (Schleifer, 2015)

the species (Mendes-Soares et al., 2014; Schleifer, 2015; Sun et al., 2015). A full list of characteristics concerning *Lactobacillus* spp. are shown below in **Table 1**.

Lactobacillus spp. are found within a variety of foods and environments, and are generally regarded as a beneficial bacterium with no pathogenic tendencies, except in individuals that already have an underlying disease (Schleifer, 2015). *Lactobacillus* spp. are found within a wide variety of environments, both food and non-food alike (Schleifer, 2015). *Lactobacillus* spp. are commonly found in sewage, soil, various species' gastrointestinal tracts, oral cavities, and vaginal canals, among other locations (Schleifer, 2015). Food products that include *Lactobacillus* spp. are various dairy products, grain products, meat and fish, beer, and various others (Schleifer, 2015). These microorganisms are also involved in a variety of food fermentation processes that include both beneficial end-products and undesirable spoilage products (Schleifer, 2015). *Lactobacillus* spp. have been studied extensively due to their suggested beneficial probiotic properties from both a food production and food safety standpoint, which will be discussed later in the "*Lactobacillus* spp. in Various Food-Production Species" section.

Table 1: The Genus *Lactobacillus* and Its Respective Characteristics

Characteristic	**Results Regarding *Lactobacillus* spp.**	**Reference**
Cell Morphology	Rod-shaped, long and slender, sometimes bent Short, often coryneform coccobacilli	(Schleifer, 2015)
Motility	Generally Non-Motile	(Schleifer, 2015)
Spore-forming	No	(Schleifer, 2015)
Gram-Stain Test	Gram-Positive	(Schleifer, 2015)
Metabolism	Obligately Heterofermentative, Facultatively Heterofermentative, Obligately Homofermentative	(Schleifer, 2015)
Growth Conditions	Facultatively Anaerobic	(Schleifer, 2015)
Nitrate Test	Reduced only when pH > 6.0	(Schleifer, 2015)
Catalase/Oxidase/ Cytochrome Test	Negative	(Schleifer, 2015)

Nutritional Requirements	Species Specific	(Schleifer, 2015)
pH Conditions	Aciduric; Optimal – 5.5 to 6.3; Growth – <5.0	(Schleifer, 2015)
Temperature Conditions	Optimal – 2 to 53°C; Growth – 2 to 53°C	(Schleifer, 2015)
Genome Size	1.2 to 4.9 Mb (Megabases)	(Mendes-Soares et al., 2014; Sun et al., 2015)
Genes Present	1,267 to 4,758 (protein-coding)	(Mendes-Soares et al., 2014; Sun et al., 2015)

Lactobacillus spp. in Various Food-Production Species

Lactobacillus spp. are found within a variety of animals, with some bacterial species being unique to certain animals. Some common *Lactobacillus* species and their hosts are shown in **Table 2** below. Within the common food-production animals (poultry, cattle, swine, goats, sheep, fish) *Lactobacillus* spp. are commonly used as a food safety and production aid. Many food-production animals use some of the same species and strains of lactobacilli-based probiotics and the emergence of host-associated *Lactobacillus* spp. are becoming more commonplace in the food-production animal industry in regard to probiotics.

Table 2: Some *Lactobacillus* Commonly Found in Food-Production Animals

Production Animal	*Lactobacillus* spp.	Reference
Chickens (Meat-type)	*L. acidophilis, agilis, animalis, aviarus, crispatus, delbreuckii, fermentum, gallinarum, gigeriorum, johnsonii, kitasatnis, salivarius, thermotolerans*	(Gusils et al., 1999; La Ragione et al., 2004; Lan et al., 2004; Morishita et al., 1997; Parte, 2018; Pascual et al., 1999; Rehman et al., 2007; Reimer et al., 2019; Manuel Jimmy Saint-Cyr et al., 2017)
Swine	*L. acidophilis, amylovorus, brevis, crispatus, curvatus, delbrueckii, fermentum, johnsonii, leichmannii, minutis, mucosae, paracasei, plantarum, reuteri, ruminis, salivarius, sobrius, vaginalis*	(Bateup et al., 1998; Fuller et al., 1978; Hoeflinger et al., 2015; Konstantinov et al., 2006; Krause et al., 2003; Leser et al., 2002; Pieper et al., 2006; Russell, 1979; Valeriano et al., 2017; Yin & Zheng, 2005)
Cattle (Beef)	*L. acetotolerans, animalis, brevis, casei, fermentum, gasseri, johnsonii,*	(Bertin et al., 2017; H. Han et al., 2007; Krause et al., 2003; Otero et al., 2006; Timsit et al., 2018)

	lactis, murinus, reuteri, ruminis, salivarius, vutulinus	
Cattle (Dairy)	*L. acetotolerans, amylovorus, animalis, casei, delbrueckii, fermentum, gasseri, johnsonii, lactis, mucosae, murinus, plantarum, pontis, reuteri, rhamnosus, ruminis, salivarius, seubicus, vitulinus*	(H. Han et al., 2007; Hernandez et al., 2008; Krause et al., 2003; Nader-Macías et al., 2008)

A comprehensive study from Wei et al (2013) used all available data from both published and unpublished sources to examine the poultry intestinal microbiome (Wei et al., 2013). They determined that the intestinal microbiome is comprised of around 117 genera, consisting mainly of *Clostridium*, *Ruminococcus*, *Lactobacillus*, and *Bacteriodes* (Clavijo & Flórez, 2018; Wei et al., 2013). Despite this, the intestinal microbiome in poultry is considered to have a lower diversity than other food-production animals, mainly due to the rapid transition of food through the digestive system (usually 4 to 5 hours) (Clavijo & Flórez, 2018; Stanley et al., 2014). In spite of the rapid transit time, the microbiome can vary heavily and is highly diverse even within flocks produced using the same parameters (Stanley et al., 2013, 2014).

The small intestine, responsible mainly for nutrient absorption, has the greatest concentration of bacterial cells, principally *Lactobacillus* (70% of the bacterial population), *Enterococcus*, and various *Clostridiaceae* (Clavijo & Flórez, 2018; Gong, Forster, Yu, Chambers, Wheatcroft, et al., 2002; G. G. Han et al., 2016; Stanley et al., 2014). The lower part of the small intestine, the ileum, is shown to consist mainly of Lactobacilli and *Enterococcus cecorum*-related groups (>70% of the bacterial population) (Gong, Forster, Yu, Chambers, Sabour, et al., 2002). Another study by Xiao et al (2017) determined that *Lactobacillus* was the dominant genus in the ileum at >35% (Xiao et al., 2017). Other studies have also shown that *Lactobacillus* spp. alone can account for

upwards of 70% of the microbiome contents in the ileum (Gong, Forster, Yu, Chambers, Sabour, et al., 2002; Lu et al., 2003; Xiao et al., 2017). Using bacterial clones, Gong et al (2002) found that 23 out of 51 cloned sequences from the ileum related to lactobacilli and 19 related to *Lactobacillus aviaries* (Gong, Forster, Yu, Chambers, Wheatcroft, et al., 2002). *L. aviarius*, *L. reuteri*, *L. johnsonii*, *L. crispatus*, *L. acidophilus*, *L. kefiranofaciens*, and *L. salivarius*, and *L. plantarum* were shown to be significantly higher than other species in the ileum such as *L. delbrueckii*, *L. paracasei*, *L. brevis*, and *L. gasseri* ($P < 0.05$) (Wang et al., 2014).

One GIT organ with the highest taxonomic diversity and a complex microbiome is the cecum (plural: ceca). This organ retains food for the longest amount of time in the digestive system at 15 to 20 hours, and plays a prominent role in host nutritional health mainly through bacterial fermentation (Clavijo & Flórez, 2018; Gong, Forster, Yu, Chambers, Wheatcroft, et al., 2002). *Lactobacillus*, *Enterococcus*, and butyrate-producing bacteria are common the ceca, as well as the ileum (Gong, Forster, Yu, Chambers, Wheatcroft, et al., 2002). A study by Xiao et al (2017) determined that *Lactobacillus* was the lowest in the ceca compared to the rest of the digestive tract ($P < 0.05$) (Wang et al., 2014; Xiao et al., 2017). Certain *Lactobacillus* spp. have been identified in the ceca, such as *L. reuteri*, *L. aviarius*, *L. johnsonii*, *L. crispatus*, *L. acidophilus*, *L. kefiranofaciens*, *L. salivarius*, and *L. delbrueckii* which were found to be significantly higher in concentration than *L. plantarum*, *L. paracasei*, *L. brevis*, and *L. gasseri* ($P < 0.05$) (Wang et al., 2014).

Probiotics and Their Ideal Characteristics

Probiotics have been defined as a “live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance” (Fuller, 1989).

Table 3: Ideal Probiotic Characteristics

- Be of host origin - Non-pathogenic - Withstand processing and storage - Resist gastric acid and bile - Adhere to epithelium or mucus - Persist in the intestinal tract - Produce inhibitory compounds for pathogens - Modulate immune response - Alter microbial activities - Generally Regarded as Safe (GRAS Status) - Commercially producible at low cost/unit

Adapted from (Patterson & Burkholder, 2003; Simmering & Blaut, 2001)

The host-association of strains isolated in this study was the first major criteria that should be met in terms of identifying an ideal probiotic strain for use within the poultry industry. The functionality of probiotics is still debated, but the mode of action for probiotics can vary greatly based on species and strains used (Pan & Yu, 2013). Since probiotics benefit the host in various ways such as 1) inhibiting colonization and multiplication of pathogenic bacteria within the host through competitive exclusion, 2) the production of bactericidal and bacteriostatic substances against pathogens, 3) formation of neutralizing enterotoxins, 4) the enhancement of gut barrier function, and 5) enhancing and modulation of the host immunity, host-associated probiotics should confer greater health benefits (Pan & Yu, 2013).

This study focuses on the indirect effects of probiotics for their food safety implications, mainly through the reduction and prevention of colonization by enteric pathogens such as *Salmonella*, *Listeria*, and other poultry-associated pathogens (Clavijo & Flórez, 2018). Probiotics are thought to work indirectly through competitive exclusion

mechanisms and the production of bacteriostatic and bactericidal substances, allowing the probiotic strains to compete with pathogenic organisms and reduce pathogen adhesion levels to the intestinal mucosa (Clavijo & Flórez, 2018; Pan & Yu, 2013; Wei et al., 2013). Competitive exclusion is regarded as the most effective way to control pathogens, such as *Salmonella*, from colonizing the GIT of broiler chickens (Chambers & Gong, 2011; Clavijo & Flórez, 2018).

Competitive exclusion states that one competitor will always dominate the other, which may be due to a physical occupation of space within the GIT, competition for resources between communities (pathogens vs commensal), or through physical or chemical confrontation with bacterial competitors, such as through produced metabolites (bacteriocins or organic acids) (Chaucheyras-Durand & Durand, 2010; Clavijo & Flórez, 2018).

A few genomic characteristics of potential probiotic strains are of interest when assessing a bacterial strain, such as the presence of bacteriocin-producing genes, antimicrobial resistance (AMR) genes, and plasmid presence.

Bacteriocins are either proteins or polypeptides produced ribosomally by bacteria during primary growth that possess antimicrobial activity, directly inhibiting competing strains or pathogens (Dobson et al., 2012; Zacharof & Lovitt, 2012). Bacteriocins restrict their activity to strains or species related to the producing species, especially strains of the same species, whereas antibiotics have a wider range of activity and do not show preference for closely related strains (Zacharof & Lovitt, 2012). *Lactobacillus* spp. have gained attention in the food industry due to their proficiency at producing bacteriocins. Bacteriocins have increased antibacterial activity at lower pH values (< 5.0), and their

adhesion to the cell surface of Gram (+) bacteria is pH dependent (Zacharof & Lovitt, 2012).

Bacteriocin production has been considered an important trait in the selection of probiotic strains, due to recent evidence that suggests bacteriocins can facilitate the introduction of a strain into an established microbiome, as well as inhibit the invasion and competition of pathogens and opposing strains (Dobson et al., 2012; Zacharof & Lovitt, 2012). Bacteriocins may also modulate the microbiota composition and influence the host immune system, giving strains an important method of inserting and establishing themselves within the microbial community of a host, relaying the usefulness of bacteriocin production within a probiotic strain (Dobson et al., 2012).

Antimicrobial resistance (AMR) is a heavily discussed topic due to antibiotic use in the past within the poultry industry, as well as in the human medicine today, which could lead to less effective antibiotics in the future and potentially compromise human and animal health. The accumulation and transfer of antibiotic resistance is a common topic of discussion within the poultry industry. Once antibiotic resistant bacteria are established within the GIT of birds, they can become deposited into the litter and recycle between the litter and the GIT in a continual cycle over multiple flocks (Pan & Yu, 2013). Since the GIT is a common reservoir for resistance genes, this cycle can increase the incidence of horizontal gene transfer of antimicrobial resistance (AMR) genes to pathogens and other adverse bacteria (Pan & Yu, 2013). Virulence genes may also transfer between enteric pathogens within poultry, making the gene recipient more pathogenic, and thus making these bacteria a greater food safety concern for consumers (Pan & Yu, 2013). The commensal bacteria within the GIT possess attributes which

allow them to outcompete with pathogenic and adverse bacteria (Pan & Yu, 2013). These competitive attributes can also be transferred to pathogens horizontally, allowing for those pathogens to increase their competitiveness within the GIT, however, horizontal transfer may also make commensal bacteria pathogenic if virulent genes are transferred in the other direction (i.e., from pathogens to commensal bacteria), thus making them harmful to host (Pan & Yu, 2013). Due to these concerns and the complex, and still not completely understood, mechanisms of direct-fed microbials such as probiotics, caution should be taken when employing these for use within food-production animals for human consumption (Pan & Yu, 2013).

Probiotic strains in this study would ideally be negative for AMR genes, as to not spread antimicrobial resistance to other bacteria or pathogens. There is a debate concerning probiotics within the food-production-animal industry, as to whether probiotic strains should be AMR positive or negative. Arguments for AMR positive strains state that if strains were to be able to resist the use of antibiotics, and a case were to arise where antibiotics would need to be used during production, the producers would not lose their probiotic benefits, as the probiotic would be able to withstand the antibiotic treatment. However, since the poultry industry is heading towards "No-Antibiotics-Ever" production practices due partly to consumer demand, the number of cases where antibiotics would need to be used is decreasing rapidly. Therefore, probiotic strains would not need to continually resist the use of antibiotics during production and would not need to be positive for AMR genes, in order to benefit the probiotic bacteria in GIT competition during these situations. Producers would simply just have to take the loss of their administered probiotic benefits if the rare occasion were to occur where antibiotics

are needed during production. These circumstances relate to the stance in this study that *Lactobacillus* spp. strains isolated in this study should be negative for AMR genes.

Lactobacillus spp. in Food Safety

Poultry make up a large sector of the food production industry today. Chicken production alone produced 55 billion pounds worth of broilers, valued at 30 billion dollars in 2017, with broiler production continually showing a steady increase over the last ten years (shown in **Figure 2** at right) (USDA National Agricultural Statistics Service, 2018a).

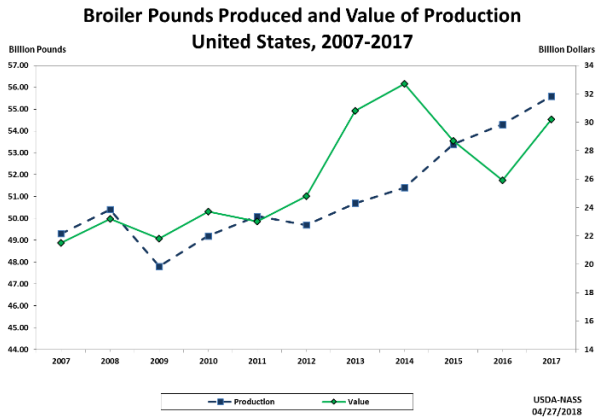

Figure 2: Broiler Pounds Produced and Value of Production, US, 2007-2017 (USDA National Agricultural Statistics Service, 2018c)

In 2017, broiler production in the US was measured just below nine billion birds, consistently increasing about 1.5 million head on average every year since 2012 (USDA National Agricultural Statistics Service, 2018b).

Probiotics have been used within the poultry industry for a variety of reasons, including increasing production traits such as feed conversion ratios and broiler weights, and as protection for both animals and consumers against harmful pathogens or diseases (Garriga et al., 1998). These harmful pathogens include *Salmonella* spp., *Campylobacter* spp., and *Clostridium perfringens*, which are common causes of human food-borne illnesses linked to poultry production (Pascual et al., 1999). There are a few main methods by which *Lactobacillus* spp. are thought to compete with pathogenic organisms and confer antagonistic effects on food-borne pathogens. These include the production of

bacteriocins, hydrogen peroxide (H_2O_2), nitric oxide, and short chain fatty acids (SCFAs), which allow strains to compete against other GIT microbes and potentially inhibit pathogenic bacteria (Clavijo & Flórez, 2018; Dobson et al., 2012).

Lactobacillus spp. are one of the most popular bacterial components of probiotics today, ranging in use from humans to animals alike. With the recent push towards "No-Antibiotics-Ever" production in the poultry industry, alternative methods have been largely studied. These alternatives, such as probiotics, can serve to hopefully achieve the same results that antibiotics were used for previously during production. *Lactobacillus* spp. have been a favorite subject of study within the industry to determine their effectiveness at increasing overall food safety of poultry products, especially raw chicken products, that are going out to market.

Lactobacillus spp. as a Generalized Poultry Probiotic

Various *Lactobacillus* spp. i.e., acidophilus, casei, crispatus, gasseri, plantarum, rhamnosus, and salivarius are used in probiotics, both in mixed-strain and single-strain probiotics (Manuel J Saint-Cyr et al., 2016). One drawback with current research on probiotics, and with *Lactobacillus* spp. in particular, is the "one-strain-fits-all" mentality, where a species or strain that has been shown to have a positive effect as a probiotic in some animal species, will then be used across many food-production species, regardless of probiotic strain-host origin. We hypothesize that identifying host-associated *Lactobacillus* spp. may allow for increased compatibility and functionality within the host, since the *Lactobacillus* spp. or probiotic strain, will already be a part of the natural microbiota present within the animal and therefore may be more effective.

Lactobacillus spp. have been proposed to have the ability to produce

bacteriostatic bacteriocin-like compounds, which have antimicrobial properties and may inhibit pathogens (Li et al., 2017). It also been stated that *Lactobacillus* spp. work through competitive exclusion and antagonism, as well as modulate the immune system, to inhibit the proliferation of pathogens in the gastrointestinal tract (Gusils et al., 1999; Li et al., 2017; Pascual et al., 1999). Lan et al (2004) determined that two *Lactobacillus* strains, *L. agilis* JCM 1048 and *L. salivarius* subsp. *salivarius* JCM 1230, act on the microbiota of the chicken intestine in two beneficial ways: by 1) "working to restore the microbial balance" and 2) "maintain[ing] the stability of the indigenous bacterial microbiota following heat-induced changes" (Lan et al., 2004). This data shows that probiotics function as a balance restorer and work to maintain microbiota stability (Lan et al., 2004).

Lactobacillus spp. and *Salmonella* spp.

Various studies have shown that *Lactobacillus* spp. are able to control *Salmonella* infections by protecting chickens directly from this pathogen, while also conferring a protective effect on raw products (Awad et al., 2009; Pascual et al., 1999; Van Coillie et al., 2007; Vicente et al., 2007). In 1999, *Lactobacillus salivarius* CTC2197 was shown to prevent *Salmonella* enterica serotype Enteritidis C-114 colonization in chickens when given orally as a probiotic (Pascual et al., 1999). The chickens that did not receive the oral probiotic, showed *Salmonella* colonization rates of 70% and 100% across two trials (Pascual et al., 1999). One study also found that *L. johnsonii* FI9785 was able to reduce *S.* Enteritidis and *E. coli* O78:K80 shedding significantly at 15 days and 1 day post-inoculation, respectively ($P < 0.001$) (La Ragione et al., 2004). Gusils et al (1999) determined that *L. fermentum in vitro* could reduce the attachment of *Salmonella*

Pullorum by 77%, while *L. animalis* inhibited *S.* Pullorum, *S.* Enteritidis, *S.* Gallinarum by 90%, 88%, and 78% respectively (Gusils et al., 1999). These studies show that lactobacilli are able to block potential binding sites that are used in the chicken intestinal tract for *Salmonella* adhesion (Gusils et al., 1999; Raja et al., 2009). In 2009, Savvidou determined that *Lactobacillus salivarius* subspecies Salivarius NCIMB 41606 was an effective means of controlling *Salmonella enterica* serovar Typhimurium Sal 1344 nal infections in poultry (Savvidou, 2009). He showed that pathogen shedding was significantly reduced in birds fed a moist feed fermented with *L.* Salivarius (48% reduction) compared to birds fed *L.* Salivarius through water (81%) or the probiotic-free group (75%) (Savvidou, 2009).

Lactobacillus* spp. and *Listeria monocytogenes

Controlling *L. monocytogenes* through Lactobacilli-based probiotics has been shown to be effective in a variety of hosts such as mice, humans, and poultry (Bambirra et al., 2007; Jacobsen et al., 1999; Neveling et al., 2017). Using protective biofilm assays, *L. lactis* 368, *L. curvatus* MBSa3, and *L. sakei* MSSa1 showed a reduction of *L. monocytogenes* biofilm formation in humans (Jacobsen et al., 1999). Neveling et al (2017) showed orally administered multi-strain Lactobacilli-based probiotics, containing both *L. salivarius* DPN181 and *L. johnsonii* DPN184, limited the transition of bioluminescent *L. monocytogenes* EDGe in the GIT of broilers *in vivo* after 3.5 hours, when compared to the control group and a group administered antibiotics (Neveling et al., 2017). *L. monocytogenes* numbers were shown to be high in the ileum (3.13×10^4) and colon (3.43×10^5), but low in the duodenum (2.79×10^3) and cecum (2.84×10^3) two hours after treatment (Neveling et al., 2017). The probiotic treated groups then showed

significant decreases in the ileum at 3.5 hours compared to the antibiotic ($P = 0.0002$) and control groups ($P = 0.0201$) suggesting the effectiveness of Lactobacilli-based probiotics to control *L. monocytogenes* colonization and growth in the GIT of broilers (Neveling et al., 2017).

Lactobacillus spp. and *Campylobacter* spp.

Many studies have been successful in showing a reduction of *Campylobacter jejuni*, using a multi-strain probiotic that contained strains with antimicrobial activity properties (Awad et al., 2009; Chaveerach et al., 2004; Fooks & Gibson, 2002). There is also evidence to support that oral administration of *L. casei* and *L. acidophilus* (among other non-*Lactobacillus* spp. strains) through feed could reduce *C. jejuni* levels up to 70% (Awad et al., 2009; Morishita et al., 1997; Willis & Reid, 2008). La Ragione et al (2004) determined that *L. johnsonii* FI9785 was successful at significantly reducing the presence of *C. jejuni* in chickens through competitive exclusion (La Ragione et al., 2004). They also showed that *C. jejuni* shedding was also significantly reduced on day 5 of the 6 days tested throughout the growout cycle, with a single dose being sufficient to suppress all aspects of colonization of *C. jejuni* (La Ragione et al., 2004).

Lactobacillus spp. and *Clostridium perfringens*

Lactobacillus spp. have also been used in birds that have been challenged with *C. perfringens* to inhibit growth and α-toxin production through oral administration (Guo et al., 2017; La Ragione et al., 2004; Stanley et al., 2013). *L. acidolphilus* and *L. fermentum* showed promise *in vitro* with a decreased levels and overall degradation of *C. perfringens* α-toxin ($P < 0.01$), proving that *Lactobacillus* spp. are potent at inhibiting the pathological effects of *C. perfringens in vitro* (Guo et al., 2017). It was also shown that *L.*

fermentum could silence the production of β2 toxins produced by *C. perfringens in vitro* (Allaart et al., 2011). Li et al (2017) discovered that *L. acidophilus* tended to decrease the mortality rate of *C. perfringens* challenged birds ($P = 0.061$), while simultaneously increasing the *Lactobacillus* spp. populations in the GIT ($P < 0.05$) (Li et al., 2017).

Overall, there has been varying success with lactobacilli-based probiotic strains, both single-strain and multi-strain probiotics, to control food-borne pathogens commonly related to poultry. Prevention of human illnesses related to food-borne pathogens, as well as a reduction in the harborage of these pathogens in the GIT of poultry, is vital to increasing food safety of poultry products present in the market (Manuel J Saint-Cyr et al., 2016). While various *Lactobacillus* spp. are associated with certain food-production animals, *Lactobacillus* spp. can be a potential aid in the "No-Antibiotics-Ever" movement that is gaining popularity within the poultry industry. Through this research, poultry could become a safer food product if possible, production-animal-specific *Lactobacillus* spp., are found.

Thesis Hypothesis and Objectives

The objective of this research was to isolate *Lactobacillus* spp. from production poultry samples and determine their potential as a probiotic for use within the poultry industry, thereby increasing food safety in final products (e.g., poultry meat). Research on probiotics is key in understanding if and how probiotics can increase food safety in the future. The main hypothesis of this study is that production broilers will contain a niche of *Lactobacillus* spp. that are unique when compared to other food-production animals, and these bacterial *Lactobacillus* species may contain exclusive, distinct characteristics, such as host-association, putative bacteriocin-producing genes, antimicrobial resistance

(AMR) genes, and plasmids, which can then be used as to determine their probiotic potential within the poultry industry to increase food safety. This central hypothesis is based on previous research concerning lactobacilli-based probiotics and the evidence of their positive effects on food-borne pathogen reduction for consumers and increased overall food safety. To test the central hypothesis the study will focus on four main objectives:

1. Identify and categorize *Lactobacillus* spp. associated with the ceca and ilea of production broilers (e.g., taxonomic classification through genetic sequencing)
2. Determine the genetic characteristics of identified *Lactobacillus* spp. (e.g., genetic diversity and host-association)
3. Determine if any identified *Lactobacillus* spp. contain potentially useful strain characteristics (e.g., putative bacteriocin-producing genes, antimicrobial resistance (AMR) genes, plasmids)
4. Challenge promising isolated *Lactobacillus* spp. with poultry-associated pathogens to determine potential antagonistic effects

These objectives will hopefully isolate host-associated, poultry-derived *Lactobacillus* spp. with positive probiotic traits that can provide insights into lactobacilli-based probiotics to increase food safety of final products (e.g., poultry meat) within the future.

CHAPTER 3

MATERIALS AND METHODS

On-Farm Sampling

On-farm sampling was done according to standardized animal sample collection techniques. Samples were taken from two anonymous commercial production-broiler farms: one "Conventional" (labeled "CV") farm, which uses antibiotics in their flock during production if necessary, and one "No-Antibiotics-Ever" (labeled "NA") farm, which did not use any antibiotics in their flock during production.

Both farms were visited seven times at set intervals where there was a feed change or a withdrawal of feed from the birds, during a single grow-out cycle (~seven weeks/flock). The CV farm was sampled on Day 0 (chicks hatched and received at farm), Day 18 (last day of "Starter" diet), Day 30 (last day of "Grower" diet), Day 44 (last day of "Finisher" diet), Day 51 (last day of "Withdrawal #1"), and twice on Day 57 (1) last day of "Withdrawal #2" before catch/transport and 2) after transport to the processing facility). The NAE farm was sampled on Day 0 (chicks hatched and received at farm), Day 19 (last day of "Starter" diet), Day 30 (last day of "Grower" diet), Day 43 (last day of "Finisher" diet), Day 49 (last day of "Withdrawal #1"), and twice on Day 54 (1) last day of "Withdrawal #2" before catch/transport and 2) after transport to processing facility). **Table 4** below summarizes both farm sampling dates and the reasons for sampling.

Table 4: Sampling Dates and Their Justifications

Farm Sample Group	Sampling Point
CV0	Day of Hatch, Receiving
CV18	Last Day of "Starter" Diet
CV30	Last Day of "Grower" Diet
CV44	Last Day of "Finisher" Diet
CV51	Last Day of Withdrawal #1
CV57	Last Day of Withdrawal #2, Before Catch and Transport
CV57-T	After Transport to Processing Facility
NA0	Day of Hatch, Receiving
NA19	Last Day of "Starter" Diet
NA30	Last Day of "Grower" Diet
NA43	Last Day of "Finisher" Diet
NA49	Last Day of Withdrawal #1
NA54	Last Day of Withdrawal #2, Before Catch and Transport
NA54-T	After Transport to Processing Facility

The following protocol was used for both farms at every time point. At each time point 15 birds were randomly chosen from the entire house and humanely euthanized through cervical dislocation by the service technician on-site. The birds entire ceca and ileum, including their contents, were aseptically collected in a labeled, sterile Whirl-Pak™ bag (Whirl-Pak™, Nasco, Milton, WI). Proper personal protective equipment (PPE) was worn by all individuals and standardized biosecurity measures were followed while sampling. Gloves were changed with every new bird received and all utensils and instruments were wiped clean, dipped in 100% ethanol, and flamed between every bird to reduce cross-contamination between samples.

The samples were kept cool and on ice (~0-4°C) during sampling, transport, and processing using an insulated cooler. Day 0 samples contained two ceca samples from two chicks to constitute "one" ceca sample, due to the small size of day-old chicks and their respective organs. This was also done with ileum samples, for a total of 30 birds

euthanized on Day 0 per farm, constituting 15 ceca and 15 ileum samples respectively. In total 420 samples were taken for the project: 210 per farm (105 ceca and 105 ilea total).

In-Lab Sample Processing

Samples were processed in Athens, GA at the University of Georgia. All samples were kept cool and on ice (~0-4°C), when they were not able to be stored in a refrigerator (4°C) during processing. Samples were processed in-lab as soon as reasonably possible. Samples were transferred to labeled, sterile, filtered stomacher bags to be weighed. All samples were minced using sterile scissors and had 15 mL of sterile Buffered Peptone Water (BPW, Difco, Sparks, MD) added regardless of sample weight (g), with the exception of the Day 0 samples that received a 10x dilution based on sample weight (g). Samples were then homogenized using a Stomacher for 1 minute at 320 rpm, with the exception of the Day 0 samples that were homogenized by hand for 2 minutes. Samples were then transferred into labeled, sterile conical 50 mL tubes (VMR®, Radnor, PA) for further use. 1.0 mL of homogenate from every sample was aseptically transferred into labeled, sterilized 1.5 mL Eppendorf tubes (Microcentrifuge Tube, VWR®, Radnor, PA) to be transported and used within this specific research project.

Sample Transportation

Samples were transferred from the site of processing at the University of Georgia in Athens, GA to the Center for Food Safety in Griffin, GA for the remainder of the experiment. During transport, the samples were kept cool and on ice (~0-4°C) in an insulated cooler and transported as quickly as reasonably possible. The 1.5 mL Eppendorf tubes containing the samples were placed in a labeled microcentrifuge tube box, covered with a lid, taped shut, labeled, and placed in the cooler. The box was surrounded by

freezer packs for the entirety of the trip (~0°C). The trip between the two facilities took between 3-6 hours depending on traffic and time of day. Upon arrival, samples were placed in the refrigerator until needed (4°C).

Naming System for Samples

Samples were named according to their sampling farm and date, as well as the corresponding organ, and the sample number. The atmospheric condition that the same was grown at is listed as well. For example: "CV18-C07-A" is a sample from the conventional farm (CV) on day 18 (18). It was a ceca sample number seven (C07) grown in atmospheric CO_2/aerobically (-A). Another example: "NA0-IL12-Cm" is a sample from the No-Antibiotics-Ever farm (NA) on Day 0 (0). It was ileum sample number 12 (IL12) and grown in an elevated/5% CO_2 using modified MRS media (-Cm).

Culturing *Lactobacillus* spp.

Only Day 0 and Day 20 samples (specifically CV0, CV18, NA0, and NA19 sample sets) were used for the remainder of the project due to the specific goals of the research regarding probiotics (120 total samples used). Every sample was grown in two different CO_2 conditions: one in aerobic CO_2 (aerobically) and one in an elevated CO_2 (5% CO_2) condition. All samples were grown at 37°C. A 10 µL loopful of every sample was added to labeled MRS broth tubes (MRS, Acumedia, Lansing, MI, USA) in duplicate (230 total tubes). MRS media was prepped according to accompanying preparation parameters on the product. Samples were placed in their respective growth conditions for approximately 24 hours. Every sample was streaked onto MRS agar plates (MRS, Acumedia, Lansing, MI, USA) and grown in their respective growth conditions for approximately 24 hours (230 total tubes; 230 total plates). Ten individual samples

were not cultured due to a lack of sample from processing and were not used for this experiment.

A smaller subset of the sample population was additionally grown using a modified MRS broth and agar plates (final conc. 0.05%; L-cysteine, Beantown Chemical, Hudson, NH, USA) (final conc. 0.002%; m-Cresol Purple, Sigma, St. Louis, MO USA) from Malashree (2012) that had increased levels of cysteine to increase the bacterial selectivity and favor acidic conditions preferred by *Lactobacillus* spp. (Malashree et al., 2012). Six samples (3 ceca and 3 ilea) from CV0, CV18, NA0, and NA19 were chosen which equally represented from all sampling dates, organs, and growth conditions present (24 samples total). These followed the same procedure and growth conditions as above (48 total tubes; 48 total plates).

Isolation of *Lactobacillus* spp.

One isolate was chosen from every streaked plate (n = 278). The isolate was then directly streaked again onto its respective MRS agar plate and incubated at its respective growth condition for approximately 24 hours.

Gram Staining

A subset of 36 samples were Gram Stained according to the standardized protocol accompanying the Gram Stain Kit (Advanced Gram Stain Kit, Hardy Diagnostics, Santa Maria, CA, USA). Samples chosen were equally represented from all sampling dates, organs, and growth conditions present. Isolates were preemptively identified via their results from the Gram Stain test and their cell morphology. Isolates that were identified as Gram positive and had rod-shaped cells were chosen to be prepared for 16S rRNA sequencing.

DNA Extraction

100 isolates were extracted for use with 16S rRNA sequencing. Samples chosen were equally represented from all sampling dates, organs, and growth conditions present. Samples were extracted via a modified microwave protocol from Cornell University (Milillo, 2005). Samples were microwaved for 4 to 6 minutes and were checked for DNA extraction via gel electrophoresis. Samples that did not extract correctly via this method were extracted via a DNA extraction kit (DNeasy®, Blood and Tissue Kit, Qiagen, Germany) using the standardized protocol accompanying the product.

PCR

A simplified PCR protocol (steps 2-3) from the Center for Host-Microbial Interactions (https://chmi-sops.github.io/mydoc_16S_Sanger.html) was performed, using 515F (5'- GTGCCAGCMGCCGCGGTAA -3') and 1492R (5'-CGGTTACCTTGTTACGACTT -3') Primers and the NEBNext® Q5® Hot Start HiFi Master Mix (NEBNext® Q5 Hot Start HiFi PCR Master Mix, New England Biolabs, Ipswitch, MA) (Beiting, n.d.). The thermocycler parameters were as follows: 95°C for 2 min; 95°C for 30 sec (x30), 55°C for 30 sec (x30), 72°C for 1:40 min (x30); 72°C for 5 min; hold at 12°C (Beiting, n.d.). PCR purification was done according to the standardized PCR protocol accompanying the PCR purification kit (QIAquick® PCR Purification Kit, Qiagen, Germany).

16S rRNA Sequencing

100 isolates were sent out to a third-party company, GeneWiz® (GeneWiz®, South Plainfield, NJ; https://www.genewiz.com/en) after being prepped to the company's preferred specifications according to the Sample Submission Guidelines on the

GeneWiz® website (https://www.genewiz.com/Public/Resources/Sample-Submission-Guidelines/Sanger-Sequencing-Sample-Submission-Guidelines?). Samples were shipped in groups of 50. 16S sequencing data from every sample was run through the NCBI BLAST system, using the nucleotide blast suite (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=MicrobialGenomes) to determine the genus of the isolates (Altschul et al., 1990). Only isolates identified as *Lactobacillus* spp. were used for the remainder of the experiment.

Whole Genome Sequencing

20 of the 100 samples that were sent out for 16S rRNA sequencing were chosen for WGS. 15 of the isolates for WGS were prepped and sequenced according to the standardized protocol from the PulseNet Nextera XT manual for the Illumina MiSeq for Gram Positive isolates (CDC, 2016). The remaining five isolates were prepped and sequenced using two protocols through a liquid culture extraction method: Steps 1-11 from the QIAamp® DNA Mini Blood Mini Handbook section "Isolation of genomic DNA from Gram-positive bacteria" (https://www.qiagen.com/us/resources/resourcedetail?id=62a200d6-faf4-469b-b50f-2b59cf738962&lang=en) using egg white lysozyme (Lysozyme from Chicken Egg, Sigma, St. Louis, MO), followed by steps 4-8 from the DNeasy® Blood and Tissue Kit protocol manual section "Purification of Total DNA from Animal Tissues (Spin-Column Protocol).

Taxonomic Identification of Isolates

Taxonomic identification of raw sequence data was done using Colorid, specifically read_id (https://github.com/hcdenbakker/colorid). The top genus and species

result were used as the identification for that isolate. All 20 WGS isolates were identified using this method. All bioinformatic work was done in Ubuntu v18.04.2 “bionic”.

Phylogenetic Tree Construction

Raw sequencing data was assembled using SPAdes v3.11.1 (https://github.com/ablab/spades) (Nurk et al., 2013). ParSNP v1.2 (https://harvest.readthedocs.io/en/latest/content/parsnp.html) was used to assemble and construct the phylogenetic trees (Treangen et al., 2014). FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) was used to visualize and midpoint root the trees. Only isolates that were identified as lactobacilli were used for the remainder of the experiment (n = 10). 84 *L. salivarius* strains that were previously sequenced in the NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/) as of March 2019 were added to the tree, along with eight *L. salivarius* strains isolated in this study (n = 92) (Benson et al., 2012). The same was done with two *L. johnsonii* strains isolated in this study and 38 strains from GenBank (n = 40). This was done to show genetic relation between strains, strain host-association, and strain-origin (Benson et al., 2012).

Host-Association Determination

GenBank was used to determine strain host-origin and colored groups representing differing clades were made on the phylogenetic trees based on the results using FigTree.

Putative Bacteriocins

Sequencing data was submitted to BAGEL4 (http://bagel4.molgenrug.nl/) and resulting putative bacteriocin-producing genes were determined for every strain (Van Heel et al., 2018).

Antimicrobial Resistance (AMR) Determination

AMR genes and gene functionality were identified in all ten *Lactobacillus* strains isolated in this study using ResFinder v3.1.0 (https://cge.cbs.dtu.dk/services/ResFinder/) and ResFinderFG v1.0 (https://cge.cbs.dtu.dk/services/ResFinderFG/) through the Center for Genomic Epidemiology (Center for Genomic Epidemiology, Lyngby, Denmark) (Pehrsson et al., 2016; Zankari et al., 2012). Default parameters were used for the assessment on the strain scaffolds.fasta files.

Plasmid Determination

Plasmids were identified in all ten *Lactobacillus* strains isolated in this study using the PlasmidFinder 2.0 (v2.0.1) (https://cge.cbs.dtu.dk/services/PlasmidFinder/) through the Center for Genomic Epidemiology using the default parameters and searching the Gram Positive Database (Carattoli et al., 2014). Default parameters were used for the assessment on the isolate scaffolds.fasta files.

Challenge Study

Five of the strains isolated in this study that were confirmed to be *Lactobacillus* spp. using WGS were chosen to participate in the challenge study against common food-borne pathogens (n = 5). Pathogens were poultry-associated strains that were on hand in the Culture Collection at the Center for Food Safety in Griffin, GA. Strains used were: *S.* Typhimurium 96037-1, *S.* Enteritidis 96037-2, *S.* Heidelberg MH27651, *S.* Enteritidis CFS039, *Listeria monocytogenes* 4c Li 2107, and *L. monocytogenes* F6854. Five strains from the Culture Collection were used in comparison to the results from the challenge study to compare probiotic potential using the same overlay technique and poultry-

associated pathogens. These strains were: *L. casei* 03, *L. fermentum* B1 28, *L. rhamnosus* BUCSAV 227, *L. salivarius* NRRL 30514, *L. malefermentans* NCBI 8516.

The protocol is a modified version from Adetoya et al (Adetoye et al., 2018). *Lactobacillus* strains isolated in this study were grown overnight in MRS broth at 37ºC in 5% CO_2. Samples were streaked simultaneously on one MRS plate by making a small 2-centimeter (cm) line on the plate with each sample. There was only one line per strain on each MRS plate, with a total of five strain lines per plate. MRS plates were incubated for 24 hours at 37ºC in 5% CO_2. On the same day, the pathogen to be tested was inoculated into TSB (Tryptic Soy Broth, Acumedia®, Lansing, MI) and incubated aerobically for 24 hours at 37ºC. The test pathogen was inoculated into molten TSA (Tryptic Soy Agar (Soybean-Casein Digest Agar), Neogen®, Lansing, MI) at a 10% final concentration. The MRS plates were overlaid with approximately 15 mL of agar-pathogen solution and allowed to set. Plates were incubated for 24 hours at 37ºC under aerobic conditions. The zones of inhibition were checked for presence/absence and radius length measured in centimeters (cm).

Freezing Isolates

Isolates were frozen at -80ºC following a simplified version of the protocol from Cornell University (Fortes, 2016). Using a sterile 1 µL loop, a single colony of the isolate was chosen and inoculated into 5.0 mL of sterile MRS broth grown overnight at 37ºC in 5% CO_2. 150 µL of warm, sterile glycerol (Glycerol, Fisher Chemical, Fair Lawn, HJ) was aliquoted into each labeled cryovial (Cryovial Tube™ vials, Nunc™, Denmark) aseptically. The overnight cultures were vortex briefly and 850 µL of each isolate was

added to the appropriate cryovial. Each vial was inverted until the glycerol and overnight culture were mixed. Vials were then frozen at -80ºC.

CHAPTER 4

RESULTS

Gram Staining

36 isolates were gram stained, all returning a positive gram stain with purple color. 27 out of 36 isolates were coccoid in shape, with small bunches clumped together into groups or chains. There was a uniform shape across all cells. Nine isolates were rod-like in shape, formed small bunches or chains, and showed mostly straight rods arranged in chains. Results are shown below in **Table 5**.

Table 5: Gram Stain Test Results from Selected Samples

Sample ID	Atmospheric Condition	Gram Stain	Cell Characteristics
CV0-C10	Atm	Positive	coccoid in shape, small bunches clumped together into groups/chains
CV0-C13	Atm	Positive	coccoid in shape, small bunches clumped together into groups/chains
CV0-IL11	Atm	Positive	coccoid in shape, small bunches clumped together into groups/chains
CV0-IL13	Atm	Positive	coccoid in shape, small bunches clumped together into groups/chains
CV0-C14	CO_2	Positive	coccoid in shape, small bunches clumped together into groups/chains
CV0-C15	CO_2	Positive	coccoid in shape, small bunches clumped together into groups/chains
CV0-IL07	CO_2	Positive	coccoid in shape, small bunches clumped together into groups/chains
CV0-IL12	CO_2	Positive	coccoid in shape, small bunches clumped together into groups/chains
CV18-C08	Atm	Positive	rod-like in shape, small bunches/chains, straight rods arranged in chains
CV18-C11	Atm	Positive	rod-like in shape, small bunches/chains, straight rods arranged in chains
CV18-IL07	Atm	Positive	rod-like in shape, small bunches/chains, straight rods arranged in chains
CV18-IL09	Atm	Positive	rod-like in shape, small bunches/chains, straight rods arranged in chains
CV18-C04	CO_2	Positive	rod-like in shape, small bunches/chains, straight rods arranged in chains
CV18-C10	CO_2	Positive	coccoid in shape, small bunches clumped together into groups/chains
CV18-IL07	CO_2	Positive	coccoid in shape, small bunches clumped together into groups/chains
CV18-IL09	CO_2	Positive	coccoid in shape, small bunches clumped together into groups/chains
NA0-C08	Atm	Positive	coccoid in shape, small bunches clumped together into groups/chains
NA0-C10	Atm	Positive	coccoid in shape, small bunches clumped together into groups/chains
NA0-IL08	Atm	Positive	coccoid in shape, small bunches clumped together into groups/chains
NA0-IL12	Atm	Positive	coccoid in shape, small bunches clumped together into groups/chains
NA0-C07	CO_2	Positive	coccoid in shape, small bunches clumped together into groups/chains
NA0-C10	CO_2	Positive	coccoid in shape, small bunches clumped together into groups/chains
NA0-IL04	CO_2	Positive	coccoid in shape, small bunches clumped together into groups/chains
NA0-IL05	CO_2	Positive	coccoid in shape, small bunches clumped together into groups/chains
NA19-C03	Atm	Positive	rod-like in shape, small bunches/chains, straight rods arranged in chains
NA19-C10	Atm	Positive	rod-like in shape, small bunches/chains, straight rods arranged in chains
NA19-IL05	Atm	Positive	rod-like in shape, small bunches/chains, straight rods arranged in chains
NA19-IL11	Atm	Positive	rod-like in shape, small bunches/chains, straight rods arranged in chains
NA19-C03	CO_2	Positive	rod-like in shape, small bunches/chains, straight rods arranged in chains
NA19-C15	CO_2	Positive	rod-like in shape, small bunches/chains, straight rods arranged in chains
NA19-IL10	CO_2	Positive	rod-like in shape, small bunches/chains, straight rods arranged in chains

NA19-IL15	CO_2	Positive	coccoid in shape, small bunches clumped together into groups/chains
CV0-C01m	CO_2	Positive	coccoid in shape, small bunches clumped together into groups/chains
CV0-C07m	CO_2	Positive	coccoid in shape, small bunches clumped together into groups/chains

16S rRNA Sequencing

100 isolates were chosen from the 278 total isolates available. 16S rRNA results using sequenced isolates from this study (n = 100) preemptively identified 40 lactobacilli (40%), potentially *L. salivarius* (37; 92.5%) and *L. johnsonii* (3; 7.5%) with the remaining 60 identified as *Enterococcus faecalis* (38), *E. faecium* (16), *E. hirae* (3), *E. durans* (2), *E. villorum* (1) using BLAST. These results are shown in **Table 6** below.

Table 6: 16S rRNA Sequencing Results with the Number of Isolates of Each Species and Their Percent Abundance (n = 100)

16S rRNA Taxonomic Classification	Number of Isolates	Number of Isolates (%)
***Lactobacillus* spp.**	**40**	**40**
L. salivarius	37	92.5
L. johnsonii	3	7.5
***Enterococcus* spp.**	**60**	**60**
E. faecalis	38	63.3
E. faecium	16	26.7
E. hirae	3	5
E. durans	2	3.3
E. villorum	1	1.7

Whole Genome Sequencing

Classification of sequencing data using ColorId determined that out of 20 isolates, eight identified as *L. salivarius* (40%), two as *L. johnsonii* (10%), four as *E. faecium* (20%), three as *E. faecalis* (15%), and one as *E. hirae* (5%). Two isolates had low quality reads and were not used for analysis. This is shown below in **Table 7**.

Table 7: WGS Results with the Number of Isolates of Each Species and Their Percent Abundance (n = 20)

WGS Taxonomic Classification	Number of Isolates	Number of Isolates (%)

L. salivarius	8	40
L. johnsonii	2	10
E. faecium	4	20
E. faecalis	3	15
E. hirae	1	5
Unidentified – Low Quality (Not Used)	2	10

Using ColorId, isolates from this study were identified at a species level. *Lactobacillus* isolates were renamed after sequencing to make more concise names for phylogenetic trees and only those isolates were used for the remainder of the experiment. AER03, AER04, AER05, AER09, AER10, AER12, AER35, and AER36 were identified as *L. salivarius*, while AER25 and AER105 were identified as *L. johnsonii*, which is summarized in **Table 8.**

Table 8: *Lactobacillus* Strains Isolated in This Study and Their Taxonomic Identification

Sample ID	Isolate/Strain ID	Taxonomic Classification
CV18-C11-A	AER03	*L. salivarius*
NA19-IL06-A	AER04	*L. salivarius*
CV18-IL09-A	AER05	*L. salivarius*
NA19-C03-A	AER09	*L. salivarius*
NA19-IL05-A	AER10	*L. salivarius*
CV18-C02-Am	AER12	*L. salivarius*
CV18-IL05-A	AER35	*L. salivarius*
CV18-IL06-A	AER36	*L. salivarius*
CV18-IL12-C	AER25	*L. johnsonii*
NA19-IL09-A	AER105	*L. johnsonii*

Phylogenetic Trees

After genetic assembly using SPAdes and tree construction and visualization using ParSNP and FigTree, analysis of the phylogenetic trees was conducted. For the first phylogenetic tree containing *L. salivarius* strains (**Figure 3** below), the tree can be

broken into three clades based on host-origin. The clades are color coded according to their host origin on the tree with pink originating from swine, blue from human, and green from avian/poultry. *Lactobacillus* strains isolated in this study are highlighted in red. Phylogenetic analysis of eight *L. salivarius* strains isolated in this study and 84 *L. salivarius* strains in GenBank (n = 92) determined that strains isolated in this study were genetically diverse, and nested in a clade of poultry-associated isolates, suggesting that the *L. salivarius* strains show an association with the host (i.e., host-associated).

For the second phylogenetic tree containing *L. johnsonii* strains (**Figure 4** below), the tree can be broken into two clades based on host-origin. The clades are color coded according to their host origin on the tree with purple originating from mammals, and green from avian/poultry. Experimental *Lactobacillus* strains isolated in this study are highlighted in red. Phylogenetic analysis of two *L. johnsonii* strains isolated in this study and 38 strains in GenBank (n = 40) determined that these strains were genetically diverse, and nested within a clade of poultry-associated isolates, suggesting that the *L. johnsonii* strains isolated in this study are host-associated as well.

These results indicate that lactobacilli strains isolated in this study are host-associated and genetically diverse.

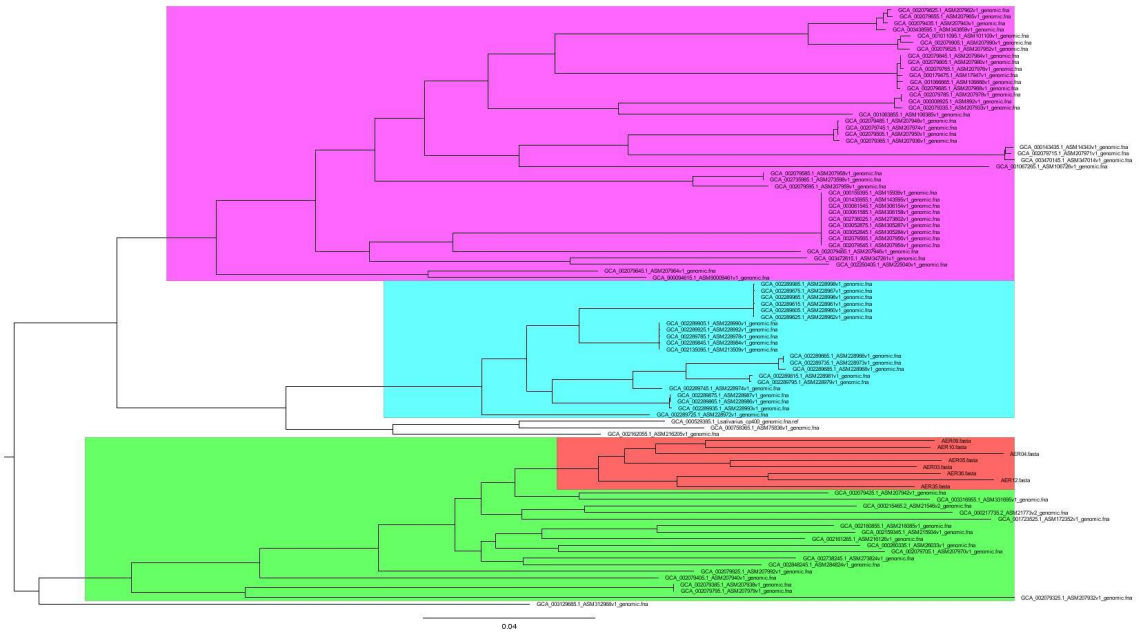

Figure 3: *L. salivarius* Phylogenetic Tree Phylogenetic tree containing 84 *L. salivarius* strains from GenBank along with eight experimental *L. salivarius* strains isolated in this study (n = 92); Host Origin: Porcine (Swine) = Pink; Human = Blue; Avian (Poultry) = Green; Experimental Strains = Red

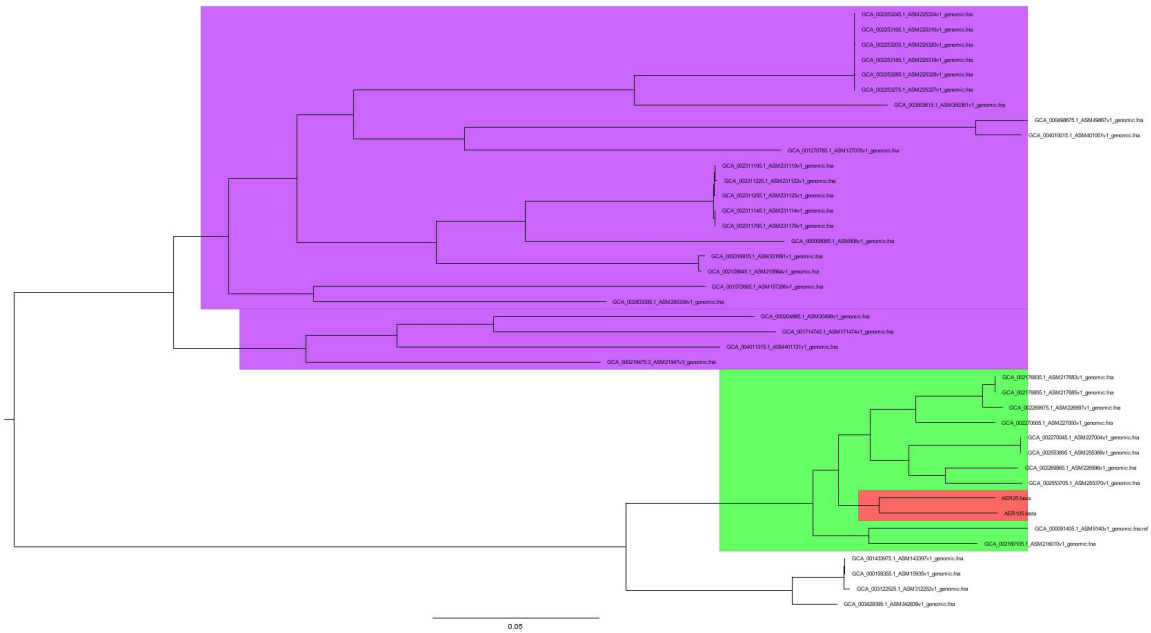

Figure 4: *L. johnsonii* Phylogenetic Tree Phylogenetic tree containing 38 *L. johnsonii* strains from GenBank along with two *L. johnsonii* strains isolated in this study (n = 40); Host Origin: Mammal = Purple; Avian (Poultry) = Green; Experimental Strains = Red

Putative Bacteriocins

Lactobacillus strains isolated in this study showed a variety of different putative bacteriocin-producing genes using BAGEL4. Bacteriocin, salivaricin, enterolysin, pediocin, nisin, and the MR10B (an enterocin)-producing genes were all identified within the *Lactobacillus* strains isolated in this study, which are summarized in **Table 9**. Strain AER03 contained an enterolysin, AER04 contained a bacteriocin and enterolysin, AER05 contained two enterolysins and a salivaricin, AER09 contained two enterolysins and a bacteriocin, AER10 contained an enterolysin and salivaricin, AER12 contained four enterolysins, a nisin, a bacteriocin, and a MR10B gene (an enterocin), AER35 contained one bacteriocin, AER36 contained a nisin, bacteriocin, enterolysin, and an MR10B gene (an enterocin), AER25 contained a bacteriocin and a pediocin, and AER105 contained an enterolysin, bacteriocin, and a pediocin-producing genes.

Table 9: *Lactobacillus* Strains Isolated in This Study and Identified Bacteriocins

Strain ID	Taxonomic Classification	Putative Bacteriocins Identified
AER03	*L. salivarius*	Enterolysin
AER04	*L. salivarius*	Bacteriocin Enterolysin
AER05	*L. salivarius*	Enterolysin (2) Salivaricin
AER09	*L. salivarius*	Enterolysin (2) Bacteriocin
AER10	*L. salivarius*	Enterolysin Salivaricin
AER12	*L. salivarius*	MR10B (Enterocin) Enterolysin (4) Nisin Bacteriocin
AER35	*L. salivarius*	Bacteriocin
AER36	*L. salivarius*	Nisin Bacteriocin Enterolysin

		MR10B (Enterocin)
AER25	*L. johnsonii*	Bacteriocin Pediocin
AER105	*L. johnsonii*	Enterolysin Bacteriocin Pediocin

Antimicrobial Resistance (AMR) Genes

Lactobacillus strains isolated in this study showed a variety of AMR genes and their respective functionality within the strain, shown in **Table 10** below. AER03 had two AMR genes, lnu(C) and vat(E) that matched at a 99.19 and 96.12% sequence identity, which encodes for Lincosamide and Streptogramin B resistance. These are shown the be non-functional. AER04 contained the lnu(C) gene at 99.19% identity, which codes for Lincosamide resistance, but were shown to be non-functional. AER05 contained two AMR genes, vat(E) and tet(M), at 96.43 and 99.69% identity which code for Streptogramin B and Tetracycline resistance. This strain was shown to have a function in tetracycline protection at 99.79%. AER09 did not have any AMR genes identified. AER10 had two AMR genes, lnu(C) and vat(E) at 99.19 and 96.43% identity, encoding for Lincosamide and Streptogramin B resistance. These were shown to be non-functional. AER12 contained four AMR genes, lnu(C), vat(E), tet(M), and tet(L) at 99.19, 96.43, 99.12, and 99.78% sequence identity, which encoded for Lincosamide, Streptogramin B, and two Tetracycline resistance genes. AER12 was shown to have tetracycline protection at 99.23% identity. AER35 had two AMR genes, lnu(C) and vat(E) at 99.19 and 99.43% identity, which encodes Lincosamide and Streptogramin B resistance. These were shown to be non-functional. AER36 contains four AMR genes, lnu(C), vat(E), tet(M), and tet(L) at 99.19, 96.43, 99.12, and 99.78% sequence identity, encoding for Lincosamide,

Streptogramin B, and two Tetracycline resistance genes, which were functional at 99.23% identity. AER25 contained a vat(E) and tet(W) genes at 96.43 and 99.01% identity shown to infer Streptogramin B and Tetracycline resistance. AER25 has a function in tetracycline protection and was shown to have a 99.90% identity match. AER105 had a lnu(A) genes at a 98.77% match encoding for Lincosamide resistance.

Table 10*: Lactobacillus* Strains Isolated in This Study and Identified AMR Genes

Strain ID	Taxonomic Classification	AMR Genes	Identity Match (%)	Resistance	Functionality	Identity Match (%)
AER03	*L. salivarius*	lnu(C) vat(E)	99.19 96.12	Lincosamide Streptogramin B	None	
AER04	*L. salivarius*	lnu(C)	99.19	Lincosamide	None	
AER05	*L. salivarius*	vat(E) tet(M)	96.43 99.69	Streptogramin B Tetracycline	tet_protection	99.79
AER09	*L. salivarius*	None				
AER10	*L. salivarius*	lnu(C) vat(E)	99.19 96.43	Lincosamide Streptogramin B	None	
AER12	*L. salivarius*	lnu(C) vat(E) tet(M) tet(L)	99.19 96.43 99.12 99.78	Lincosamide Streptogramin B Tetracycline Tetracycline	tet_protection	99.23
AER35	*L. salivarius*	lnu(C) vat(E)	99.19 96.43	Lincosamide Streptogramin B	None	
AER36	*L. salivarius*	lnu(C) vat(E) tet(M) tet(L)	99.19 96.43 99.12 99.78	Lincosamide Streptogramin B Tetracycline Tetracycline	tet_protection	99.23
AER25	*L. johnsonii*	vat(E) tet(W)	96.43 99.01	Streptogramin B Tetracycline	tet_protection	99.90
AER105	*L. johnsonii*	lnu(A)	98.77	Lincosamide	None	

Plasmids

10 *Lactobacillus* strains isolated in this study contained no plasmids using PlasmidFinder2.0, shown below in **Table 11**.

Table 11: *Lactobacillus* Strains Isolated in This Study and Identified Plasmids

Strain ID	Taxonomic Classification	Plasmids

AER03	*L. salivarius*	None Identified
AER04	*L. salivarius*	None Identified
AER05	*L. salivarius*	None Identified
AER09	*L. salivarius*	None Identified
AER10	*L. salivarius*	None Identified
AER12	*L. salivarius*	None Identified
AER35	*L. salivarius*	None Identified
AER36	*L. salivarius*	None Identified
AER25	*L. johnsonii*	None Identified
AER105	*L. johnsonii*	None Identified

Challenge Study

Five *Lactobacillus* strains isolated in this study were challenged against six poultry-associated pathogens in triplicate using an overlay technique. This was done to test the antagonistic effects of five *Lactobacillus* strains isolated in this study against pathogens that would naturally occur, or are associated with, poultry *in vivo*. Zones of inhibition were measured in cm, with an average radius length taken across all three replicates. Greater radius length average suggests a greater inhibitory effect on the challenge pathogen via the tested *Lactobacillus* strains isolated in this study. All poultry-associated pathogens were gathered from the culture collection at the Center for Food Safety in Griffin, GA, specifically *S.* Typhimurium 96037-1, *S.* Enteritidis 96037-2, *S.* Heidelberg MH27651, *S.* Enteritidis CFS039, *Listeria monocytogenes* 4c Li 2107, and *L. monocytogenes* F6854. Results are shown in **Table 12** below along with **Images 1-6**. When AER04 was challenged with the six pathogens, zones of inhibition were shown against all six pathogens with all three replicates showing inhibition indicating antagonistic effects against the pathogen tested. AER04 showed zone of inhibition radius averages of 0.8 cm, 0.8 cm, 0.6 cm, 0.7 cm, 0.4 cm, 0.6 cm against *S.* Typhimurium 96037-1, *S.* Enteritidis 96037-2, *S.* Heidelberg MH27651, *S.* Enteritidis CFS039, *L.*

monocytogenes 4c Li 2107, and *L. monocytogenes* F6854 respectively. AER25 only showed growth against *S.* Heidelberg MH27651 in two replicates and *L. monocytogenes* F6854 in three replicates, and a zone of inhibition radius of 0.2 cm on average against both pathogens. AER35 showed zones of inhibition against all six pathogens with all three replicates showing inhibition. AER35 showed a zone of inhibition radius average of 0.6 cm, 0.7 cm, 0.5 cm, 0.5 cm, 0.5 cm, 0.9 cm against *S.* Typhimurium 96037-1, *S.* Enteritidis 96037-2, *S.* Heidelberg MH27651, *S.* Enteritidis CFS039, *L. monocytogenes* 4c Li 2107, and *L. monocytogenes* F6854 respectively. AER36 showed zones of inhibition against all six pathogens with all three replicates showing inhibition. AER36 showed a zone of inhibition radius average of 0.7 cm, 0.8 cm, 0.7 cm, 0.4 cm, 0.6 cm, 0.8 cm against *S.* Typhimurium 96037-1, *S.* Enteritidis 96037-2, *S.* Heidelberg MH27651, *S.* Enteritidis CFS039, *L. monocytogenes* 4c Li 2107, and *L. monocytogenes* F6854 respectively. AER105 showed no zones of inhibition against any of the pathogens. This results from the comparison strains was used to determine the probiotic potential compared to type strains or strains that are commonly used within probiotics (**Table 13**). All strains showed inhibition across multiple replicates with the exception of *L. malefermentans* NCBI 8516 against *L. monocytogenes* F6854. *L. casei* 03 showed growth against all pathogens with 3, 3, 3, 2, 3, and 2 replicates showing inhibition respectively. *L. fermentum* B1 28 showed growth against all pathogens with 3, 1, 2, 2, 3, and 1 replicate showing inhibition respectively. *L. rhamnosus* BUCSAV 227 showed growth against all pathogens with 3, 3, 3, 3, 3, and 2 replicates showing inhibition respectively. *L. salivarius* NRRL 30514 showed growth against all pathogens with 3, 3, 3, 3, 3, and 1 replicate showing inhibition respectively. *L. malefermentans* NCBI 8516 showed growth

against all pathogens, except against *L. monocytogenes* F6854, with 3, 3, 3, 1, and 3 replicates showing inhibition respectively.

Table 12: Select *Lactobacillus* Strains Isolated in This Study Challenged with Poultry-Associated Pathogens

	S. Typhimurium 96037-1	*S.* Enteritidis 96037-2	*S.* Heidelberg MH27651	*S.* Enteritidis CFS039	*L. monocytogenes* 4c Li 2107	*L. monocytogenes* F6854
L. salivarius AER04	Y / 3 / 0.8 cm	Y / 3 / 0.8 cm	Y / 3 / 0.6 cm	Y / 3 / 0.7 cm	Y / 3 / 0.4 cm	Y / 3 / 0.6 cm
L. johnsonii AER25	N	N	Y / 2 / 0.2 cm	N	N	Y / 3 / 0.2 cm
L. salivarius AER35	Y / 3 / 0.6 cm	Y / 3 / 0.7 cm	Y / 3 / 0.5 cm	Y / 3 / 0.5 cm	Y / 3 / 0.5 cm	Y / 3 / 0.9 cm
L. salivarius AER36	Y / 3 / 0.7 cm	Y / 3 / 0.8 cm	Y / 3 / 0.7 cm	Y / 3 / 0.4 cm	Y / 3 / 0.6 cm	Y / 3 / 0.8 cm
L. johnsonii AER105	N	N	N	N	N	N

Y - Yes (Green), Zones of Inhibition Shown; N - No (Red); No Zones of Inhibition Shown
Number: Out of three replicates, the number that showed Zones of Inhibition
Size: Average radii (cm) of Zone of Inhibition across all positive replicates

Table 13: Select Comparison Strains Challenged with Poultry-Associated Pathogens

	S. Typhimurium 96037-1	*S.* Enteritidis 96037-2	*S.* Heidelberg MH27651	*S.* Enteritidis CFS039	*L. monocytogenes* 4c Li 2107	*L. monocytogenes* F6854
L. casei 03	Y / 3	Y / 3	Y / 3	Y / 2	Y / 3	Y / 2
L. fermentum B1 28	Y / 3	Y / 1	Y / 2	Y / 2	Y / 3	Y / 1
L. rhamnosus BUCSAV 227	Y / 3	Y / 3	Y / 3	Y / 3	Y / 3	Y / 2
L. salivarius NRRL 30514	Y / 3	Y / 3	Y / 3	Y / 3	Y / 3	Y / 1
L. malefermentans NCBI 8516	Y / 3	Y / 3	Y / 3	Y / 1	Y / 3	N

Y - Yes (Green), Zones of Inhibition Shown; N - No (Red); No Zones of Inhibition Shown
Number: Out of three replicates, the number that showed Zones of Inhibition

Image 1: *Lactobacillus* strains isolated in this study challenged with *S.* Typhimurium 96037-1

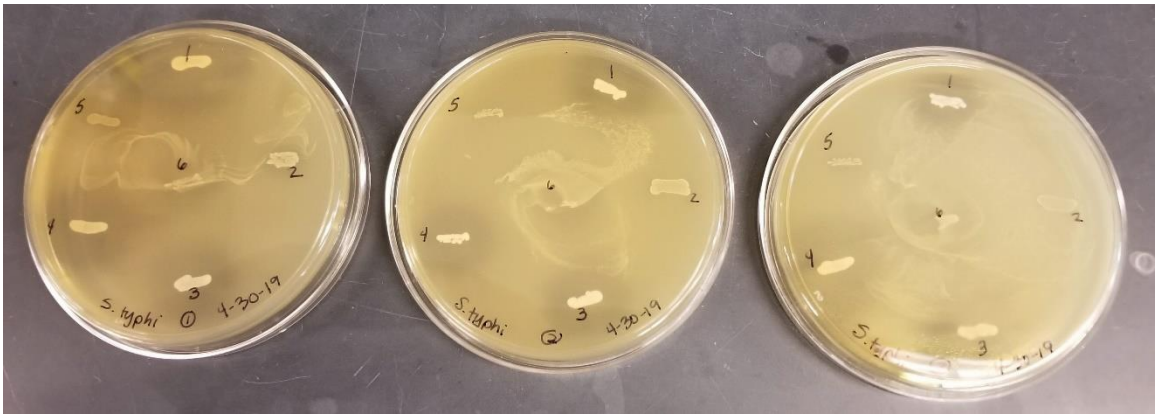

Image 2: *Lactobacillus* strains isolated in this study challenged with *S.* Enteritidis 96037-2

Image 3: *Lactobacillus* strains isolated in this study challenged with *S.* Heidelberg MH27651

No Images Taken

Image 4: *Lactobacillus* strains isolated in this study challenged with *S.* Enteritidis CFS039

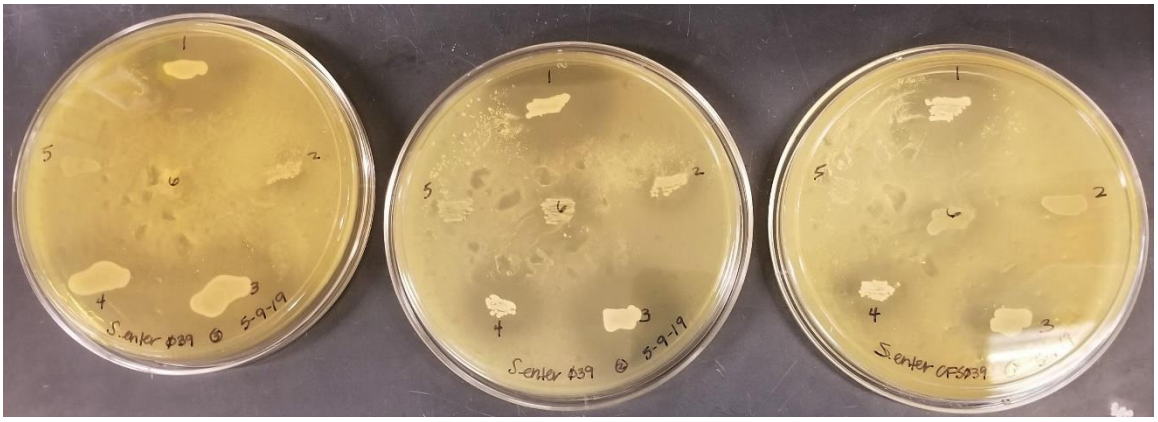

Image 5: *Lactobacillus* strains isolated in this study challenged with *L. monocytogenes* 4c Li 2107

Image 6: *Lactobacillus* strains isolated in this study challenged with *L. monocytogenes* F6854

CHAPTER 5

DISCUSSION

Lactobacilli-based probiotics are an important aid in controlling food-borne pathogens in poultry and are a key aspect of study within the field. *Lactobacillus* spp. have shown their potential as a probiotic in poultry by controlling pathogens such as *Salmonella* spp., *Campylobacter* spp., *Listeria monocytogenes,* and *Clostridium perfringens* (Awad et al., 2009; Guo et al., 2017; Gusils et al., 1999; Lei et al., 2012; Pascual et al., 1999; Van Coillie et al., 2007; Vicente et al., 2007). In order to identify new lactobacilli-based probiotic strains that are host-associated and poultry-derived this exploratory study was conducted. Strains would need to have positive characteristics to be considered potential probiotics such as: be of host-origin, non-pathogenic, persist in the intestinal tract, alter microbial activities, have the ability to resist processing and storage, among other characteristics (Patterson & Burkholder, 2003; Simmering & Blaut, 2001). An up and coming issue with probiotics that are used within the industry is the "one-strain-fits-all" mentality, that does not necessarily use host-associated, host-derived probiotic strains within food-production animals. We hypothesize that host-originated and host-associated probiotic species will have greater synergistic effects within the host, such as easier establishment and increased persistence within the microbial community of the GIT, as well as elevated antagonistic effects against pathogenic bacteria. Within this study, the objective was to isolate, identify, and categorize *Lactobacillus* spp. from poultry samples, determine their probiotic potential, and run a small challenge study

against poultry-associated pathogens. These results serve to increase food safety of poultry products in the future using probiotics as an aid.

Lactobacillus Strains, Specifically *L. salivarius* and *L. johnsonii*, were Successfully Identified and Categorized at a Species Level from The Ceca and Ileum of Production Broilers Using Selective Media and WGS

Using selective media and WGS both *L. salivarius* and *L. johnsonii* were successfully isolated from ceca and ilea samples from production broilers.

16S rRNA sequencing data from this study revealed that 40% of the 100 chosen isolates were *Lactobacillus* spp, while the remaining 60% were *Enterococcus* spp. which is to be expected considering the high populations of *Lactobacillus* spp. and *Enterococcus* spp. commonly found in the ileum and ceca of production broilers. Out of 40 *Lactobacillus* spp., *L. salivarius* was the most prominent result from 16S rRNA sequencing at 92.5%, with the remaining 3 potentially identified as *L. johnsonii* (7.5%). Although isolates equally represented the two organs, selective media and growth conditions could have swayed the *Lactobacillus* spp. numbers found in each organ, with 19 of the experimental isolates from this study originating from the ileum, and 21 from the ceca, with the latter being quite high compared to previous literature.

Several studies have examined *Lactobacillus* species associated with both the ceca (Clavijo & Flórez, 2018; Gong, Forster, Yu, Chambers, Sabour, et al., 2002; Wang et al., 2014; Xiao et al., 2017) and ileum (Gong, Forster, Yu, Chambers, Wheatcroft, et al., 2002; Lu et al., 2003; Wang et al., 2014; Xiao et al., 2017), identifying a variety of

species, such as *L. aviarius*, *L. reuteri*, *L. johnsonii*, *L. crispatus*, *L. acidophilus*, *L. salivarius*, and *L. plantarum* (Wang et al., 2014).

The isolate numbers were relatively unusual concerning both numbers from the ceca and ileum when examining them separately. 40% *Lactobacillus* spp. identified out of 100 isolates from the intestinal tract is somewhat low, compared to the >70% reported for the GIT, but this could be due to the small sample size and growth conditions using selective media. However, all 100 isolates were either *Lactobacillus* spp. or *Enterococcus* spp. and this high percentage is to be expected given the selective media and organ sampling locations.

Fallbacks with this data include the selectivity of the media and growth conditions, which could have skewed the numbers of isolates of each *Lactobacillus* species isolated as well as the specific species isolated.

Lactobacillus Strains Isolated in This Study were Genetically Diverse and Host-Associated

Through phylogenetic analysis, *Lactobacillus* strains isolated in this study were determined to be genetically diverse from one another and host-associated determining that there are *Lactobacillus* species, specifically *L. salivarius* and *L. johnsonii*, that are associated with poultry.

As mentioned previously in the above section, *L. salivarius* and *L. johnsonii* are normally present in the GIT of poultry, specifically in both the ceca and ileum of broilers. Through the construction of phylogenetic trees containing all *L. salivarius* from GenBank and eight *L. salivarius* strains isolated from this study, AER03, AER04, AER05, AER09,

AER10, AER12, AER35, and AER36 (n = 92). **Figure 3** showed that the *L. salivarius* strains isolated in this study were genetically diverse from one another given their branch length on their tree (shown in red) and are located within avian-associated clade (shown in green), suggesting that these isolates are host-associated.

Construction of a phylogenetic tree using two *L. johnsonii* strains isolated in this study, AER25 and AER105, and 38 strains from GenBank (n = 40). This tree, shown in **Figure 4**, determined that the *L. johnsonii* strains isolated in this study were genetically diverse based on branch length and nested within a clade of poultry-associated isolates, suggesting that strains isolated in this study are host-associated.

Adhesion reduction ability is highly dependent on probiotic organism source, where strains that are from chicken intestinal mucosa (i.e., host-associated) will have a greater capacity for adhesion and therefore a greater likelihood to displace pathogens within the intestinal tract, serving to ultimately increase food safety (Clavijo & Flórez, 2018; Collado et al., 2005). *Lactobacillus* strains isolated in this study were poultry-derived and host-associated which should allow for increased effectiveness against pathogenic bacteria, and therefore be a more effective probiotic strain within poultry in terms of increasing the safety of poultry products.

To further confirm host-association and build upon these findings, methods such as tissue culture would allow for morphological and physiological tests to be run to further examine specifics related to how this bacteria fits into the microbial community, interacts with the tissues within the GIT, and the benefits and mechanisms of host-associated probiotics within their corresponding hosts and environments.

Lactobacillus Strains Isolated in This Study Contained A Wide Variety of Characteristics of Interest in Potential Probiotic Strains

The ideal probiotics should have certain genetic characteristics in order to be the most productive and beneficial within the host including host-association, non-pathogenic properties, and freedom from plasmids or other transferable elements, are just a few of the ideal characteristics. This study examined *Lactobacillus* strains isolated in this study for putative bacteriocin-producing genes, AMR genes, and plasmid presence.

Bacteriocins

Lactobacillus strains isolated in this study displayed a wide variety of putative bacteriocin-producing genes, encoding for Bacteriocins, Salivaricins, Enterolysins, Pediocins, and Nisin, as well as the MR10B gene (an Enterocin). Putative bacteriocins found in *Lactobacillus* strains isolated in this study are summarized in **Table 9**.

Various bacteriocin classes are typically associated with certain bacterial genus and species, due to these bacteriocins being produced or studied more frequently in certain bacteria. Salivaricins are commonly associated with *Streptococcus* spp., such as *S. salivarius* K12 and CRL1328 (Gillor et al., 2008). These have also been identified within *Lactobacillus* spp., such as some *L. salivarius* strains (Ayala et al., 2019). Enterolysins are commonly produced by *Lactobacillus* species such as *L. helviticus and L. salivarius*, as well as some species of *Enterococcus* spp. (Ayala et al., 2019; Mokoena, 2017). Pediocins have been previously linked with *Pediococcus* spp, such as *P. acidilactici*, and also with *Leuconostoc gelidum* (Gillor et al., 2008; Mokoena, 2017). Pediocins are somewhat unique in that these bacteriocins can inhibit a broad range of bacteria, such as *Streptococcus aereus* and vegetative cells of *Clostridium* spp., and *Listeria* spp. (Gillor et

al., 2008). Nisin is the one of the most widely used and exploited bacteriocins in the food industry and is currently used worldwide (Zacharof & Lovitt, 2012). It is active against Gram (+) bacteria and pathogenic organisms such as *Staphylococcus aereus* and *L. monocytogenes*, and has been approved by the FDA for use within the United States since 1988 (Zacharof & Lovitt, 2012). Nisin production has been previously seen in, and is highly associated with, *Lactococcus lactis* spp. (Jacobsen et al., 1999; Zacharof & Lovitt, 2012). MR10B is an enterocin gene that is typically associated with *Enterococcus faecalis* and other *Enterococcus* spp. (Martín-Platero et al., 2006). Enterocin production is commonly linked to *E. faecium*, such as *E. faecium* EK13, and *L. acidolphilus* (Gillor et al., 2008; Mokoena, 2017).

L. salivarius strains have been shown previously to contain a multiple bacteriocins including salivaricins and enterolysins, with Ayala et al (2019) finding a salivaricin P and two enterolysin A bacteriocins associated with *L. salivarius* L-28 (Ayala et al., 2019). This supports the data found in this study concerning the presence of putative salivaricins and enterolysins within the *L. salivarius* strains isolated in this study (Ayala et al., 2019). Other bacteriocins such as pediocins, enterocins, and nisin production are not typically associated with *L. salivarius* and *L. johnsonii* specifically, however they are usually associated with closely related bacterial species that are classified as lactic acid bacteria.

There are three potential explanations for the appearance of these bacteriocin producing genes in the experimental *Lactobacillus* strain isolated in this study: 1) the genes observed in these isolates are native to *Lactobacillus*, however they have not been associated with this genus before, 2) there was cross-contamination within the isolated

strains in this study before sequencing, or 3) these genes have possibly transferred over to the *Lactobacillus* strains isolated in this study from other bacteria that are heavily associated with the bacteriocins (e.g., *Pediococcus* spp. and Pediocins).

Further studies isolating and categorizing the specific substrates that are produced by these strains to examine the putative bacteriocin-producing genes in further detail are needed. Determining bacteriocin substrate origin, functionality, composition, and usability were not examined due to time constraints, and the mechanism that works against poultry-associated pathogens (see results from the challenge study below) needs to be examined further. Isolation and categorization of a potentially new bacteriocins could be marketable within the food industry, as well as increase the overall food safety of poultry products in the future.

Overall, the *Lactobacillus* strains isolated in this study showed a large variety of bacteriocins commonly associated with various lactic acid bacteria, a positive characteristic for a potential probiotic use within the industry. Increased research into the specifics of the bacteriocins produced is needed if future studies were to move forward using the *Lactobacillus* strains isolated in this study.

AMR Genes and Functionality

Lactobacillus strains isolated in this study displayed a multitude of AMR genes corresponding to resistance to three antibiotics, Lincosamide, Streptogramin B, and Tetracycline.

While *Lactobacillus* strains isolated in this study contained genes for Lincosamide, Streptogramin B, and Tetracycline resistance, the genes for Lincosamide resistance (lnu(C) and lnu(A)) and Streptogramin B (vat(E)) resistance were shown to be

potentially non-functional using ResFinderFG, which identifies functional metagenomic antibiotic resistance determinants (shown in **Table 10**). While these are non-functional in the *Lactobacillus* strains isolated in this study, the high sequence identity of all strains that contain these (>96%) is concerning regarding the potential of these genes to transfer to other bacteria, both commensal and pathogenic. Tetracycline resistance genes (tet(M), tet(L), and tet(W)) were found in strains AER05, AER12, AER36, and AER25. These genes were potentially functional with a >99% sequence identity, providing tetracycline protection to these lactobacilli strains. Concern over AMR genes transferring horizontally to other bacteria or pathogens is an issue.

Previous studies have determined that poultry-derived *Lactobacillus* spp. strains usually contain a variety of AMR genes (Dec et al., 2017; Kmeť & Piatnicová, 2010), which contrasts with the findings in this study which conferring resistance to three antibiotics. Dec (2017) determined that their *Lactobacillus* spp. strains contained a large variety of AMR genes, testing 36 common AMR genes, showing results that contrast the findings in this study, which only identified six genes, lnu(C), lnu(A), vat(E), tet(M), tet(L), and tet(W) (Dec et al., 2017). They showed that >70% of their strains exhibited tetracycline resistance genes, which is lower than numbers found in this study with four out of ten strains with these genes (40%), but greater than Kmet and Piatnicova (2010) at 28% of their strains containing *tet* genes (Dec et al., 2017; Kmeť & Piatnicová, 2010). The presence of tet(M) and tet(L) are common within *L. salivarius*, and tetracycline resistance is high among strains of *L. reuteri*, *L. gallinarum*, *L. crispatus*, and *L. salivarius*, with high amount of strains containing both tet(M) and tet(L) (>68%), which

is in line with the findings in this study, with three out of four experimental strains containing one or both genes (Dec et al., 2017).

These results offer insight into the ability of the *Lactobacillus* strains isolated in this study to affect the flock, the environment, and human health due to the potential for AMR genes to transfer between hosts, potentially creating greater AMR resistance. Antibiotics are classified by their importance in treatment of human disease as either "medically important" or "not medically important," with "medically important" antibiotics classified further on their level of importance and use as either "critically important," "highly important," or "important" (FDA, 2018; Geneva: World Health Organization, 2017). Lincosamides, Streptogramins, and Tetracyclines are all "medically important" antibiotics, classified as "highly important" as of 2016 (FDA, 2018; Geneva: World Health Organization, 2017). However, two of the most commonly used antibiotics, Virginiamycin, a Streptogramin, and Chlortetracycline, a Tetracycline, were phased out for use as growth promoters in 2016 based on FDA guidelines (National Chicken Council, 2019).

Lincosamides have grown in popularity within food-production animals by 63% between 2009 and 2017, while Tetracyclines have shown a 33% decrease during that same period (FDA, 2018). Within poultry, medically important antimicrobial drugs made up a very small portion (5%) of total sales in 2017 (5,559,212 kg of active ingredient total), with estimated annual total of 268,047 kg sold, which is a 46% decrease difference from 2016 (FDA, 2018). Lincosamide usage decreased 7% between 2016 and 2017 from 8,874 kg to 8,213 kg, making up 5% of the total Lincosamide usage in all food-production-animals (FDA, 2018). Streptogramins are not individually reported, but in

combination with Diaminopyrimidines and Polymyxins, totaled 76,440 kg sold for use in all food-production animals (FDA, 2018). In chickens, Tetracycline usage decreased 46% between 2016 and 2017 from 285,513 kg to 153,621 kg, and made up for 4% of all Tetracycline usage in food-production-animals (FDA, 2018).

While the numbers concerning these three antibiotics and their use within food-production-animals is large when examining the amounts sold in 2017 alone, the percentage of their use within poultry is relatively small (5% for Lincosamides and 4% for Tetracyclines) (FDA, 2018). This data, the increased shift towards "No-Antibiotics-Ever" production in the poultry, and the non-functional AMR genes (for Lincosamide and Streptogramin B) found in the *Lactobacillus* strains isolated in this study all help to screen the strains for their use as a probiotic based on their AMR characteristics. The number of AMR genes and the potential functionality of the Tetracycline genes in the *Lactobacillus* strains isolated in this study are mildly concerning due to AMR transfer between bacteria and the medical importance of the antibiotics in question, however, the large scale impacts should be minimal within the industry and if used correctly, should be of minimal concern to human health.

Overall, while all three antibiotic types (Lincosamides, Streptogramins, and Tetracyclines) are medically important, their use within the poultry industry specifically, coupled with the trend towards lesser antibiotic use within production, decreases the threat level of AMR transfer within the *Lactobacillus* strains isolated in this study. *Lactobacillus* strains isolated in this study need to be studied further in terms of AMR gene transference (either directly via transformation or through plasmids via conjugation) *in vitro* possibly using bacterial cloning techniques.

Plasmids

Lactobacillus spp. strains isolated in this study did not contain any plasmids, a preferable strain trait due to the possibility of AMR transfer through plasmids from one bacterium to another.

Plasmids presence within *Lactobacillus* strains isolated in this study are a focus because these can be horizontally transferred (i.e., through conjugation) and potentially increase the virulence or resistance of other bacteria based on the genes that are present in the transferred plasmids. None of the *Lactobacillus* strains isolated in this study contained any plasmids using PlasmidFinder2.0 (**Table 11**) which is a positive trait that this study aimed to identify.

These findings are positive since there are implications that AMR genes may transfer within plasmids further spreading antibiotic resistance.

Lactobacillus Strains Isolated in This Study, Especially *L. salivarius*, Showed Preferable Antagonistic Effects Against Poultry-Associated Pathogens

Lactobacillus strains isolated in this study, mainly *L. salivarius* strains AER04, AER35, and AER36, had positive antagonistic effects against poultry-associated pathogens, displaying zones of inhibition across multiple replicates with zone radii lengths consistently greater than 0.4 cm.

Since several lactobacilli strains can decrease non-beneficial bacterial groups within the chicken GIT, such as *Salmonella* spp. and *Campylobacter* spp., a small challenge study was conducted using five *Lactobacillus* strains isolated in this study

challenged against six poultry-associated pathogens using an overlay technique (Pan & Yu, 2013). The results are summarized in **Table 12**.

Many studies have shown *Lactobacillus* spp. are capable of controlling Salmonella infections in poultry, though the exact mechanism of protection is unclear (Awad et al., 2009; Pascual et al., 1999; Van Coillie et al., 2007; Vicente et al., 2007).

Five experimental strains isolated in this study, three *L salivarius* (AER04, AER35, AER36) and two *L. johnsonii* (AER25, AER105), showed preferable antagonistic effects against four *Salmonella* and two *Listeria* strains. The *L. salivarius* strains isolated in this study showed greater zones of inhibition radius lengths across all challenge pathogens compared to the experimental *L. johnsonii* strains isolated in this study, with a greater number of replicates inducing inhibition and greater zone of inhibition radii lengths comparatively.

The *L. johnsonii* strains isolated in this study did not compete well with the pathogens overall, with only one strain, AER25, inducing inhibition, though the inhibition zones were small at radii length of 0.2 cm on average. La Ragione (2004) showed *L. johnsonii* FI9785 can significantly reduce *S. enteritidis* shedding at 15 days post-inoculation in poultry, which contradict the findings in this study, however, the results may differ due to the use of GIT versus fecal samples ($P < 0.001$) (La Ragione et al., 2004).

All three *L. salivarius* strains isolated in this study induced growth inhibition in all three replicates, with zone of inhibition radii lengths greater than 0.4 cm (0.4 to 0.9 cm). Savvidou (2009) determined that *L. salivarius* NCIMB 41606 was effective at controlling *S.* Typhimurium Sal 1344 nal infections and reduced pathogen shedding when

compared to a probiotic free group, which supports findings in this study (Savvidou, 2009). All three L. *salivarius* strains isolated in this study, AER04, AER35, and AER36, showed large zones of inhibition (>0.6 cm average) potentially contributing to the results seen by Savvidou (2009) (Savvidou, 2009). *L. salivarius* CTC2197 has been shown to decrease *S.* Enteritidis C-114 colonization when given as an oral probiotic, which is consistent with *L. salivarius* strains isolated in this study when challenged against *S.* Enteritidis 96037-2 and *S.* Enteritidis CFS039 (Pascual et al., 1999).

When the challenge study results of the *Lactobacillus* species isolated in this study are compared to the outside strains (**Table 13**), the *L. salivarius* strains isolated in this study, AER04, AER35, and AER36, showed equal or better results (equal or greater number of replicates with inhibition) against multiple strains. These included *S.* Enteritidis 96037-2, *S.* Enteritidis CFS039, and *L. monocytogenes* F6854, with the latter showing the greatest difference in results. The *L. salivarius* strains isolated in this study showed inhibition across all three replicates, whereas the comparison strains did not show inhibition across all three replicates in some cases. The comparison strains are all either type strains or strains that are commonly used in other probiotic studies, which suggests that the *Lactobacillus* strains isolated in this study, specifically the *L. salivarius* strains, were just as good as, or better than, the comparison strains using this method against these poultry-associated pathogens. This evidence shows promising results for the *Lactobacillus* strains isolated in this study in a comparative setting when measuring their overall antagonistic effect against poultry-associated pathogens across multiple replicates.

The antagonistic effects seen in this study could be attributed to a variety of mechanisms such as bacteriocin production, hydrogen peroxide production, organic acid production, or combination of many mechanisms via the *Lactobacillus* strains isolated in this study.

As discussed in previous sections, bacteriocin production via *Lactobacillus* strains isolated in this study could possibly explain the antagonistic effects shown within the challenge study against poultry-associated pathogens.

Hydrogen peroxide (H_2O_2) production is a well-known method of bacterial control against pathogens for a variety of *Lactobacillus* spp. including *L. johnsonii* and *L. acidophilus* (Hertzberger et al., 2014). However Dec et al (2018) determined that H_2O_2 production did not directly correlate with pathogen inhibition, with *L. johnsonii* exhibiting strong H_2O_2 production levels, but the inhibitory effect on *C. jejuni* was weak (Dec et al., 2018). Moreover, some of the *L. salvarius* isolates used showed weak levels of H_2O_2 production, but strongly inhibited growth of pathogens when challenged, serving to state that while many *Lactobacillus* spp. are shown to produce H_2O_2, it may not solely contribute to the inhibition of pathogens in poultry (Dec et al., 2018). This method however, may partially contribute to the inhibitory effects seen in this study from the *Lactobacillus* strains isolate in this study when challenged with poultry-associated pathogens.

In vitro studies have shown that the fermentation of carbohydrates by lactic acid bacteria produces lactic acid, which can then lower the pH of the surrounding environment within the GIT and serve to inhibit pathogens such as *E. coli*, *Salmonella* Typhimurium, and *Clostridium perfringens* (Hinton et al., 2016; Pan & Yu, 2013). It has

also been stated that SCFAs in an undissolved form can diffuse freely across the cell membrane into the cell, dissociating and lowering the intracellular pH that can then inhibit essential enzymes or metabolism of bacteria, including pathogens (Pan & Yu, 2013; Van Der Wielen et al., 2000; Van Immerseel et al., 2004, 2006). Due to the ability of *Lactobacillus* spp. to ferment carbohydrates and produce organic acids and SCFAs, this serves as another alternative as to the explain the results shown in the challenge study using *Lactobacillus* strains isolated in this study.

While each of the mechanisms mentioned (bacteriocins, H_2O_2 production, organic acids) have shown to be effective in some capacity, the exact mechanism of inhibition of *Lactobacillus* spp. is still unclear. Potentially a combination of multiple mechanisms work coherently to produce the antagonistic effects seen in this study, but more research is needed. If the antagonistic mechanism could be understood fully, it could then be modified or enhanced to be more effective at increasing the safety of food products in the future, and therefore decrease illnesses related to food-borne pathogens.

Since specific substrates were not examined within this study due to time constraints, the mechanism that works against poultry-associated pathogens needs to be examined further. Specific studies to examine what substrates are produced and relate these substrates to antagonistic results would be beneficial. Understanding the mechanism behind this effect can serve to further increase overall food safety of poultry products if it were able to be understood and manipulated to maximize the antagonizing effects on food-borne pathogens.

Overall, the *Lactobacillus* strains isolated in this study showed very promising antagonistic effects when challenged against poultry-associated pathogens, especially the

L. salivarius strains (AER04, AER35, and AER36). These results provide many more areas of study for future research and are points of interest for host-associated probiotic research in the area of food safety.

Future Research

The research on probiotics is vast and a rapidly growing field, especially within poultry practices where "No-Antibiotics-Ever" production systems are rapidly becoming popular and are pushed heavily within the industry. Probiotics vary wildly in their effectiveness and method of action based on animal species, animal age, feed composition, route of probiotic administration, strain composition, production method, and many other factors. With the positive information obtained within this study, future research within the *Lactobacillus* strains isolated in this study and their probiotic potential *in vivo* is needed. Increased knowledge regarding *Lactobacillus* spp. and their ability to be cultured easily and quickly, could be useful due to the positive effects seen *in vitro* of *Lactobacillus* spp. as probiotics in the food-production industry. Further information regarding the specific functions of putative bacteriocin-producing and AMR genes is needed. Lastly, the mechanism behind the antagonistic effects seen within the challenge study against the poultry-associated pathogens could be further examined to gain further knowledge about the mechanisms by which *Lactobacillus* spp. work against the test pathogens used in this study.

CHAPTER 6

CONCLUSION

WGS data indicated that a variety of species and strains were present in isolates found in this study, including *L. salivarius*, *L. johnsonii*, *E. faecium*, *E. faecalis*, and *E. hirae*. Through WGS, eight *L. salivarius* and two *L. johnsonii* strains were identified. The *Lactobacillus* strains isolated in this study were determined to be host-associated, poultry-derived, and showed preferable genomic elements, such as a variety of bacteriocin-producing genes and few, mostly non-functional AMR genes. Bacteriocin, enterolysin, salivaricin, pediocin, nisin, and a MR10B (an enterocin)-producing genes were all identified within *Lactobacillus* strains isolated in this study. No plasmids were identified within any strains.

A variety of AMR genes were identified within the *Lactobacillus* strains, including genes encoding for Lincosamide, Streptogramin B, and Tetracycline resistance, with Tetracycline protection being the only potentially functional gene in four out of ten strains. This is concerning due to the possibility of horizontal gene transfer of these resistance genes to other bacteria, including both commensal and pathogenic. In a challenge study against six poultry-associated pathogens, five *Lactobacillus* strains isolated in this study showed relatively promising results, with *L. salivarius* strains, AER05, AER35, and AER36 showing inhibition against all six pathogens across multiple replicates. One *L. johnsonii* strain, AER05, showed weak inhibition against *S.* Heidelberg MH27651 and *L. monocytogenes* F6854, while the other strain, AER105, showed no

inhibition against any pathogen. Overall, these results indicate that a small number of the experimental *Lactobacillus* strains isolated in this study display strong and promising characteristics, such as host-association and putative bacteriocin-producing genes that will substantiate their use as future probiotics to increase the safety of final products (e.g., poultry meat) within the poultry industry.

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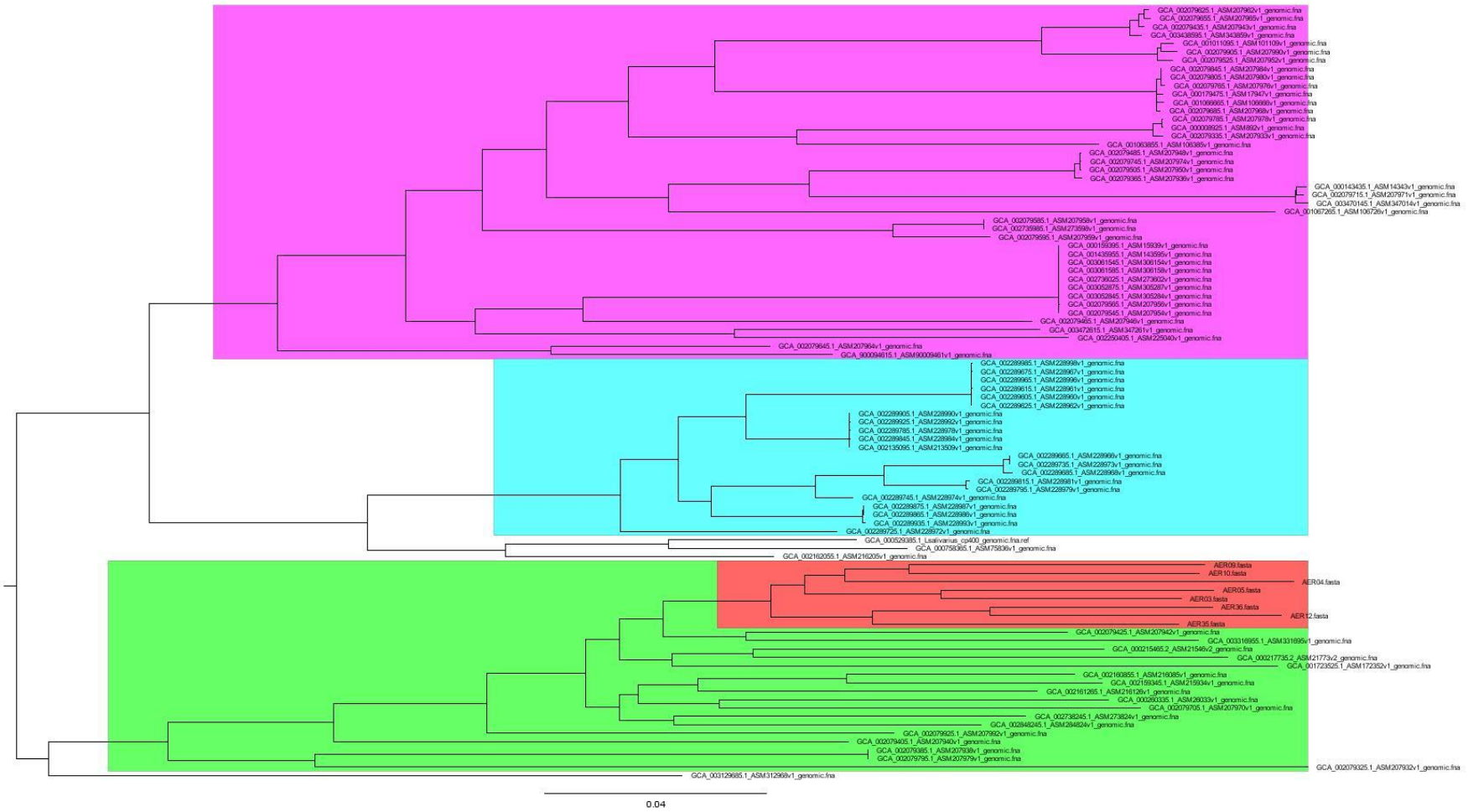

Figure 3: *L. salivarius* Phylogenetic Tree Phylogenetic tree containing 84 *L. salivarius* strains from GenBank along with eight *L. salivarius* strains isolated in this study (n = 92); Host Origin: Porcine (Swine) = Pink; Human = Blue; Avian (Poultry) = Green; Experimental Strains = Red

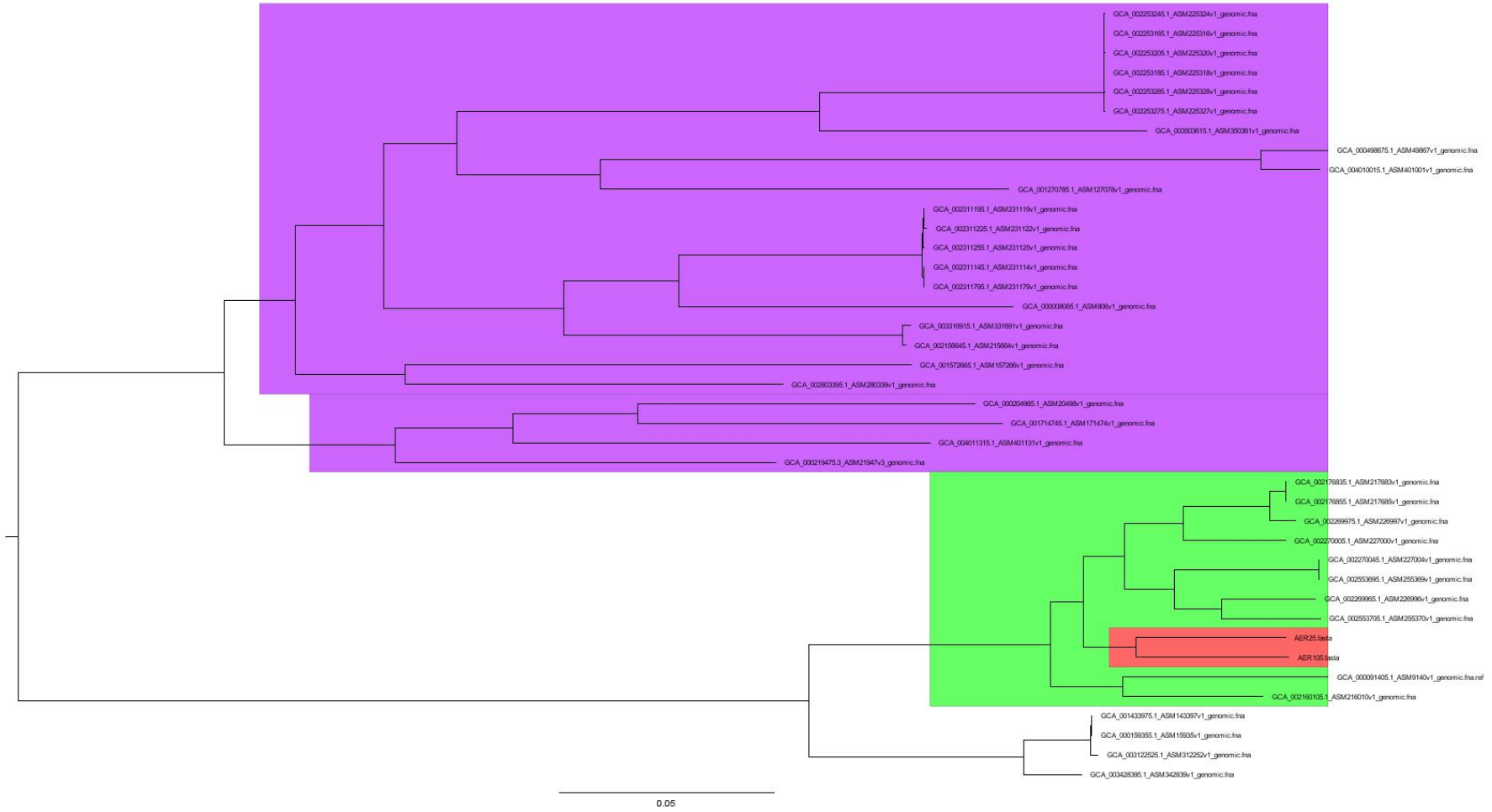

Figure 4: *L. johnsonii* Phylogenetic Tree Phylogenetic tree containing 38 *L. johnsonii* strains from GenBank along with two *L. johnsonii* strains isolated in this study (n = 40); Host Origin: Mammal = Purple; Avian (Poultry) = Green; Experimental Strains = Red