ABSTRACT

DARON, CAITLYN SUZANNE. Characterization of *Lactobacillus* Isolates from Chickens. (Under the direction of Dr. Hosni M. Hassan).

The purpose of this research has been to identify and characterize previously isolated *Lactobacillus* strains for their probiotic potential (bacterial strains ability to impart beneficial health impacts on the host). Characterization of the selected isolates was achieved through 16s rRNA sequencing, physiological and biochemical testing, and shotgun whole genome sequencing. 16s rRNA sequencing revealed the identities of six selected *Lactobacillus* strains as: C25 (*L. crispatus*), P17 (*L. animalis*), P28 (*L. animalis*), P38 (*L. animalis*), P42 (*L. acidophilus*), and P43 (*L. reuteri*). When appropriate, these strains were cultured in commercial MRS under anaerobic conditions and subjected to testing such as final growth concentration in aerobic versus anaerobic conditions, carbohydrate utilization, catalase activity, growth in semi-defined media with specific sugars, acid and bile salt tolerance. Shotgun whole genome sequencing was performed on three strains: C25 (*L. crispatus*), P38 (*L. animalis*), and P43 (*L. reuteri*). In-silico analysis of the annotated genes of the three sequenced genomes was conducted in an attempt to identify genes for oxidative stress, sugar utilization, acid tolerance, bile salt tolerance, and bacteriocins. While *L. crispatus, L. acidophilus,* and *L. animalis* did display a strong performance in the carbohydrate utilization and growth in semi-defined media with specified sugars, they underperformed in the bile and acid tolerance portions of the study. Conversely, *L. reuteri* tolerated the two conditions. The findings of this study determined strain P43 (*L. reuteri*) to be the strongest candidate for use as a potential probiotic, due to its high acid and bile tolerance.

Characterization of *Lactobacillus* Isolates from Chickens

by
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BIOGRAPHY

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I love you.

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Literature Review

I.1 Introduction

Lactic acid bacteria (LAB), are found naturally in the oral cavity, gastrointestinal tract, and urinary tract of humans and animals [1]. They are useful in food production and food preservation practices and often times convey a biological advantage, such as disease prevention and immune system improvements, in human and animal physiology [2]. Certain LAB have been identified as being able to promote human health, specifically in the gastrointestinal tract, due to their ability to reduce pathogen colonization and survive to reproduce in bile and gastric acid environments [3]. The World Health Organization and the Food and Agriculture Organization of the United Nations defines probiotics as, "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [4]. A small number of lactic acid bacteria have been identified and are currently being used as probiotics to improve the gut health of humans and animals, however; more research is needed to identify additional probiotic strains for improvements in health as well as other applications.

I.2 Background of Lactic Acid Bacteria

Lactic acid bacteria were first described by Orla-Jensen in 1919 according to their, "morphology and cultural features, sources of energy, nutritive material utilized and the manner of utilization, temperature relations, and agglutination and other specific properties" [5]. Using these classification criteria, it was determined that lactic acid bacteria are Gram

positive rods or cocci, acid tolerant, non-respiring, non-spore forming, facultative anaerobic bacteria that produce short chain carboxylic acids, which reduces the surrounding pH, and ferments carbohydrates with lactic acid as the major end product of this process [6], [7], [8], [9]. Lactic acid bacteria can be characterized as obligate homofermenters, facultative heterofermenters, or obligate heterofermenters based on their utilization of the aldolase enzyme. Obligate homofermenters produce greater than 85% lactic acid from the utilization of hexoses. Facultative heterofermenters mainly break down hexoses to generate lactic acid, however; they can utilize some pentoses to generate lactic acid, acetic acid, and/or ethanol. Obligate heterofermenters can degrade hexoses and pentoses, generating lactic acid, acetic acid and/or ethanol, and CO_2 as byproducts of metabolism [10]. *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* are the traditional group members known as lactic acid bacteria, though there are others such as: *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* [9], [11]. They can grow at temperatures as low as 5˚C and as high as 53˚C , with an optimum temperature range of between 30˚C and 40˚C [12]. Lactic acid bacteria are able to grow and remain viable within a pH range of 4.5 to 7.0, with cells incurring damage at pH levels below this range [13]. LAB are found in plant matter, fermented foods (yogurt, cheese, olives, pickles, salami, etc.), the mouth, gastrointestinal tracts, and vaginas of both humans and animals [1]. LAB have established themselves as part of the normal microbiota of humans and animals, are non-pathogenic, and have been classified as generally recognized as safe (GRAS) [2].

I.3 Industrial Applications

I.3.1 Food and Beverage

For centuries, lactic acid bacteria have been used in food production and food preservation practices in dairy, meats, alcoholic beverages, and vegetables, through the process of fermentation. Fermentation is the use of microorganisms, their enzymes, and byproducts, for altering the taste, smell, texture, nutritional component, etc. of a food product [14]. Fermentation is initiated by microorganisms already present on substrates, or by those added as a starter culture [15]. A starter culture is a large inoculum of a microorganism(s) that are added to a food product to initiate the fermentation process [16]. In dairy products, lactic acid fermentation conveys an acidic flavor, aids in curd coagulation, contributes to texture in the cheese making process, and the low pH helps retard the growth of pathogens and spoilage microorganisms [17]. Examples of fermented milk products include: yogurt, cheeses, and sour cream. In meat fermentation, LAB contributes to reddening, flavor development, sliceability and spoilage resistance [18]. An example of fermented meat includes the many varieties of sausages. Lactic acid bacteria are also important in the development of many alcoholic beverages. In wine for example, malolactic fermentation is performed by LAB after the initial alcohol fermentation performed by yeast. L-malic acid is decarboxylated to L-lactic acid and CO_2, which reduces the acidity, contributes to the aroma, and improves microbial stability of the wine beverage [19]. Lactic acid bacteria are also used in the malting process of brewing beer; starter cultures are added to the malting process to improve its quality and safety, as well as improve the fermentation process, stability, color, and flavor of the beer [19]. In vegetable fermentations, lactic acid bacteria are important for improving

aspects of safety, nutrition, sensory, and shelf-life [20]. Fermentation processes can happen in one of two ways; spontaneously, by manipulating the vegetables' environment so as to encourage the growth of the lactic acid bacteria already present on the freshly harvested produce, or by using starter cultures [20]. Examples of fermented vegetable products include: pickles, sauerkraut, and olives.

I.3.2 Probiotics

In addition to food production and food preservation, lactic acid bacteria are used as probiotics to convey health benefits to humans and animals. As mentioned previously, probiotics are live microorganisms that, when introduced into a host, contribute positively to its overall health. In order for a microorganism to be classified as a probiotic, it must include an assessment of strain identity, in vitro tests to screen potential probiotics, an assessment of safety, and in vivo studies for substantiation of effects [21]. Specifically, the microorganism must not be pathogenic or carry antibiotic resistance genes, must survive in the gastrointestinal tract and be acid and bile tolerant, adhere to mucosal surfaces and colonize the intestine, produce antimicrobial substances such as lactic acid, hydrogen peroxide, and bacteriocin, and must be shelf stable [3], [22]. Not all strains with probiotic potential are created equal and each strain contributes its own range of beneficial health effects. Using analytical methods, such as: 16s rRNA gene sequencing (ribosomal ribonucleic acid), DNA-DNA hybridization (deoxyribonucleic acid), pulsed-field gel electrophoresis, and multilocus sequence typing, proper strain identification as well as strain-specific benefits of probiotics can be determined [23]. Doses of probiotic lactic acid bacteria can come from food products

or from oral pharmaceutical tablets or capsules. The daily recommended dose is five billion colony forming units a day (5.0×10^9 CFU/day) for at least five days [24]. Prebiotics are substrates that select for and stimulate the growth of an exclusive list of microbial species [25]. When prebiotics are administered along with probiotics, the preferential growth of these probiotic strains is induced. Thus, the use of probiotics with prebiotics forms a synbiotic relationship, which benefits the host.

I.4 Lactic Acid Bacteria- Beneficial for Human Health

The overarching goal of probiotics is to improve the health of its host, specifically in humans. The improvement in human's health may be accomplished in three ways by: (i) modulating the host's immune responses, (ii) effecting other microorganisms, either commensal and/or pathogenic, and (iii) neutralizing microbial and host metabolic byproducts, such as toxins and bile salts [26]. Different species and strains of lactic acid bacteria elicit different immune responses. LAB can stimulate both the innate and the adaptive immune responses by binding to specific receptors on immune cells, resulting in the production of cytokines, chemokines, T-cells, dendritic cells, and macrophages [11]. Lactic acid bacteria can impact the survivability of other bacteria occupying the same niche through secretion of antibacterial substances and competition of resources. Some probiotic strains have also been recognized as possessing the ability to bind to certain toxins and mutagen inducers and in turn, inactivating their detrimental effects on the host [26]. Probiotics have been recognized for their broad range of beneficial effects on human health. Figure 1 summarizes the specific health impacts probiotics have conveyed when studied in humans.

The beneficial effects of probiotic bacteria documented in human intervention studies.

Effect	Strain(s)	Comments	References
Treatment of acute diarrhoea in children, especially caused by rotavirus	META	Positive correlation	[18,19]
Reduction of the risk of antibiotic-associated symptoms in children and in adults	META	Positive correlation	[22]
Reduction of the recurrence of *Clostridium difficile* enterocolitis	299v	Preliminary, more research needed	[20,21]
Reduction of the risk of acute diarrhoea in children	LGG; BB12	Documented for certain strains	[79,80]
Relief of milk allergy/atopic dermatitis in infants	LGG; BB12; combination of *L. rhamnosus* 19070-2 and *L. reuteri* DSM 122460	Documented for certain strain(s), more research needed	[30–32,57,81]
Relief of allergic rhinitis	*L. paracasei* 33	Preliminary, more research needed	[33]
Reduction in the risk of atopic diseases in infants	LGG	4 years single study — repetition needed	[29]
Reduction in the risk of respiratory infections	LGG	Preliminary, more research needed	[23,24]
Amelioration of the immune response	Various	Documented for certain strains, more research needed	[82–85]
Reduction in the risk of dental caries	LGG	Preliminary, repetition needed	[86]
Suppression of *Helicobacter pylori*	Various	Preliminary, more research needed	[25–28]
Reducing the recurrence of pouchitis	VSL#3; LGG	Documented for certain combination(s), more research needed	[35,36]
Relief of IBS symptoms	299v	Preliminary, more research needed	[34]
Relief of rheumatoid arthritis symptoms	LGG	Preliminary, more research needed	[37]

The strains used are indicated, as are specific comments on the trials. META, indicates meta-analyses and LGG, 299v and BB12 refer to *L. rhamnosus* GG, *L. plantarum* 299v and *B. lactis* BB12, respectively.

Figure 1: Positive health impacts of probiotic bacteria on human health, from reference [27].[a]

[a] The references listed in the figure are found in the original publication, not in this manuscript.

I.4.1 Oral Health Impacts

The focus of most research centered around probiotics as of late, has been on the health impacts on the oral and gastrointestinal tract environments. The first component of the gastrointestinal tract begins in the mouth. In the oral cavity, *Lactobacillus* species comprise less than 1% of microbiota sampled from saliva, with *L. paracasei*, *L. plantarum*, *L. rhamnosus*, and *L. salivarius* being the most commonly isolated species [28]. To increase the number of *Lactobacillus* spp. present in the oral cavity, a probiotic supplement must be consumed. Recent studies suggest that certain strains of probiotic lactic acid bacteria can

exert antimicrobial actions against oral pathogens, such as *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Streptococci mutans*, which are associated with tooth decay and gum disease [28], [29]. In order for a probiotic microorganism to thrive in the oral cavity, they must possess defense mechanisms to combat harsh conditions in this environment, adhere to and colonize saliva coated tissue, and limit the growth of oral pathogens [29]. Inhibition of oral pathogens occurs through competition for nutrients, growth factors, and secretion of antimicrobials such as: acids, hydrogen peroxide, organic peroxide, diacetyl, and bacteriocins to name a few [7]. Researchers have found a strong association with LAB's ability to treat periodontal diseases, such as gingivitis and periodontitis. Reduced bleeding from the gums and lower levels of pro-inflammatory cytokines and other inflammatory markers measured in saliva lead to these findings [28]. These health effects are only observed with regular use of probiotics, as permanent colonization in the oral cavity is unlikely [7].

I.4.2 Gastrointestinal Tract Health Impacts

While contributions to the improvement of oral health are important, probiotics' claim to fame has long been associated with their improvements in the human gastrointestinal tract (GIT). The GIT is home to more than a trillion microbes, and while most have not been cultured in vitro, those that have are overwhelmingly Gram positive, just like probiotic lactic acid bacteria [3], [27]. In order for LAB to survive and colonize in the gut niche, they must possess some biological advantages to outcompete normal microbiota and pathogenic microorganisms in the environment. Some of these biological advantages, in addition to

aiding in the survival of probiotic LAB, also convey health advantages to the human host. Positive health effects from probiotics include: boosting intestinal epithelial cell function, regulating cytokine, T-lymphocyte, and antibody secretion, and curbing physiologic stress [30]. The human GIT is comprised of epithelial cells which make up the mucosal barrier; the first line of defense against pathogens. If the structure of the mucosal barrier is compromised, by inflammation for example, then pathogens can permeate through the mucosal barrier and travel on to infect the blood, spleen, etc. [11]. Probiotic bacteria possess structural and genetic features which can combat pathogens. Specifically, the structural components of lactic acid bacterial cells as well as certain byproducts they secrete, end up modulating the human host's immune response. For example, many Gram positive bacteria contain teichoic acid and lipoteichoic acid in their cell walls which serves to effect membrane permeability, influence extracellular relations, stabilize the plasma membrane, and assist with cell wall growth [31]. Within the human gastrointestinal tract, the teichoic acids present within the probiotic's cell wall become bound and recognized by pattern recognition receptors on epithelial cells. This results in the stimulation of the immune system as it works to determine if the bacteria is foreign or native, and subsequent mounting of an appropriate response [27]. When these probiotic bacteria are bound by receptors on epithelial cells, the immune response often times results in the activation of cell signaling pathways, such as the production of pro and/or anti-inflammatory cytokines and chemokines, depending on the probiotic strain. Activation of pro-inflammatory signaling molecules results in maturation of critical immune cells and inflammation of the intestinal epithelia to rid of pathogens in the area, while activation of anti-inflammatory signaling molecules results in repair to the

intestinal epithelial mucosal barrier after an inflammatory response [32]. Moreover, the act of probiotic bacteria binding to epithelial cells is an immunity boosting tactic, as this adhesion blocks pathogenic bacteria from being able to adhere to epithelial cells in the gastrointestinal tract, colonize, and cause infection.

I.4.3 Antimicrobial Secretions

Some probiotic strains secrete antimicrobials, such as short-chain fatty acids, hydrogen peroxide, and bacteriocins [33], [34]. Short-chain fatty acids, such as acetic and lactic acids, are byproducts of anaerobic carbohydrate metabolism. When these acids accumulate to high amounts, they lower the pH of the surrounding environment and inhibit microbial growth. Microbial inhibition can occur by several mechanisms; acids passively diffuse across the cell membrane and once inside, dissociate and acidify the cytoplasm, or the cell experiences osmotic stress due to acid anion generation by anaerobic carbohydrate metabolism [34]. The human GIT is primarily anaerobic, although there are pockets of oxygen near mucosal surfaces [35]. When lactic acid bacteria become exposed to oxygen (O_2), the NADH oxidase system takes over, which reduces O_2 to either hydrogen peroxide (H_2O_2) or water (H_2O) [36]. H_2O_2 is one of three partially reduced oxygen intermediates that can form when O_2 is reduced to H_2O. This conversion of O_2 to H_2O_2 can cause H_2O_2 to build up in the environment and can have an inhibitory effect on nearby cells, namely cells containing high amounts of iron ions. When H_2O_2 interacts with iron, it gets converted to a reactive hydroxyl radical ($HO^{\cdot}$) in a process known as the Fenton reaction. For those nearby cells containing iron (but no catalases or peroxidases) that interact with the H_2O_2 produced by LAB, deoxyribonucleic

acid (DNA) damage and eventual cell death occur [37]. Some lactic acid bacteria possess NADH peroxidase which directly converts H_2O_2 into H_2O, thus preventing progression towards the Fenton reaction [38]. In addition to short-chain fatty acids and hydrogen peroxide, lactic acid bacteria can secrete bacteriocins, another type of antimicrobial. Bacteriocins are produced by both Gram positive and Gram negative bacteria, are made by the ribosome of cells, and act like toxins towards other bacteria as a killing agent, with a limited killing capacity [34], [39]. Bacteriocins may function as colonizing peptides, facilitating the introduction and/or dominance of a producer into an already occupied niche. Alternatively, bacteriocins may act as antimicrobial or killing peptides, directly inhibiting competing strains or pathogens. Lastly, bacteriocins may function as signaling peptides, either signaling other bacteria through quorum sensing and bacterial cross talk within microbial communities or signaling cells of the host immune system (figure 2) [39]. Bacteriocins secreted by Gram positives are primarily effective against other Gram positive bacteria, though there are exceptions. Gram positive bacteria that may be encountered in the GIT and susceptible to bacteriocins produced by lactic acid bacteria include: *Bacillus*, *Clostridium*, *Enterococcus*, *Lactococcus*, *Listeria*, *Micrococcus*, and *Staphylococcus*, to name a few [34].

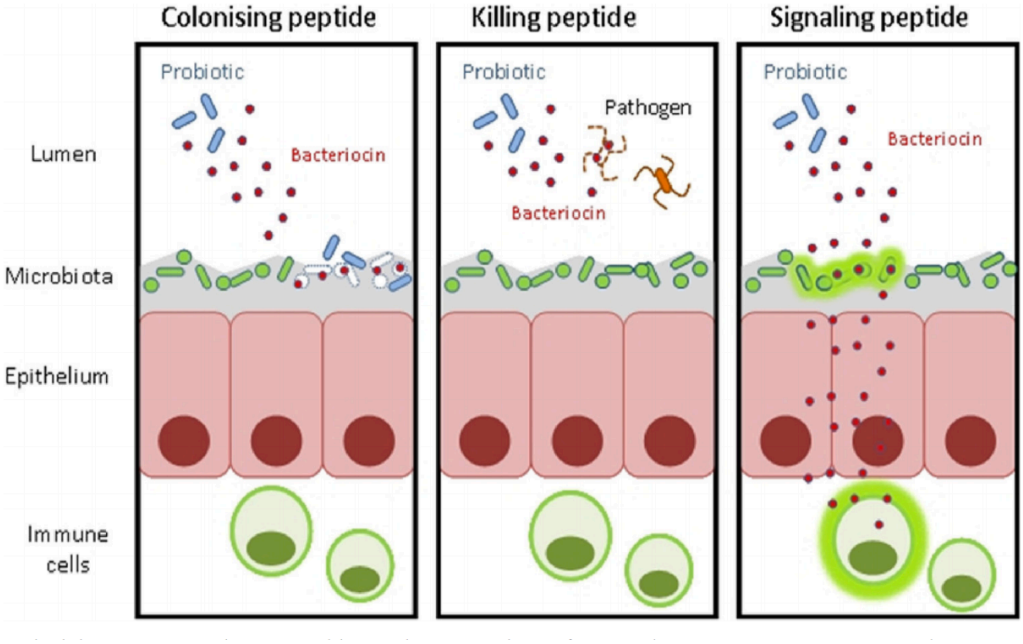

Figure 2: Mechanism of action of bacteriocins, from reference [39].

Gram negative bacteria encountered in the GIT that are susceptible to bacteriocins produced by LAB include *Campylobacter*, *Helicobacter,* and *Salmonella typhimurium* [34], [40]. Furthermore, bacteriocins, such as nisin, have been deemed safe for human consumption by the Food and Drug administration, and are an acceptable method for ensuring the preservation of foods by inhibiting the growth of pathogenic and spoilage microorganisms [34], [41].

I.4.4 Lactose Intolerance and the Health Impacts of Probiotics

These biological advantages and survival mechanisms elicited by lactic acid bacteria also aid in the treatment and prevention of a variety of illnesses and ailments in the human host.

Research studies have found that LAB can help lactose-intolerant individuals digest lactose. Individuals who can digest lactose have the enzyme lactase (β-galactosidase) present in copious amounts in their small intestine. Lactose is commonly consumed in dairy products and β-galactosidase serves to help the body break it down into glucose and galactose [42]. Lactose-intolerant individuals have low levels of β-galactosidase; this results in the buildup of lactose, which causes uncomfortable symptoms such as bloating, cramping, and watery diarrhea [43]. Most probiotic strains produce β-galactosidase as a metabolic byproduct, so ingesting foods with these probiotic strains added to them can promote lactose digestion, and eliminate the uncomfortable symptoms. It has been extensively documented that strains of lactic acid bacteria are able to treat diarrhea caused by antibiotics and foodborne pathogens, due to their ability to compete with pathogens for nutrients, prevent binding to epithelial cells, and secrete antimicrobials, such as bacteriocins [44]. Irritable bowel diseases, such as Crohn's disease and ulcerative colitis, may also be treated with probiotic LAB. People suffering from irritable bowel diseases possess microbiota that have diminished numbers of lactic acid bacteria and increased numbers of *Escherichia coli*, coliforms, and bacteroides in the colon [45]. In response to this unfavorable microbiota, the body signals continuous inflammation in the gastrointestinal tract. Probiotics containing lactic acid bacteria may restore the beneficial host gut microbiota and limit inflammation in the gastrointestinal tract [45].

I.4.5 Allergy Health Impacts

Studies have shown that lactic acid bacteria may also have an effect on allergies. Between 30-40% of the world's population have an allergy [46]. Allergies are the result of the body's immune system responding to some environmental factor that is harmless. Common allergens are pollen, mold, and foods. When the body encounters an allergen, the immune system increases the production of immunoglobulins to eliminate the allergen. Probiotic LAB can modulate the host's allergic reaction response by influencing the production rate of the immunoglobulins.

I.4.6 Cardiovascular Health Impacts

There have also been improvements to cardiovascular health with probiotic supplements. Cholesterol is important in maintaining cardiovascular health; there are two types of cholesterol, low density lipoprotein (bad for health) and high density lipoprotein (good for health). Studies have shown that through mechanisms such as, bile salt deconjugation, absorption of cholesterol by bacterial cell membranes, and production of short chain fatty acids, probiotics can decrease low density lipoprotein and total cholesterol, increase high density lipoprotein cholesterol, lower systolic blood pressure, and upregulate antioxidant activity [47]. These health impacts observed as a result of exposure to probiotics reduces the chances of developing cardiovascular disease. Additionally, probiotic lactic acid bacteria also manage and improve hypertension, body weight, and Diabetes mellitus [47].

I.5 Physiological and Biochemical Properties of Lactic Acid Bacteria

I.5.1 Carbohydrate Metabolism

Bacteria can be classified according to their oxygen requirements: obligate aerobes, microaerophiles, facultative anaerobes, or obligate anaerobes. Their oxygen requirements are directly related to their ability to respire or ferment carbohydrates. LAB tend to be facultative anaerobes, growing to the same concentration in the presence or absence of oxygen. LAB can also either be homofermentative or heterofermentative. Homofermentative LAB utilize glycolysis. Glycolysis is characterized by the splitting of a hexose sugar (glucose, fructose, mannose, etc.) into two triose sugar molecules; the triose sugars are converted to 2 pyruvate that are further reduced to 2 lactate [48]. This pathway generates two moles of ATP for every mole of sugar consumed and relies on the aldolase enzyme to breakdown hexoses. Homofermentative LAB generate lactic acid as their only end product. Heterofermentative LAB utilize the pentose phosphate pathway. In this pathway, a hexose sugar is converted to a pentose sugar and CO_2. The pentose sugar is then converted to lactate and acetate or ethanol as end products of the fermentation process [6]. The pentose phosphate pathway generates one mole of ATP for every mole of hexose sugar utilized.

I.5.2 Defense Against Reactive Oxygen Species

Bacteria are exposed to a wide array of environmental conditions and in order for them to have the best chance at survival, they must possess a variety of physiological and biochemical properties to cope with the stress. When exposed to oxygen (O_2), most LAB are subjected to oxidative stress, as the oxygen intermediates generated during oxygen reduction

are toxic to the cells. The reduction of oxygen to water requires the addition of 4 electrons and 4 protons. In aerobic organisms, the reduction of oxygen to water is carried out by the cytochrome oxidase system; and ~95% of the oxygen consumed is tetravalently reduced to water without the release of partially reduced oxygen intermediates. The remaining ~5% of the oxygen consumed is reduced via the univalent pathway which generates the toxic partially reduced oxygen species (i.e., O_2^-, H_2O_2, and $HO^\cdot$) [49], [50]. These intermediates, called reactive oxygen species (ROS), can pose a threat to cells. Reactive oxygen species induce oxidative stress in the cell and can damage cellular proteins, lipids, and DNA, causing cell death [51]. Sources of ROS include: autoxidation of reduced metabolites with low redox potential and enzymes capable of donating electrons to oxygen (O_2) [51]. Certain species of lactic acid bacteria can possess NADH, pyruvate, and/ or lactate oxidase which have the potential to carry out electron transfers that generate O_2^- and H_2O_2 [52]. $HO^\cdot$ is formed when H_2O_2 and ferrous iron salts react with one another in a process known as the Fenton reaction. This hydroxyl radical is extremely toxic and damages proteins, DNA, and the plasma membrane [51]. In addition to oxidases, superoxide dismutase is an enzyme some species of LAB possess that converts the superoxide anion radical into hydrogen peroxide or water. Additionally, intracellular concentrations of manganese ions can be used by the cell to accomplish the same reduction of O_2^- to H_2O_2 when superoxide dismutase is absent [53]. When high amounts of H_2O_2 are generated in the presence of oxygen, some species of LAB prematurely enter into stationary phase and have a reduced concentration [51]. Special enzymes can further convert H_2O_2 into a harmless byproduct; catalase converts H_2O_2 into oxygen and water, whereas peroxidase converts H_2O_2 to only water, though catalase is

typically absent in lactic acid bacteria. Additionally, H_2O_2 is permeable like water and can diffuse out of the cell into the surrounding environment where it becomes diluted. Finally, LAB also lack iron containing components within the cell, which blocks the Fenton reaction and subsequence generation of $HO^{\cdot}$. In the absence of an iron ion catalyst, the hydrogen peroxide reacts too slowly with other biological materials to cause irreparable damage to the cell [54].

I.5.3 Acid Tolerance

As lactic acid bacteria make their way through the gastrointestinal tract, they encounter extremely acidic conditions in the stomach (pH 1-2). As part of their normal metabolic processes, LAB produce high amounts of acid into the surrounding environment. Low pH environments are harmful to most cells, so lactic acid bacteria must be able to adapt under acid stress conditions. When the pH is low, protonated organic acids can passively diffuse through the cell membrane and into the cytoplasm, where they dissociate into protons and charged byproducts that can no longer leave the cell without assistance. This results in a substantial accumulation of protons in the cytoplasm, which lowers the internal pH of the cell. Any change to the cytoplasmic pH effects the electrochemical gradient of protons and the proton motive force. The proton motive force is the energy source used in various transmembrane transports. Additionally, the acidification of the cytoplasm lowers the activity of acid-sensitive enzymes and damages proteins and DNA [55]. One method lactic acid bacteria use to combat this is by lowering the cytoplasmic pH as the extracellular pH decreases, so as to shorten the severity of the gradient, however; once a critical value acidic

pH is reached (strain dependent), cellular functions cease [56]. The ATPase enzyme is responsible for synthesizing ATP using protons or expelling protons out of the cell with the energy provided by ‘ATP hydrolysis. In LAB, the latter activity increases at low pH and is crucial to maintaining a stable pH gradient [55]. Lactic acid bacteria can also utilize the arginine deaminase pathway to manage pH homeostasis. Three enzymes are involved in the arginine deaminase pathway: arginine deaminase, ornithine cabamoyltransferase and carbamate kinase. They are responsible for catalyzing and converting arginine into ornithine, ammonia, and carbon dioxide. This reaction yields 1 mol of ATP per 1 mol of arginine. The ammonia then reacts with H+ protons to assist in the alkalization of the environment. The ATP that was generated can then be utilized by ATPase to expel excess H+ out of the cytoplasm, thus raising the intracellular pH [55]. Decarboxylation reactions are another way LAB can use up excess protons that have accumulated in the cytoplasm. When a carboxylic acid compound enters into the cell, a proton is required to decarboxylate it. The decarboxylated compound is then shifted to an electrogenic transporter, which generates a molecule of ATP and exports the decarboxylated compound from the cell [55]. In the event that a cell is injured from high acid environments, repair mechanisms can be called upon to fix the damage. Chaperone proteins can help repair and refold acid damaged proteins and an acid-inducible RecA-independent DNA repair system can be called upon to repair DNA damaged by low pH environments [55].

I.5.4 Bile Salt Tolerance

Stomach acid (composed of chloric acids) and lactic acid are not the only acids LAB encounter in the gastrointestinal tract; bile acids are also present and pose a threat. Bile, produced in the liver, stored in the gallbladder, and released during digestion into the small intestine, aids in the solubilization and absorption of dietary fats [57], [58]. Bile is composed of bile acids which are produced in the liver. When bile acids become conjugated with amino acids glycine and taurine, they form bile salts. Acids are in the unconjugated form, while salts are in the conjugated form [58]. Cholic acid and deoxycholic acid are the most predominant bile acids in the human intestine and under normal physiological conditions, the concentration ranges between 2% and 0.05% [57],[58]. "Bile acids are surface active, amphipathic molecules, that act as detergents and disassemble biological membranes [55]." As happens with stomach and lactic acid, bile acids can passively diffuse into the cell and cause acidification of the cytoplasm. In addition, bile acids and salts can stimulate oxidative stress and DNA repair processes, changes in sugar metabolism, and improper folding of proteins [58]. To combat these lethal effects from bile, LAB have developed resistance mechanisms, such as bile salt hydrolases, active efflux of bile acids/salts, and changes in the architecture/composition of the cell membrane and cell wall. Bile salt hydrolases are enzymes that de-conjugate the glycine and taurine amino acids from bile salts, yielding unconjugated acids, which other gut bacteria can metabolize [58]. This also decreases detergent properties and the solubility of bile at low pH [55]. Since the unconjugated form is weaker than the conjugated form, the proton can be recalled, thus raising the pH in the immediate vicinity [58]. Bile-efflux systems are common in bacteria and make use of

multidrug transporters, whose activity depends on the proton motive force or ATP hydrolysis, to pump out bile acids and salts actively accumulating in the cytoplasm [55], [58]. LAB may also change the lipid composition of their bacterial membranes through changes in the production of proteins involved in fatty acid metabolism. These changes reduce bile's capability of passively diffusing into the membrane and improve the hydrophobicity of the cell membrane [55]. In addition, LAB can produce external exopolysaccharide layers that act as a protective coating against bile and other unfavorable environmental conditions [55]. These physiological and biochemical properties allow for the persistence and survival in unfavorable environmental conditions.

I.6 Lactic Acid Bacteria in Poultry

Lactic acid bacteria are also indigenous in poultry. There can be species variation between lactic acid bacteria found in the different sections of the gastrointestinal tract, however; they all contribute to the symbiotic interactions with others in the microbial community and to the overall health of the chicken [59]. The variability in species is related to the age, growth environment, and diet of the chickens [59]. Studies have found that *Lactobacillus* strains predominate in the ileum and jejunum of the small intestine at 68.7% as compared to *Streptococcus* (6.6%) and *Enterococcus* (6.4%). In the cecum, these numbers rapidly decline, with *Lactobacillus* at 8.2%, while *Streptococcus* and *Enterococcus* fall to 0.7% and 1% respectively [59]. As happens in humans, poultry are susceptible to being colonized by pathogenic bacteria, such as *Campylobacter jejuni*, *Salmonella spp*., *Escherichia coli*, and *Clostridium perfringens* [59], [60]. While *C. jejuni* can cause a systemic infection in poultry,

this does not result in any pathological symptoms, however; the same cannot be said for the other three pathogens [61]. For example, *Salmonella* can cause diarrhea, intestinal lesions, damage to villi, drop-head, wing prolapse, white loose stool, and high mortality, *C. perfringens* can cause necrotic enteritis of the small intestine, and Avian pathogenic *Escherichia coli* can cause aerosacculitis, polyserositis, septicemia, and other diseases [60], [62], [63]. Lactic acid bacteria can reduce the disease potential and colonization rate of these pathogens by disrupting cell-cell communication [59]. Some LAB strains produce antimicrobials, such as organic acids and bacteriocins, which impact colonization, proliferation, and survival [64]. In fact, incorporating probiotics into chicken feed also promotes the health of poultry by preventing colonization of unwanted microorganisms through increased competition for space and nutrients, improving the immune system of the host, and strengthening the gut barrier [65]. As an added benefit, with this decrease in pathogen numbers in poultry, those who consume these animal products should have a reduced chance of ingesting a pathogen and becoming sickened. Finally, the use of probiotics in the poultry industry has yielded welcome results, such as enhancement of chicken body weight, feed conversion ratio, and egg weight [64].

I.7 Conclusion

Lactic acid bacteria have been used successfully as probiotics in improving the health of both humans and animals. The World Health Organization has imposed strict guidelines for the classification of bacteria as probiotics. While many LAB meet the requirements and are considered to be probiotic bacteria, the *Lactobacillus* genera is of particular interest. In fact,

the species with the greatest potential for probiotic properties are: *Lactobacillus rhamnosus, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus delbrueckii subsp. bulgaricus,* and *Lactobacillus plantarum* [46]. In the present study, six 16s rRNA sequence-confirmed strains of lactic acid bacteria of the *Lactobacillus* genera were isolated from the gut of a chicken; (1) *L. crispatus*, (3) *L. animalis*, (1) *L. acidophilus*, and (1) *L. reuteri*. *L. crispatus* was isolated from a chicken fed a normal diet, while the others were isolated from chickens fed a normal diet supplemented with 1% of the prebiotic galactooligosaccharide (GOS). Physiological and biochemical characterizations were conducted to determine the probiotic potential of these isolates.

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CHAPTER II

Physiological and Biochemical Tests Characterizing *Lactobacillus* isolates from Chickens

II.1. Abstract:

The probiotic potential (bacterial strains ability to impart beneficial health impacts on the host) of six identified *Lactobacillus* isolates was assessed based on their physiological and biochemical characteristics. The strains were sequenced using 16s rRNA gene sequencing and subjected to a series of biochemical tests including: Gram stain and cellular morphology, growth kinetics and final pH in aerobic versus anaerobic conditions, carbohydrate utilization, growth in semi-defined media with specified sugars, catalase activity, acid tolerance, and bile tolerance. Additionally, the whole genome of three strains underwent shotgun sequencing. The sequencing results detailing the possible possession of specific proteins and enzymes, coupled with the strains' performance in high acid and bile conditions ultimately led to the determination of strain *L. reuteri* as exhibiting the most probiotic potential.

II.2. Significance:

Humans and animals are susceptible to many diseases, which then get treated or managed with antibiotics, antifungals, antivirals, and the like. Probiotic bacteria are a natural means to prevent as well as treat certain diseases; however, probiotics are species specific and are native to a specific host [1]. Therefore, it is important to use probiotic isolates relevant to the host to be applied for health benefits. By identifying new potential probiotic bacteria specific for poultry, these could be used in commercial poultry applications to prevent the spread of

antibiotic resistance and foodborne illnesses. This study utilized several methods, through physiological testing and genome evaluations, to identify chicken specific *Lactobacillus* isolates with probiotic potential.

II.3. INTRODUCTION:

The characterization of *Lactobacillus* isolates through physiological and biochemical testing serves to provide useful information for commercial application and/ or future studies. *Lactobacillus* species are part of the group known as lactic acid bacteria, which are naturally found associated with gastrointestinal tracts of humans and animals and have been shown to convey beneficial health impacts to these hosts, such as stimulating the host's immune response and inhibiting competing pathogens [2], [3], [4]. In fact, these positive health impacts are so important that these bacteria are being willfully consumed and marketed as probiotics. The World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations define probiotics as, "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [5]. In order for a bacterial strain to be characterized as a probiotic, it must pass a series of assessments and guidelines specified by the WHO (be non-pathogenic, acid and bile tolerant, produce antimicrobials, etc) [3], [5]. The ultimate goal is to isolate chicken-specific probiotic organisms. In a previous study, 102 lactic acid bacteria strains were isolated from chicken gut (Rezvani and Hassan – unpublished). The objective of this study was to narrow down the list of the 102 potential probiotic strains and select the best potential candidates for future application in poultry. After further characterizations, six isolates belonging to the

Lactobacillus genera were selected for further studies to determine their probiotic potential. Tests performed in this study include: sequencing of 16s rRNA genes, Gram stain and cellular morphology, final cell concentration and final pH in aerobic and anaerobic conditions, carbohydrate utilization, growth in semi-defined media with sugars and prebiotic, catalase activity, acid tolerance and survival, bile salt tolerance and survival, and whole genome sequencing.

II.4. MATERIALS AND METHODS

II.4.1. Bacterial Strains:

The cultures used in this study are listed in Table 1. The cultures used were from a previous study aimed at enriching and isolating lactic acid bacteria (LAB) from cecal samples of 4-weeks old layer chickens (M. Rezvani and H. Hassan). The Cecum samples from control chicken (#337) and from $GOS_{56\%}$ (0.5%) fed chicken (#365) were collected and suspended in PBS containing 25% glycerol at a final concentration of 100mg/mL; samples were stored at -80^{o}C. $GOS_{56\%}$ is composed of 56% GOS and 44% glucose.

Table 1: Bacterial Strains Used

Strain #	Phenotype/Genotype	Source
C-Group	G^+ isolates from Control Birds	Rezvani and Hassan
P-Group	G^+ isolates from $GOS_{56\%}$ Fed birds	Rezvani and Hassan
C25	*Lactobacillus crispatus*	Present study
P17	*Lactobacillus animalis*	Present study
P28	*Lactobacillus animalis*	Present study
P38	*Lactobacillus animalis*	Present study
P42	*Lactobacillus acidophilus*	Present study
P43	*Lactobacillus reuteri*	Present study

II.4.2. GOS Enrichment:

Fifty µL aliquots from the frozen samples were spread anaerobically on modified deMan-Rogosa-Sharp (MRS-$GOS_{90\%}$) (where glucose in the MRS media was replaced with 0.5% $GOS_{90\%}$-90% GOS purity), and the plates were incubated anaerobically for 48 hours at 37^{o}C in a Coy Anaerobic Chamber (Coy Lab Products, Grass Lake, MI). Cells growing on the surface of each plate were collected anaerobically in 1.00 mL PBS containing 25% glycerol. The cell suspensions were diluted to 10^{-3} and 50 µL aliquots were re-enriched anaerobically on MRS- $GOS_{90\%}$ plates. After 48 hours of incubation the cells from each plate were collected in 1.00 mL of MRS- $GOS_{90\%}$ containing 25% glycerol and stored at -80^{o}C.

II.4.3. Purification of the Different Isolates:

MRS plates (~20) were each T-streaked with a loop-full of the GOS-enriched cultures and incubated anaerobically at 37^{o}C for 48 hours. Single colony isolates with various colony shapes and morphologies were selected, and re-streaked for several transfers till pure colonies were obtained. Preliminary screening of the selected colonies was conducted to determine colony morphology, Gram stain, cell shape/morphology using phase microscopy, production of acid, and coagulation of skim-milk. A Total of 102 isolates were selected and stored at -80^{o}C in MRS-25% glycerol. Six isolates were selected for further studies as reported in this manuscript (Table 1).

II.4.4. Media Growth Conditions and Measurements:

MRS media (Becton Dickinson, NJ), modified MRS (MMRS) per liter of water: Bacto peptone- 10g, Beef extract- 10g, Yeast extract-5g, Dextrose-20g, Ammonium citrate dibasic-2g, Magnesium sulfate-0.1g, Manganese sulfate-0.05g, and Dipotassium phosphate-10g, and altered MRS (AMRS) per liter of water: Tryptone- 10g, Yeast extract- 2g, Sodium acetate-3g, Dipotassium phosphate- 2.6g, Ammonium citrate- 2g, Magnesium sulfate- 0.1g, Manganese sulfate- 0.05g, and Citric acid- 0.75g [Conda Lab, Spain) were used in this study. Solid media were prepared by adding 1.5% agar to MRS or MMRS. Cultures were grown at 37^{o}C under anaerobic conditions in a Coy Anaerobic Chamber (H_2 10%, CO_2 5%, and N_2 85%) (Coy Lab Products, Grass Lake, MI). For aerobic growth, static (without shaking) cultures were grown in 20mL of media/ in 125mL flasks at 37^{o}C in the specified media in an aerobic incubator. Cell concentration was measured as Optical Density at 600nm (OD_{600}) using a BioRad Smartspec 3000 with a 1 cm light path (BIO-RAD, SmartSpec 3000, PA). Theoretically, there is a positive correlation between the OD_{600} and the concentration of the cell suspension; the higher the OD reading, the higher the concentration of cells in a sample. When a sample is placed inside a spectrophotometer, a beam of light is shined at the sample. As the light passes through the cell suspension, some of the light energy is converted to a different form of energy while some is scattered in different directions. Since most bacteria are colorless, less light is absorbed and more light is scattered. A photosensor measures this light scattering and assigns a value, reflecting the amount of biomass present in that sample [6], [7]. If the cell density in the suspension is too high, the scattered light will bounce from cell to cell and the relationship between cell density and OD_{600} becomes non-linear.

Therefore, we diluted the cultures to make sure the OD_{600} readings are in the linear range (OD_{600} ~0.6).

II.4.5. Gram Stain and Cellular Morphology:

From the -80˚C freezer stock, each strain was T-streaked onto MRS agar plates and incubated in the anaerobic chamber overnight at 37˚C. A colony of each isolate was then aseptically transferred to a pre-moistened microscope slide, allowed to air dry, and then heat fixed. Using the reagents provided in the Gram staining kit (Becton Dickinson, NJ), a Gram stain was performed on each isolate. Gram stained slides were then observed under 100x oil immersion (Cargille, NJ) using a Nikon Alphaphot-2 YS2 compound microscope (Nikon, Japan). Gram stain results were recorded as positive if purple in color or negative if pink in color. Cellular morphology was also determined.

II.4.6. Relationship between OD_{600} and viable count (CFU/mL):

It was necessary to determine the colony forming units per milliliter (CFU/mL) in 1 OD_{600}. Knowing this information would help to adjust and control bacterial densities and inoculum amounts in a specific range for use in subsequent microbiological studies. To assess the relationship between OD_{600} and CFU/mL, each of the *Lactobacillus* isolates were cultured in a 125mL flask and incubated anaerobically in 20mL of deMan-Rogosa-Sharp (MRS) broth in the anaerobic chamber overnight at 37˚C. The Optical Density at 600nm (OD_{600}) of *E. coli* K-12 strain NC4468 was also determined in the same manner using the MMRS media. The OD_{600} was read. The spectrophotometer was blanked using phosphate-buffered saline (PBS)

at pH 7.4 to minimize any increase in cell concentration. The culture flask was vortexed before the reading. Following the OD_{600} reading, the culture was serially diluted 10-fold in PBS and plated (100µL) on MRS agar at a 1.5% agar composition in duplicate. Plates were incubated anaerobically for 24-48 hours and colonies counted. This procedure was carried out three times, the readings averaged, and 1 OD_{600} standards determined. 1 OD_{600} standards for each strain communicate the expected concentration of viable cells in 1mL of an overnight culture. This information could then be used to customize starting OD_{600} values for subsequent experiments. This procedure was carried out for all six *Lactobacillus* isolates as well as an *E. coli* K-12 strain NC4468 to be used as a control in future experiments.

II.4.7. Growth Kinetics, Final Cell Concentration (OD_{600}), and final pH in MRS Media in Presence and Absence of Atmospheric Oxygen:

From the -80˚C freezer stock, each *Lactobacillus* strain isolate was cultured in a 125mL flask in 20mL of MRS and incubated statically and anaerobically overnight at 37˚C. The Optical Density at 600nm (OD_{600}) was read using the BioRad spectrophotometer. The culture flask was vortexed before the reading. The OD_{600} was adjusted to approximately 1.0×10^7 CFU/mL and then this concentration was inoculated into six separate 125mL flasks each containing 30mL of MRS broth (pH 6.8) (total final cell concentration of each flask was 3.0×10^8 CFU), and incubated statically; three flasks were incubated aerobically and three flasks were incubated anaerobically at 37˚C. OD_{600} readings of each flask were taken every hour for eight hours, including a time zero reading, with a final reading taken at 24 hours. The OD readings from 0-8 hours were used to calculate the average specific growth rate [$k.h^{-1}$] and

generation time [g in minutes] with standard deviation of each strain in aerobic versus anaerobic conditions. Maximum growth concentration (final OD_{600}) and final pH (Fisher Scientific pH meter, NH) were measured for each replicate at 24 hours and averaged. This procedure was repeated for all six *Lactobacillus* isolates.

II.4.8. Carbohydrate Utilization:

API 50 CH (bioMerieux, NC) is a test strip of 49 carbohydrates and 1 control used for identifying microorganisms' ability to metabolize specific carbohydrate substrates. API 50 CH was used for assessing carbohydrate utilization in this study. The following is a summary of the protocol as per the instruction of the supplier. Each *Lactobacillus* strain isolate was streaked onto an MRS agar plate and incubated overnight at 37˚C in the anaerobic chamber. A cell suspension with an OD_{600} of 0.4 was prepared for each of the 6 isolates by scrapping cells from the overnight plates and suspending in 10mL of sterile deionized water placed in sterile 15mL conical tubes. In a new sterile conical tube, 5mL of the bacterial suspension was combined with 5mL of 2x bromocresol purple media to make this a 1x solution. The API 50 CH strips were prepared by moistening the wells with water and the excess water dumped out. The strips were laid on top of the wells in numerical order. 100µL of the bacterial suspension with the bromocresol purple was added to each of the wells in the test strip kit. The wells were then overlaid with 100µL of mineral oil. Any air bubbles that remained were removed by gently tapping the strips against the bench. The lid was then placed over the kit and incubated in the Coy chamber at 37˚C for 48 hours. The test strips were read at 24 and 48 hours. This procedure was carried out for all six *Lactobacillus* isolates.

II.4.9. Growth on Glucose, Galactose, Lactose, and GOS in semi-defined media (final OD_{600} concentration and final pH):

AMRS was used for this study. This AMRS formulation was selected because we are interested in GOS utilization and the API 50 CH did not contain this sugar. We used this modified media that gives minimum growth in absence of added sugars. We included glucose, galactose and lactose for comparison. This AMRS eliminated polysorbate 80, sugar content, and all potentially nutritive ingredients from the media composition. AMRS was transferred to slip cap tubes in 10mL volumes and autoclaved. The sugars tested include: glucose, galactose, lactose, and galactooligosaccharide (GOS). 90% GOS was gifted courtesy of Dr. José Bruno-Bárcena (Department of Plant and Microbial Biology, North Carolina State University). Stock solutions of the sugars were prepared at the following final concentrations: glucose- 37% w/v, galactose- 33.3% w/v, lactose- 49% w/v, and GOS- 48% w/v. Glucose and galactose were heat sterilized in the autoclave; while lactose and GOS were filter sterilized. Sugars were then added to the sterile AMRS broth tubes at final concentrations of 0.2%, 0.5%, 1%, and 2%. From the -80˚C freezer stock, the *Lactobacillus* isolates were inoculated into sterile MRS broth and incubated statically overnight in the anaerobic chamber at 37˚C. The cells were spun down in the centrifuge (Sorvall RC-5B Refrigerated Superspeed Centrifuge, MA) for 15 minutes at 13,880xg, resuspended in 10mL of sterile PBS, and the OD_{600} measured. 10µL of the overnight culture was then inoculated into the sugar tubes in duplicate. The OD_{600} of the inoculated sugar tubes were read at 0, 24 and 48 hours; the readings at each time point were averaged. The pH was also read at 48 hours and averaged. This procedure was carried out for all six *Lactobacillus* isolates.

II.4.10. Catalase Activity:

From the -80˚C freezer stock, each *Lactobacillus* or *E. coli* strain isolate was T-streaked onto a sterile MRS plate (MMRS for *E. coli*) and incubated between 24-48 hours in the anaerobic chamber at 37˚C. A colony from each isolate plate was transferred to a microscope slide, and one drop of 3% hydrogen peroxide was applied. A positive test resulted in the formation of bubbles within 15-30 seconds, while a negative test resulted in no bubbles formed.

II.4.11. Acid Tolerance and Survival:

Phosphate-buffered saline (PBS) was used for this part of the study (0.2645 g, Sodium phosphate Monobasic dihydrate; 1.15 g, Sodium phosphate dibasic; and 9.0 g, Sodium chloride per liter of Deionized H_2O). All ingredients and chemicals used were from Fisher Scientific. The pH of PBS was read (Fisher Science Education pH meter, NH) and adjusted to 7.0, 4.0, 3.0, or 2.0, using 1N HCL, 9mL volumes of the pH adjusted PBS solutions were transferred to slip cap tubes, and the tubes autoclaved. *E. coli* K-12 strain NC4468 grown in MMRS was used as a control in this experiment. The experiment was performed in the anaerobic chamber. From the -80˚C freezer stock, the *Lactobacillus* strain isolate was inoculated into a 50mL sterile conical tube containing 20mL of sterile MRS broth and incubated statically overnight anaerobically at 37˚C. The cells were spun down in the centrifuge for 15 minutes at 13,880xg. The supernatant was discarded, and cells were resuspended in 10mL of PBS at pH 7.4. The Optical Density at 600nm (OD_{600}) was adjusted to yield $1.0x10^8$ CFU/mL. From the 10^8 CFU/mL tube, 1mL was transferred into 9mL of PBS adjusted to pH 7.0, 4.0, 3.0, or 2.0 (i.e., initial CFU/mL equals $1.0x10^7$). At time

intervals 0-, 2- , 4-, 6-, 8- , and 24-hours, 1mL from the acid treatments were transferred into 9mL of PBS adjusted to pH 7.4 for acid neutralization purposes, resulting in a final CFU/mL of $1.0x10^6$. New neutralization tubes containing PBS at pH 7.4 were used for each pH and each time point. Spot plating (10µL) was carried out in duplicate on MRS plates and incubated anaerobically for 24 hours at 37˚C. Viable cell counts were also conducted from the overnight culture tubes exposed to the different pHs ($T_0 = 1.0x10^8$ CFU/mL). 10-fold serial dilutions were carried out in PBS and plated (100µL) on MRS agar plates in duplicates. Plates were incubated anaerobically for 18-48 hours at 37˚C. This procedure was carried out for all six *Lactobacillus* isolates and the *E. coli* K-12 strain NC4468 control.

II.4.12. Bile Salt Tolerance and Survival:

E. coli K-12 strain NC4468 was used as a control in this experiment and had to be grown in MMRS medium. MacConkey agar utilizes bile salts and crystal violet to select for Gram negative bacteria and inhibit Gram positive bacteria, so the rationale was that Gram negative *E. coli* would grow to high cellular concentrations even in the presence of bile salts. All *Lactobacillus* strains were incubated in MRS media. From the -80˚C freezer stock, each *Lactobacillus* strain isolate was inoculated into a 50mL sterile conical tube containing 20mL of sterile MRS broth and incubated statically overnight in the anaerobic chamber at 37˚C. The cells were spun down in the centrifuge for 15 minutes at 13,880xg. The supernatant was discarded, and cells were resuspended in 10mL of MRS for *Lactobacillus*, and MMRS for *E. coli* K-12 strain NC4468. The OD_{600} was adjusted to 0.5 using the BioRad spectrophotometer and blanked with MRS or MMRS, depending on the strain used. From the 0.5 OD_{600} tube,

1mL was transferred into 9mL of MRS containing Oxgall bile salts (Sigma-Aldrich, MO) at 0%, 0.3%, 0.4%, and 0.5% in duplicates (starting OD was 0.05). Bile salt tubes were incubated in the Coy chamber at 37˚C and the OD_{600} read at 0- and 3-hours. Viable cell counts were also determined for each concentration of the bile salt at 0- and 3-hours. This procedure was carried out for all six *Lactobacillus* isolates and the *E. coli* K-12 strain NC4468 control.

II.4.13 Shotgun Whole Genome Sequencing:

The whole genomes of *L. crispatus* (C25), *L. animalis* (P38), and *L. reuteri* (P43) isolates were sequenced in collaboration with RTI International. DNA was extracted from cells, which were grown anaerobically for 20 hours in MRS media, using the Promega Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI). Paired-end libraries were created for each of C25, P38, and P43 with an average insert size of 251bp. Libraries were sequenced on Illumina MiSeq (Illumina, San Diego, CA) at Argonne National Laboratory (Lemont, IL). Modal kmer coverage were 1060x, 1100x, and 2200 x for C25, P38, and P43, respectively. After error correction, reads were assembled using MIRA v4.9.5 (Open source: http://genome.cshlp.org/content/14/6/1147.full). Final reported coverages were 79x for C25, 90x for P38, and 60x for P43. After assembly, contigs with less than 20x coverage or length of less than 200 bp were discarded. The length of the draft genome of *L. crispatus* C25, *L. animalis* P38, and *L. reuteri* P43 are 22,341,728 bp, 151,063 bp, and 1,940,664 bp with G+C content of 36.8%, 41.1%, and 38.7%, respectively.

Distance Tree generation:

Phylogenetic trees were built for each strain using other *Lactobacillus* genomes for comparison. The results placed each strain closest to its presumed species: C25 with *L. crispatus*, P38 with *L. animalis*, and P43 with *L. reuteri*, giving evidence that the three strains were indeed the expected species, and that the assembly was of high quality. In addition, we assessed assembly quality by comparing known metabolism of each strain to both hand- and RAST-annotated functionality. The draft genomes were annotated using the NCBI Prokaryotic Genome Annotation Pipeline (www.ncbi.nlm.nih.gov/genome/annotation_prok/).

II.5. RESULTS AND DISCUSSION

II.5.1 Sequencing of 16s rRNA genes:

Purified colonies from 41 isolates were sent to Genewiz for 16S rRNA gene sequencing. Genewiz uses the V1-V8 hypervariable region of the 16S rRNA gene. The sequences were compared to other 16S rRNA gene sequences available in GenBank using NCBI blast library (Table 2). Six isolates (highlighted in yellow) were selected for further studies: C25, *Lactobacillus crispatus*; P17, *L. animalis;* P28, *L. reuteri*; P38, *L. animalis*; P42, *L. acidophilus*; and P43, *L. reuteri*. The highlighted strains were selected based on their *Lactobacillus* genera identification, predicted high acid production, and high level of confidence in the strain identification from GenBank based on the forward and reverse strand results generated from the 16s rRNA sequencing. The six isolates (1) *L. crispatus*, (3) *L. animalis*, (1) *L. acidophilus*, and (1) *L. reuteri* were then studied and characterized according

to their physiological and biochemical features. The confirmed 16S rRNA gene sequences for the six isolates used in this study are listed in Appendix A.

Table 2: Lactic Acid Bacteria Strains selected for 16s rRNA Gene Sequencing[a]

[a] Rows highlighted in yellow indicate a *Lactobacillus* strain selected for additional studies. C groups represent isolates from the control bird while P groups represent isolates from the prebiotic treated bird.

LAB ID	FWD/ query cover %	REV/ query cover %	BLAST ID
C2	87%	-	*Enterococcus faecium (durans* also*)*
C3	88%	-	*Enterococcus faecium (durans* also*)*
C4	92%	-	*Enterococcus faecium (durans* also*)*
C5	88%	-	*Enterococcus faecium (durans* also*)*
C7	96%	-	*Enterococcus faecium (durans* also*)*
C8	83%	81%	*Enterococcus faecium*
C9	91%	91%	*Enterococcus hirae*
C10	85%	-	*Enterococcus faecium (durans* also*)*
C11	85%	85%	*Enterococcus hirae*
C12	98%	-	*Enterococcus faecium (durans* also*)*
C17	91%	-	*Enterococcus faecium (durans* also*)*
C20	96%	91%	*Enterococcus faecium (durans* also*)*
C25	99%	98%	*Lactobacillus crispatus*
C26	96%	96%	*Enterococcus avium*
C27	97%	96%	*Enterococcus avium*
C28	95%	92%	*Enterococcus sp. (possibly avium)*
C29	80%	80%	*Lactobacillus crispatus*
P3	86%	96%	*Enterococcus faecium*
P8	89%	83%	*Enterococcus faecium*
P12	97%	91%	*Enterococcus faecium*
P13	97%	91%	*Enterococcus faecium*
P15	98%	86%	*Lactobacillus animalis*
P17	97%	97%	*Lactobacillus animalis*
P18	79%	83%	*Enterococcus faecium*

Table 2 Continued

P19	84%	82%	*Enterococcus faecium*
P20	93%	87%	*Enterococcus faecium*
P26	76%	81%	*Lactobacillus animalis* or *murinus*
P28	99%	99%	*Lactobacillus animalis*
P29	98%	97%	*Lactobacillus animalis* or *murinus*
P31	98%	99%	*Lactobacillus animalis*
P32	82%	81%	*Lactobacillus animalis*
P33	88%	93%	*Lactobacillus animalis* or *murinus*
P36	81%	85%	*Lactobacillus animalis* or *murinus*
P37	98%	97%	*Lactobacillus animalis* or *murinus*
P38	98%	97%	*Lactobacillus animalis* or *murinus*
P39	81%	81%	*Lactobacillus animalis* or *murinus*
P40	98%	97%	*Lactobacillus animalis* or *murinus*
P41	99%	96%	*Lactobacillus reuteri*
P42	81%	78%	*Lactobacillus acidophilus*
P43	85%	95%	*Lactobacillus reuteri*
P48	87%	87%	*Enterococcus faecalis*

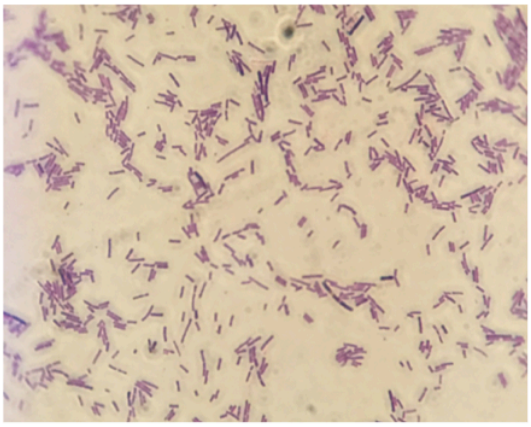
II.5.2. Gram Strain and Cellular Morphology:

Figure 1 displays the Gram stain and cellular morphology of the isolates examined. The *E. coli* control used in this study was used a Gram negative control and stained pink. All *Lactobacillus* isolates were confirmed to be Gram positive because all of the isolates stained purple. All of the isolates were rod shaped, but the look of the rods varied for some strains. The rod shaped cells for C25, P17, P28, and P38 all appeared as classic rods with similarity in length and thickness. P42 appeared as long, thin, and rope-like rod-shaped cells. P43 appeared as short, thick, and oval/ oblong shaped, verging on slightly cocci shaped cells. P17, P28, and P43 formed large grouped clusters of cells, with few singlets or doublets in the

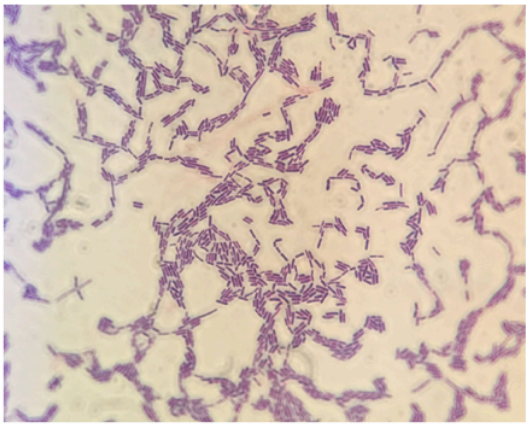
field of view. C25, P38, and P42 displayed some grouping and clustering of cells, but with higher amounts of cells as singlets and doublets. This finding is aligned with previous studies which have also reported *Lactobacillus* as being Gram positive, rod shaped (ranging from long to short) bacteria [8], [9], [10].

Figure 1: Gram Stain and Cellular Morphology of *Lactobacillus* isolates[a].
[a] From the -80˚C freezer stock, each strain was T-streaked onto MRS agar and incubated anaerobically overnight at 37˚C. One colony was Gram stained and examined under a compound light microscope; Gram stain and cellular morphology were determined.

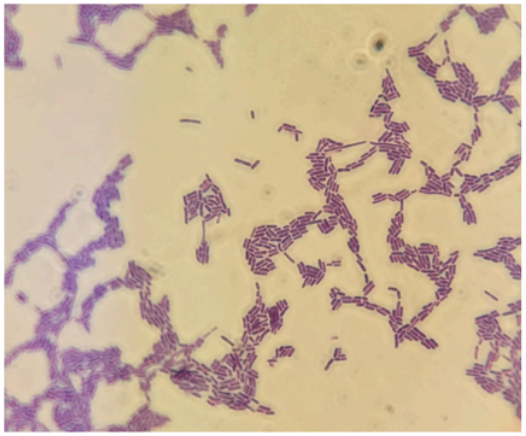
C25- *L. crispatus*



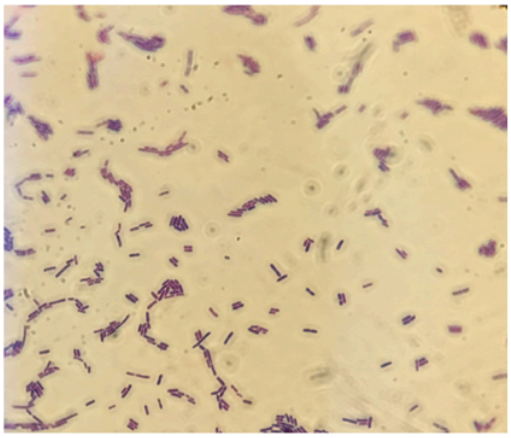
P17- *L. animalis*



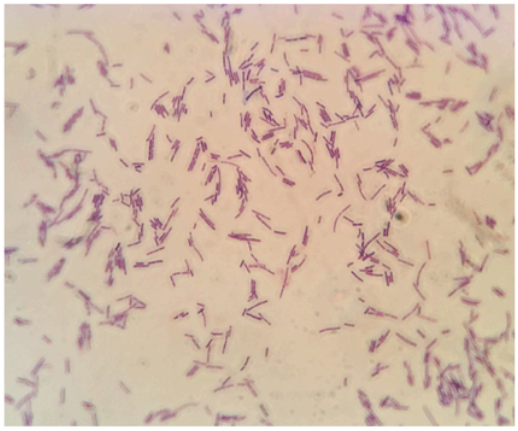
P28- *L. animalis*



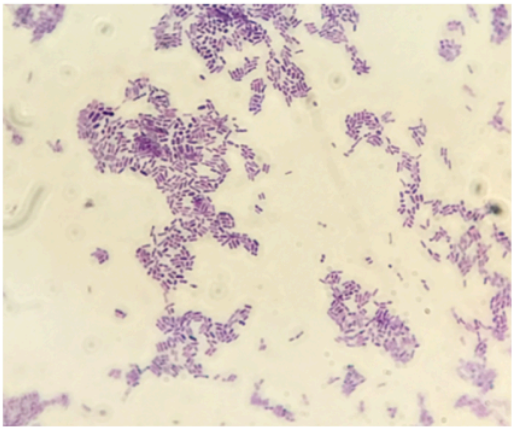
P38- *L. animalis*



P42- *L. acidophilus*



P43- *L. reuteri*



II.5.3. Growth Curves, Final Cell Concentration (OD_{600}), and final pH for Cells Grown in MRS Media in Presence and Absence of Atmospheric Oxygen:

For most bacteria, conditions in their natural environment do not allow for unrestricted growth; common limiting growth factors include depletion of nutrients, diminished availability of oxygen, and an overabundance of acids and other byproducts of metabolism. In terms of oxygen requirements, bacteria may be classified as obligate aerobes, obligate anaerobes, facultative anaerobes, or microaerophilic/ aerotolerant anaerobes. For some bacterial species, oxygen can be toxic to cellular functions, which could also have an impact on growth concentration. The growth concentration of a bacterial culture in the presence or absence of oxygen can convey useful information pertaining to that bacterial strain's physiological and metabolic machinery. The objective of this part of the study was to illuminate the growth kinetics, metabolic efficiency, and oxygen tolerance of the different *Lactobacillus* isolates. Tables 3 and 4 and Figures 2 and 3 summarize the data.

Table 3: Growth Parameters Under Aerobic Conditions[a]

	L. crispatus C25	*L. animalis* P17	*L. animalis* P28	*L. animalis* P38	*L. acidophilus* P42	*L. reuteri* P43
k (h^{-1})	0.597 ± 0.05	0.875 ± 0.04	1.05 ± 0.12	0.735 ± 0.06	0.737 ± 0.06	0.554 ± 0.04
g (min)	70.04 ± 5.25	47.54 ± 2.0	40.08 ± 4.19	56.78 ± 4.22	56.7 ± 4.68	75.26 ± 4.56
Maximum concentration* (OD_{600}nm) at 24 hours	7.10 ± 0.38	1.65 ± 0.56	3.53 ± 0.28	3.31 ± 0.11	7.01 ± 0.37	5.86 ± 0.14
pH at 24 hours	3.84 ± 0.03	4.47 ± 0.14	4.15 ± 0.03	4.07 ± 0.02	3.89 ± 0.02	4.21 ± 0.02

*Maximum concentration is maximum OD_{600}

[a] All *Lactobacillus* strains were grown aerobically in MRS broth at 37˚C. For each strain tested, an overnight culture was used to inoculate three 125mL flasks containing 30mL of MRS medium. The initial (T_0) cell density was ~ 10^7 CFU/mL for each strain. The cultures were incubated aerobically, OD_{600} readings were measured from 0-8 hours, and a final reading at 24 hours. The pH was read at 24 hours. Values are reported from one independent experiment with three replicates and averaged with standard deviation.

Table 4: Growth Parameters Under Anaerobic Conditions[a]

	L. crispatus C25	*L. animalis* P17	*L. animalis* P28	*L. animalis* P38	*L. acidophilus* P42	*L. reuteri* P43
k (h^{-1})	0.690 ± 0.04	0.934 ± 0.11	1.19 ± 0.03	0.933 ± 0.12	0.694 ± 0.05	0.586 ± 0.01
g (min)	60.40 ± 3.46	44.88 ± 4.98	34.94 ± 0.82	45.12 ± 6.12	60.16 ± 4.42	71.02 ± 1.02
Maximum concentration* (OD_{600}nm) at 24 hours	7.41 ± 0.32	5.49 ± 0.04	5.84 ± 0.26	5.64 ± 0.15	6.68 ± 0.10	6.65 ± 0.58
pH at 24 hours	3.72 ± 0.01	3.88 ± 0.02	3.89 ± 0.02	3.91 ± 0.02	3.70 ± 0.02	4.16 ± 0.04

*Maximum concentration is maximum OD_{600}

[a] All *Lactobacillus* strains were grown anaerobically in MRS broth at 37°C. Experimental procedures were the same as in Table 3 except it was performed under anaerobic conditions in a Coy Anaerobic Chamber as outlined in Materials and Methods.

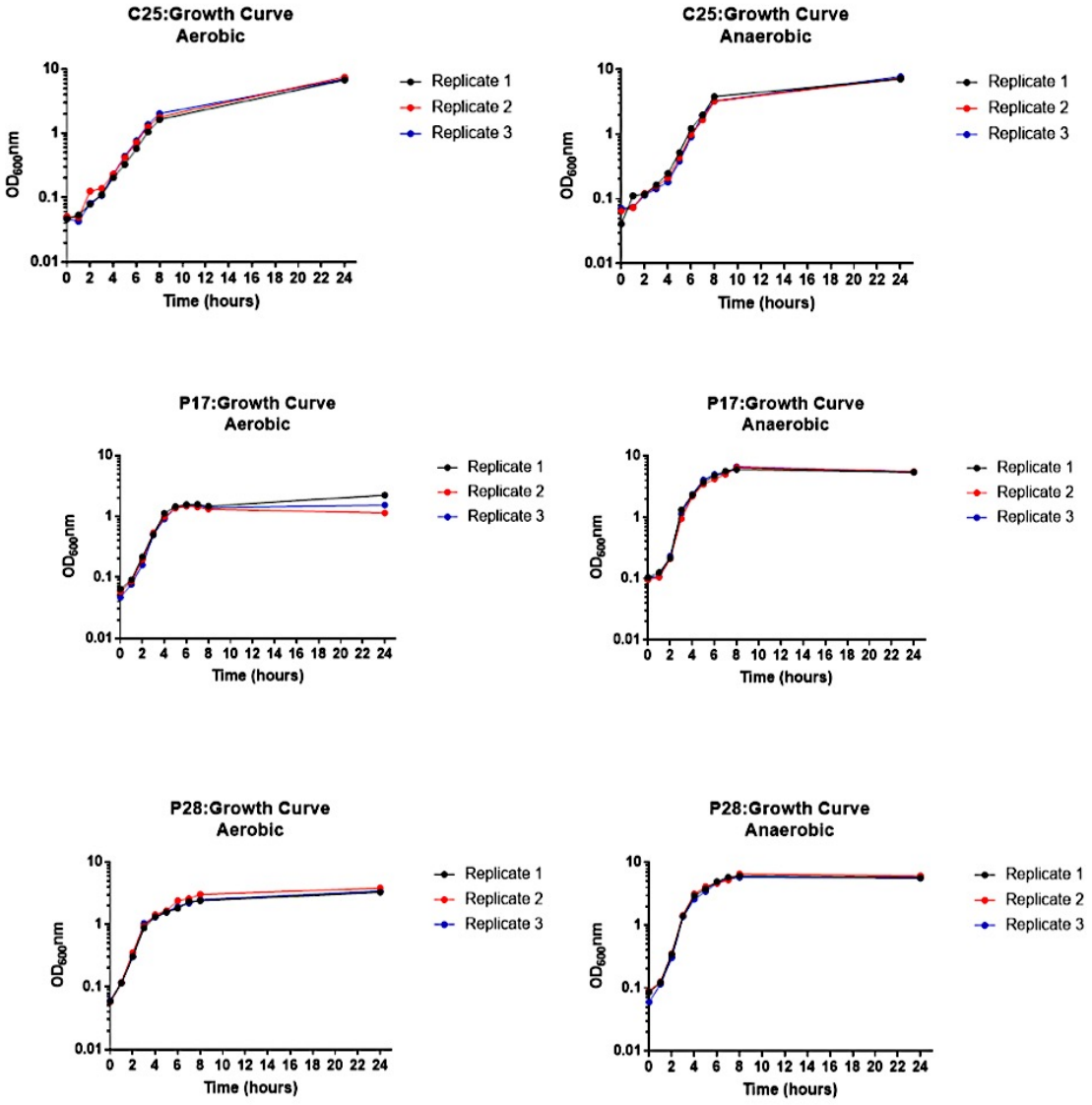


Figure 2: Bacterial Growth Curves of Strains C25, P17, and P28 grown Aerobically and Anaerobically. Strains were prepared as in Tables 3 and 4. The curve lines represent growth (OD_{600}). Replicate values are represented by different colored points and lines. • indicate the OD_{600} reading at a particular sampling time point.

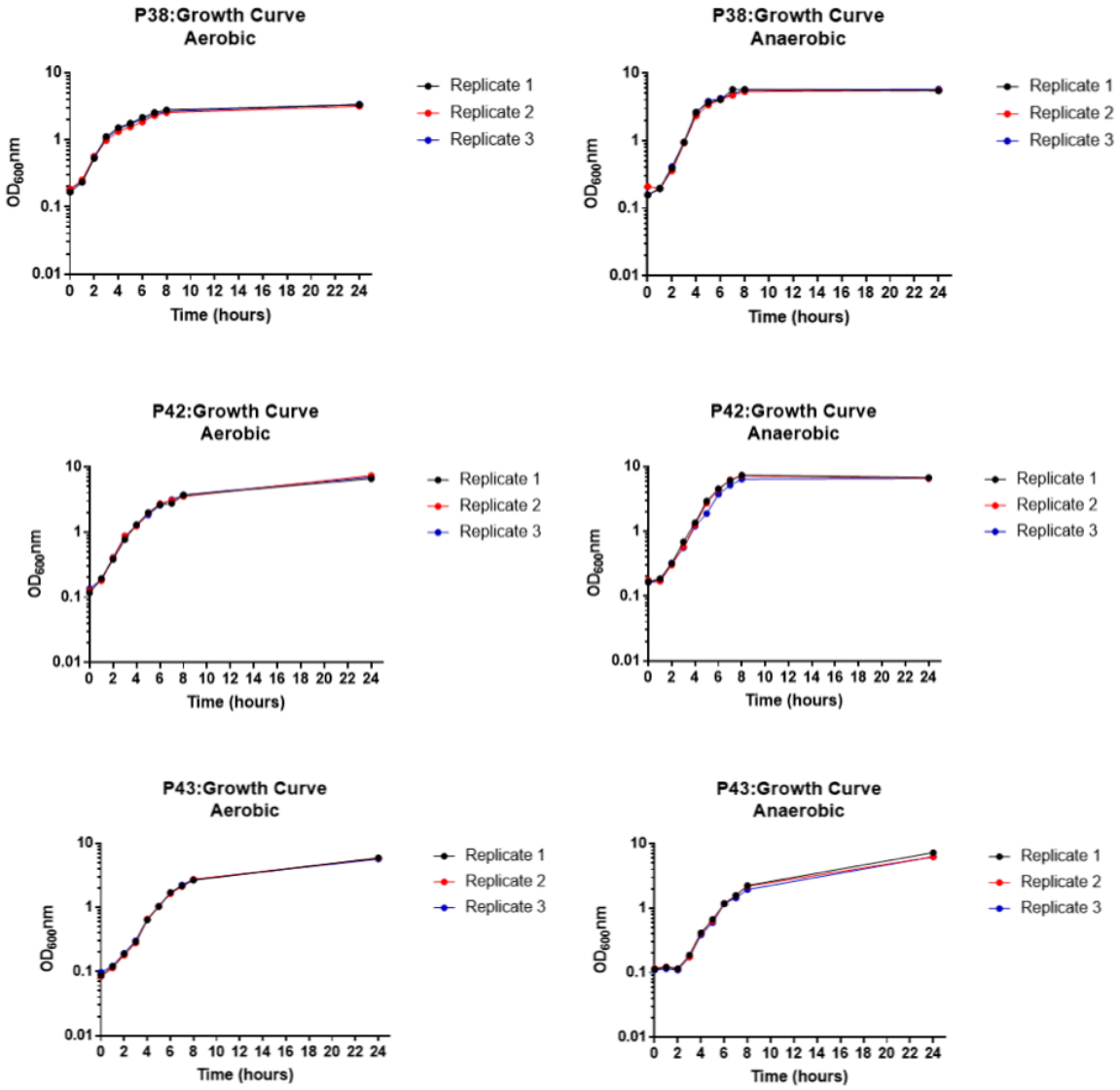


Figure 3: Bacterial Growth Curves of Strains P38, P42, and P43 grown Aerobically and Anaerobically. Strains were prepared as in Tables 3 and 4. Cultures were grown and growth curves analyzed as described in Figure 2.

Specific growth rate ($k.h^{-1}$) and generation time, g (doubling time) are interrelated ($g = 0.693/k.h^{-1}$), but for comparison, only doubling time is discussed. The maximum OD_{600} value was read at 24 hours. For all strains except P42 (*L. acidophilus*), the maximum concentration at 24 hours was higher when grown anaerobically. The generation time was faster in anaerobic conditions for all strains with the exception of P42 (*L. acidophilus*). In addition, C25 (*L. crispatus*) and P43 (*L. reuteri*) were somewhat similar to each other in their growth characteristics and responses to oxygen; the difference in maximum concentration between the two growth conditions was less than 1 OD. However; the difference in doubling time in anaerobic versus aerobic conditions for C25 was approximately ten minutes, but less than five minutes for P43. All *L. animalis* (i.e., P17, P28, and P38) displayed similar growth characteristics in response to the presence and absence of oxygen, with few differences. All three strains experienced faster doubling times when grown in anaerobic conditions. P17 and P28 were similar in that the difference in the doubling time for both growth conditions was around five minutes, while for P38, the gap extended beyond ten minutes, as was observed in C25. In general, the maximum concentration at 24 hours was higher when grown anaerobically compared to aerobically, but P28 and P38 experienced virtually the same difference in maximum growth concentration between the two growth conditions, approximately a 2-fold difference. P17 experienced an almost 4-fold difference in maximum growth concentration between the two conditions. P42 (*L. acidophilus*) was a nonconformist in that the generation time was faster when grown in aerobic conditions; the difference in doubling time was less than five minutes. The maximum concentration at 24 hours was higher when grown aerobically; there was a difference of less than 1 OD between the two

growth conditions. The final pH at 24 hours showed that all strains produced higher amounts of acid when grown in anaerobic conditions. C25 was the only strain that did not enter stationary phase during the first eight hours in either growth condition. According to Figures 2 and 3, in both anaerobic and aerobic conditions, the maximum concentration at 24 hours was almost identical for C25 and P43, indicating an indifference to oxygen in terms of the maximum concentration. The duration of the experiment permitted P17, P28, P38, P42, and P43 to enter into stationary phase; the time points at which stationary phase began varied, indicating the possibility of oxygen impacting the maximum specific growth rate. For P17, P28, and P38, the peak and ending data points were positioned closer to 10 ODs for the anaerobic condition compared to the aerobic condition, confirming a higher maximum concentration in environments devoid of oxygen. For P42, the opposite occurred, showing a slight affinity for aerobic growth conditions over anaerobic conditions. For P17, P28, and P38, exposure to oxygen more than likely effected the cells' metabolic processes, contributing to the slower generation times and lower maximum concentration values, even though the pH values were virtually the same. Some lactic acid bacteria are capable of reducing oxygen. It is possible that *L. animalis* reduced oxygen to hydrogen peroxide. If this happened, this partial reduction of oxygen likely led to an overproduction of hydrogen peroxide that caused oxidative stress and reduced biomass. Under certain growth conditions, bacterial growth concentration is lowered due to uncoupled growth in favor of energy utilization for cell repair and maintenance [11]. It is possible that uncoupled growth occurred in these three *L. animalis* strains. Where, under aerobic conditions, the fermentation pathway can still be used for energy generation and ATP synthesis, as well as acid byproducts, but the

ATP is used for energy of maintenance and DNA/cellular repair of oxidative damage by ROS. Thus, oxygen may have induced oxidative stress in which cells prioritized survival mechanisms over continued growth. This could have caused the cells in survival mode still using all available nutrients in the media to produce ATP for survival processes' while also producing lactic acid to the same level as cells grown in anaerobic conditions. Conversely, the ATP generated in anaerobic conditions, in the absence of oxidative stress, would have been diverted towards more cell growth. This could explain the difference in the final maximum growth concentration and the similar final pH values observed in P17, P28, and P38 under the influence of the two growth conditions. For C25, P42, and P43, the difference in maximum concentration for strains grown aerobically versus anaerobically was less than 1 OD. These strains also grew to similar final pH values with slightly more acidic pH reads when grown in the absence of oxygen. The slim difference in maximum growth concentration and final pH at 24 hours between aerobic and anaerobic conditions makes it likely that growth did not uncouple, that cellular metabolism occurred independently of oxygen availability, and that little, if any oxidative stress took place in the cells. Few, if any studies exist which analyze the growth concentration and metabolic performance of these specific strains' (*L. crispatus*, *L. animalis*, *L. acidophilus*, and *L. reuteri*) in aerobic versus anaerobic conditions. Historically, *Lactobacillus* strains have been found to prefer anaerobic environments. Based on the results of the bacterial growth curves, the oxygen requirements could be summed up as: C25 (*L. crispatus*), P42 (L. acidophilus), and P43 (L. reuteri) facultative anaerobes and P17, P28, and P38 (all *L. animalis*) microaerophilic/ aerotolerant anaerobes.

II.5.4. Carbohydrate Utilization:

For this part of the study, we utilized the commercially available API 50 CH test strips which screened the different strains for their abilities to utilize approximately 49 different sources of carbohydrates. The data obtained from the *Lactobacillus* strains' carbohydrate utilization is summarized in Table 5.

Table 5: *Anaerobic Carbohydrate Utilization after 48 Hours[a, b, c]

Sugar	C25	P17	P28	P38	P42	P43	Sugar	C25	P17	P28	P38	P42	P43
Temoin (control)	-	-	-	-	-	-	Esculine citrate de fer	+	+	+	+	+	+
Glycerol	-	-	-	-	-	-	Salicine	+	+	+	+	+	+
ERYthritol	-	-	-	-	-	-	D-cellobiose	+	+	+	+	+	+
D-arabinose	-	-	-	-	-	-	D-maltose	+	+	+	+	+	+
L-arabinose	+	-	-	-	-	+	D-lactose	+	+	+	+	+	+
D-ribose	+	+	+	+	-	+	D-melibiose	+	+	+	+	+	+
D-xylose	-	-	-	-	-	-	D-saccharose	+	+	+	+	+	+
L-xylose	-	-	-	-	-	-	D-trehalose	+	+	+	+	+	V
D-adonitol	-	-	-	-	-	-	Inuline	-	-	-	-	-	-
Methyl-betaD-xylopyranoside	-	-	-	-	-	-	D-melezitose	-	-	-	-	-	V
D-galactose	+	+	+	+	+	+	D-raffinose	+	+	+	+	+	+
D-glucose	+	+	+	+	+	+	Amidon	+	-	-	-	-	-
D-fructose	+	+	+	+	+	+	Glycogene	+	-	-	-	-	-
D-manose	+	+	+	+	V	+	Xylitol	-	-	-	-	-	-
L-sorbose	-	-	-	-	-	-	Gentiobiose	-	-	-	-	+	+
L-rhamnose	-	-	-	-	-	-	D-turanose	+	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	D-lyxose	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	D-tagatose	-	-	-	-	-	+
D-mannitol	+	-	-	-	-	-	D-fucose	-	-	-	-	-	-
D-sorbitol	-	-	-	-	-	-	L-fucose	-	-	-	-	-	-
Methyl-alphaD-mannopyranoside	-	-	-	-	-	-	D-arabitoL	-	-	-	-	-	-
Methyl-alphaD-glucopyranoside	V	-	-	-	-	+	L-arabitoL	-	-	-	-	-	-
N-acetylglucosamine	+	+	+	+	+	+	potassium gluconate	-	-	-	-	-	-
Amygdaline	+	+	+	+	+	V	potassium 2-cetogluconate	-	-	-	-	-	-
Arbutine	+	+	+	+	+	+	potassium 5-cetogluconate	-	-	-	-	-	-

[a] V is variable/ weak utilization (yellow/green color change on the API 50 CH test strip), + is positive (yellow color change on the test strip), and – is negative (green color change on the test strip) for carbohydrate utilization

[b] One culture at a time was grown on an MRS agar plate overnight anaerobically. Cells were scraped off the plate into a sterile conical tube filled with 10mL DI H_2O, then the OD_{600} was adjusted to 0.4. 5mLs of the cells were combined with 5 mLs of 2x bromocresol purple media to make a 1x solution. Of that suspension, 100µL was added to each sugar compartment of the API 50 CH test strips. This was then overlaid with mineral oil and incubated anaerobically; final readings were reported at 48 hours. This was carried out for all six *Lactobacillus* strains.

[c] Symbols highlighted in teal represent a difference in utilization for a specific carbohydrate compared to the majority of the other strains. Symbols highlighted in lavender represent variability or a weak positive utilization result.

Organisms do not remain in a constant environment; environmental conditions continually change, one of which is the type of carbohydrates available to bacteria for metabolism and

energy generating processes. It is important to know which carbohydrates bacteria can and cannot utilize as this can have implications on energy output, growth rate, competition with other organisms in the same environment, etc. While the weak/ variable readings are included in Table 5 for comparison, only definitive positive and negative readings are discussed. All *Lactobacillus* strains were able to utilize the most common carbohydrates found in the GIT: glucose, fructose, and lactose. Nine of the forty-nine carbohydrates tested showed variance among the six isolates. P17, P28, and P38, i.e., all *L. animalis,* exhibited the same carbohydrate utilization. C25 was the only strain to utilize D-mannitol, amidon, glycogene, and D-turanose, all of which are six carbon sugars, or disaccharides that can be broken down into six carbon sugar units. P42 was the only strain unable to utilize D-ribose, a pentose sugar. P42 and P43 were the only strains able to utilize gentiobiose, a disaccharide composed of two linked glucose molecules. P43 was also found to utilize Methyl-alphaD-glucopyranoside, a seven carbon monosaccharide and D-tagatose, a six carbon monosaccharide. C25 and P43 were also the only two strains capable of utilizing L-arabinose, a pentose sugar. *Lactobacillus* strains can be classified as being obligate homofermenters, facultative heterofermenters, or obligate heterofermenters, and this distinction becomes important when dealing with the breakdown of five or six carbon sugars. Bacteria with the enzyme aldolase are able to break down hexoses, and those with phosphoketolase are able to break down pentoses [12]. When fructose is broken down during heterofermentation, D-mannitol, a hexose sugar alcohol forms. In addition to mannitol, lactic and acetic acid, carbon dioxide, and ethanol are also produced [13]. C25 (*L. crispatus*) was able to utilize fructose as well as D-mannitol, indicating the potential for this strain to be an

obligate heterofermenter. This was further substantiated by the fact that C25 is able to metabolize the following pentose sugars: L-arabinose and D-ribose. Furthermore, C25 was also the only strain to utilize amidon (French for 'starch'). C25 was also able to utilize the highly branched glucose polysaccharide, glycogene, and the disaccharide analogous to sucrose, D-turanose. In the gastrointestinal tract, exposure to and abundance of specific carbohydrates fluctuates. *L. crispatus* is a strain commonly found in the vagina of humans and animals, however; this C25 strain's ability to utilize numerous carbohydrate substrates, both simple and complex, may suggest that it has adapted to gastrointestinal environmental conditions. P42 (*L. acidophilus*) was unable to use D-ribose or L-arabinose, both of which are pentose sugars. It is likely that P42 is an obligate homofermenter. Like C25, P43 (*L. reuteri*) was also able to utilize D-ribose and L-arabinose in addition to many hexose carbohydrate substrates, suggesting it is also an obligate heterofermenter. P17, P28, and P38 were all able to utilize D-ribose, but not L-arabinose, suggesting facultative heterofermentation capabilities. These conclusions mostly identify with the findings described in Bergey's Manual of Systematic Bacteriology for each of the *Lactobacillus* strains studied; *L. animalis* is a facultative heterofermenter [14]. *L. reuteri* is an obligate heterofermenter according to Bergey's and the same conclusion was reached for the P43 isolate. *L. acidophilus* is an obligate homofermenter according to Bergey's and the same conclusion was reached for P42. *L. crispatus* is an obligate homofermenter capable of hydrolyzing starch and esculin according to Bergey's; however, a conclusion of obligate heterofermenter was reached. In our study, *L. crispatus* exhibited metabolism similar to that of an obligate heterofermenter due to the successful utilization of L-arabinose and D-ribose,

both pentose sugars, along with successful utilization of several hexose sugars, such as D-galactose, D-glucose, D-fructose, and D-mannitol. Previous studies using the API 50 CH test strips have been unable to narrow down expected phenotypes for particular *Lactobacillus* strains. For example, a study conducted by Boyd *et. al.* (2005) examined eighteen *L. crispatus* strains, none of which displayed the same phenotype [15]. Another study by Charteris *et. al.* (2001) assessed three strains of *L. acidophilus* and found none of the phenotypic patterns matched [16]. This owes to the conclusion that carbohydrate utilization is strain dependent. While several of the complex carbohydrates were unable to be fermented by these strains alone; in the GIT, where there exists a community of bacterial species, these complex carbohydrates may be broken down through a group effort [17]. Since the GIT is naturally low in mono- and disaccharides, this innate ability of *Lactobacillus* spp. to ferment multiple sources of carbohydrates is beneficial not only for the bacterial cell, but for the host as well [18].

II.5.5. Growth on Glucose, Galactose, Lactose, and GOS in semi-defined media (final OD_{600} concentration and final pH):

The data obtained from the *Lactobacillus* strains' growth in semi-defined media with specified sugar or prebiotic is summarized in Figure 4.

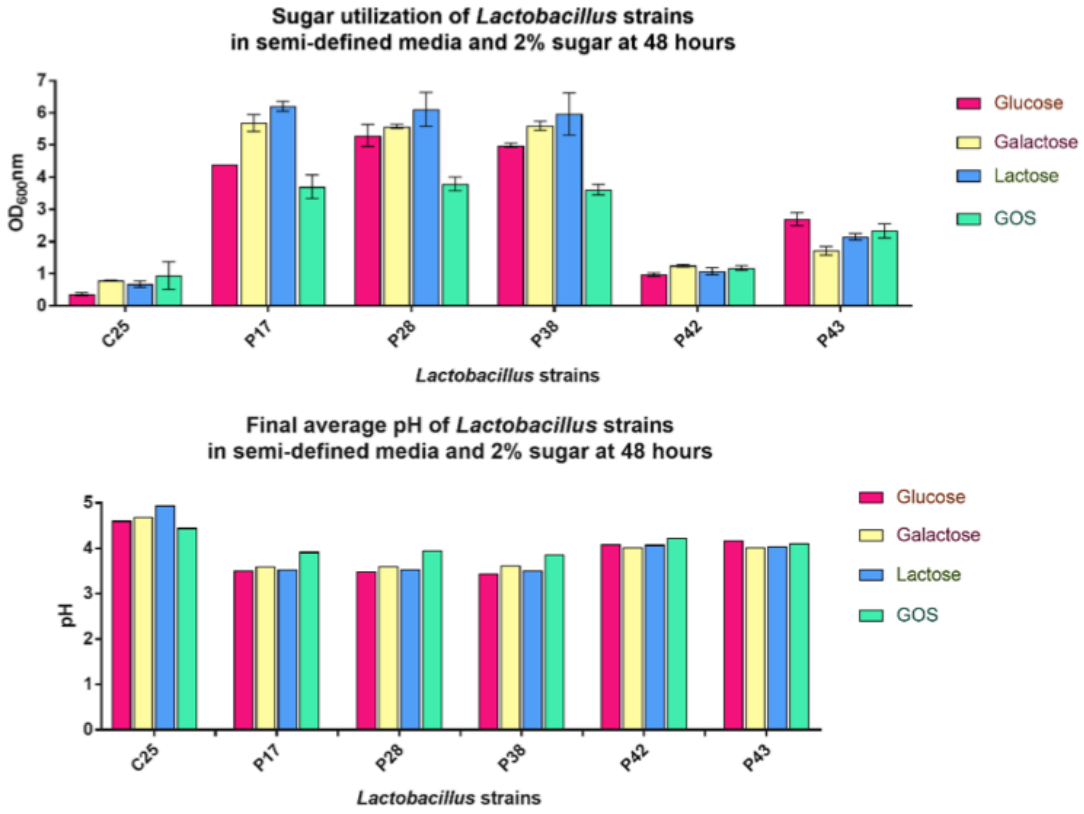


Figure 4: Final Concentration and pH Values of *Lactobacillus* Strains grown in Semi-Defined Media with 2% Sugar. Altered MRS (AMRS) was prepared in house and sterilized. Sugars were dissolved in house, reported as final weight/volume, and sterilized. The sugars glucose, galactose, lactose, and GOS were added to AMRS at 2% concentrations. One *Lactobacillus* strain was inoculated into an MRS broth tube and incubated (static) anaerobically at 37˚C overnight. Cells were centrifuged and resuspended in phosphate-buffered saline. The OD_{600} was recorded and 10µL of cells were added to all AMRS sugar tubes. OD_{600} readings and the final pH were measured at 48 hours post inoculation. This was performed with all six strains. Data was generated from one independent experiment with two replicates and averaged with standard deviation.

All microorganisms need an energy source to carry out metabolism and much of the time, this energy is in the form of sugars. One group of sugars, prebiotics, have been shown to

facilitate the growth of microbes, especially probiotic bacteria. High populations of probiotic bacteria in the GIT of humans and animals can result in a number of beneficial effects on the host. Galactooligosaccharides (GOS) are a type of prebiotic that may stimulate microbial growth, which may induce these beneficial effects. Upon supplementation of glucose, galactose, lactose, and GOS, the three *L. animalis* strains (P17, P28, and P38) displayed the highest growth concentrations amongst all strains tested; the highest growth concentration was achieved in lactose > galactose > glucose > GOS. After *L. animalis*, P43 (*L. reuteri*) followed by P42 (*L. acidophilus*) and lastly, C25 (*L. crispatus*) achieved modest growth concentration results. For P42, the growth concentration appeared to be even amongst all metabolic stimulants tested, with a slight preference for galactose followed by GOS. P43 achieved its highest growth concentration in glucose, followed by GOS. C25 exhibited the smallest total growth concentration when cultured in each of the four sugars, with the highest growth concentration achieved in GOS. The final pH values support these data; C25 displayed the lowest overall growth concentration which resulted in the highest and most basic pH readings. P17, P28, and P38 had the lowest and most acidic pH readings. The final pH readings for P42 and P43 were almost identical. A previous study conducted by Cardelle-Cobas *et al.* (2010) looked at the influence of 0.3% (w/v) of GOS derivatives on the growth of *L. animalis*, *L. reuteri,* and *L. acidophilus. L. animalis* and *L. reuteri* were incubated aerobically, while *L. acidophilus* was incubated anaerobically. *L. animalis*, *L. reuteri,* and *L. acidophilus* all grew to a final OD_{600} of less than 1.0 in the presence of all GOS derivatives at 48 hours [19]. Only the results achieved by *L. acidophilus* closely matched the findings from our study; if *L. animalis* and *L. reuteri* had been incubated anaerobically, perhaps the final

OD_{600} values would have been closer to the values determined in the present study. A different study conducted by Rousseau *et al.* (2005) evaluated the growth of *L. crispatus* in response to GOS. Their findings suggested weak growth, with an average OD_{600} of approximately 0.3 in the presence of GOS derivatives at 48 hours [20]. Our strain of *L. crispatus* exhibited the weakest growth in GOS compared to the other five strains tested, however; the final maximum concentration at 48 hours was slightly greater than 1.0 OD_{600} compared to OD_{600} of 0.3 obtained by Rousseau's group. This could suggest possible genomic fitness acquired by our strain, which allowed for slightly better growth concentrations in the presence of GOS. In general, all six strains tested could sustain their metabolism and growth in GOS. While GOS did stimulate growth in the *L. animalis* strains, the growth concentrations did not surpass those of the other three sugars commonly encountered in the GIT. As a stand-alone nutritive material in semi-defined media, GOS did not appear to stimulate cell growth to higher maximum concentrations compared to glucose, galactose, or lactose.

II.5.6. Catalase Activity:

Table 6: Presence of Catalase Activity in *Lactobacillus and E. coli* [a]

Strain	Presence of catalase + or -
C25- *L. crispatus*	-
P17- *L. animalis*	-
P28- *L. animalis*	-
P38- *L. animalis*	-
P42- *L. acidophilus*	-
P43- *L. reuteri*	-
E. coli K-12 strain NC4468	+

[a] Each strain was T-streaked onto sterile MRS agar plates and incubated between 24-48 hours anaerobically at 37˚C. A single colony was transferred to a microscope slide and one drop of 3% hydrogen peroxide was applied to the slide. The immediate formation or nonappearance of bubbles was then observed. If bubbles- test was positive and if no bubbles- test was negative. This procedure was carried out for all six *Lactobacillus* strains; one experiment with one replicate.

Catalase is an enzyme which degrades hydrogen peroxide into oxygen and water. The possession of catalase benefits cells in that it neutralizes hydrogen peroxide before it can be converted into the harmful hydroxyl radical ($HO^{\cdot}$). *E. coli* K-12 strain tested positive for catalase activity, forming bubbles immediately upon exposure to H_2O_2. All *Lactobacillus* strains tested in our study were negative for catalase activity. While it does not appear that these strains possess the catalase enzyme, this does not mean they are without other means to

neutralize hydrogen peroxide; NADH peroxidase is an enzyme common to *Lactobacillus* which accomplishes the same feat. Independent studies performed by Banina *et al.* (1997) and Aween *et al.* (2012) tested a total of 34 different strains of *L. acidophilus* and found all to be catalase negative [21], [22]. Previous studies were also conducted on strains *L. animalis*, *L. reuteri*, and *L. crispatus* and all were determined to be catalase negative [23], [24].

II.5.7. Acid Tolerance and Survival:

Lactic acid bacteria ingested by humans and chickens encounter high acid conditions as they travel through the gastrointestinal tract, particularly in the stomach. Low pH environments are harmful to most cells; possessing adaptations that allow for survival in acid would be advantageous. In order to ascertain the ability of the protocol as outlined in the material and methods section to detect reduction in cell viability after exposure to different pHs, we conducted a muck experiment using isolate P17 (Figure 5). The data obtained regarding acid tolerance and survival of *Lactobacillus* and *E. coli* strains' is summarized in Figure 6.

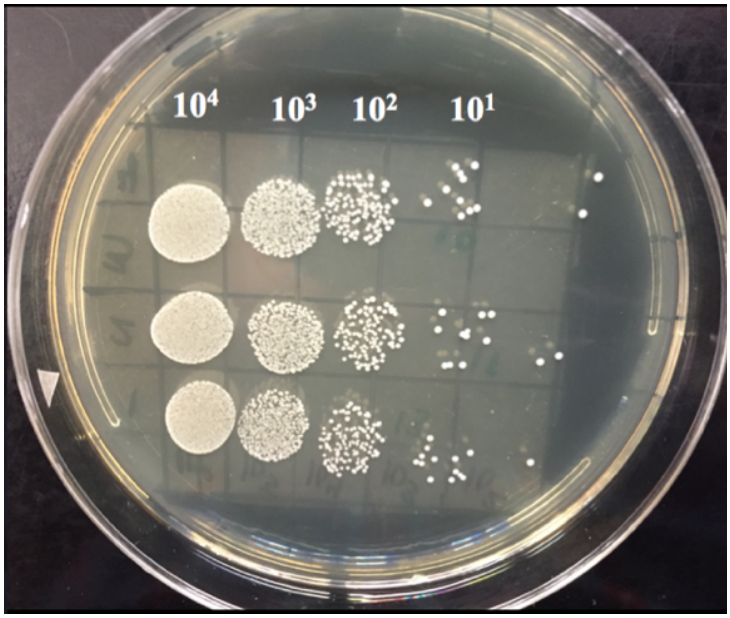
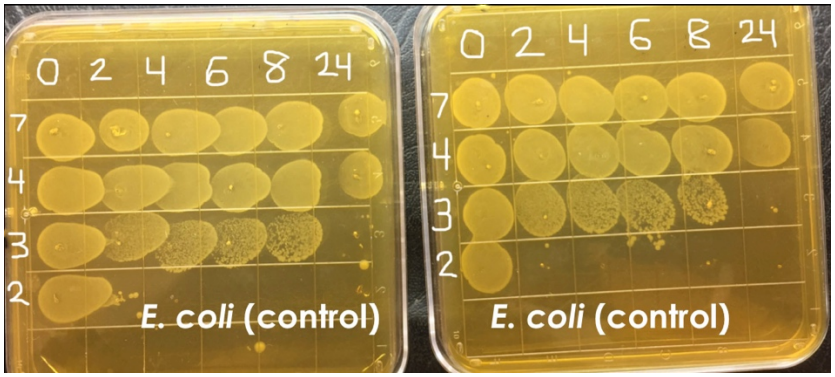


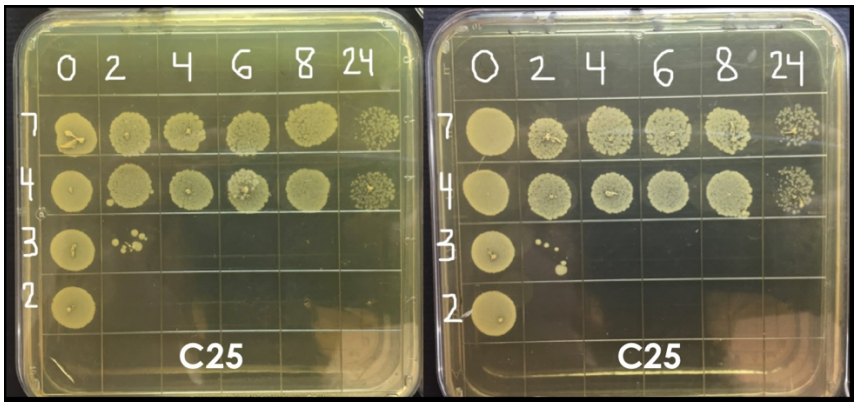
Figure 5: Mock Log Reduction Gradient[a].

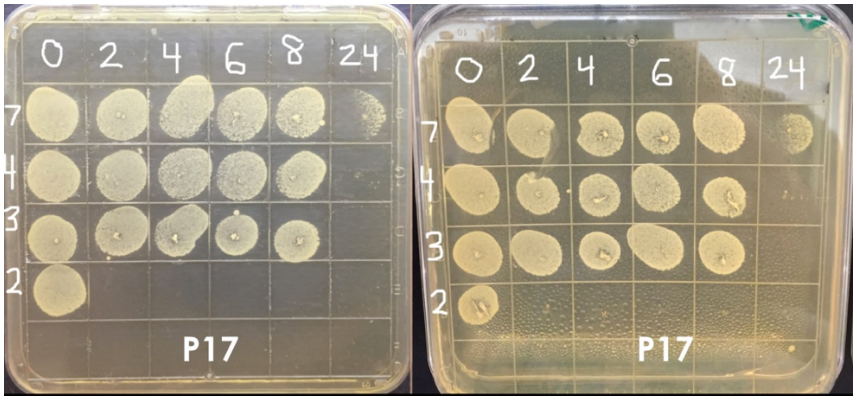
[a] An overnight culture of P17 was adjusted to an OD_{600} of $1.0x10^8$ CFU/mL. This culture was exposed to mock dilutions in absence of any acid conditions (i.e., PBS – pH 7.0) then serially diluted in PBS and 10µL aliquots were spot plated in triplicate. The dilutions written in white represent the log concentration of cells in the 10µL spots in each column. This was used as a reference to visualize the extent of log reduction in the acid tolerance and survival study for all strains.

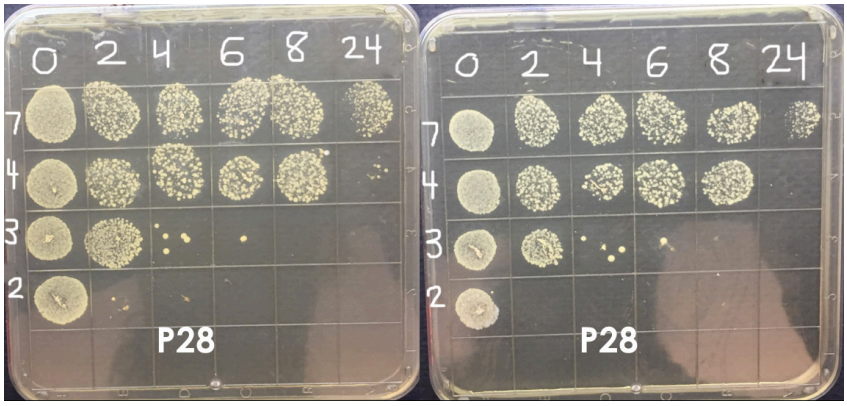
Figure 6: Acid Tolerance and Survival for *Lactobacillus* and *E. coli* Strains[a].

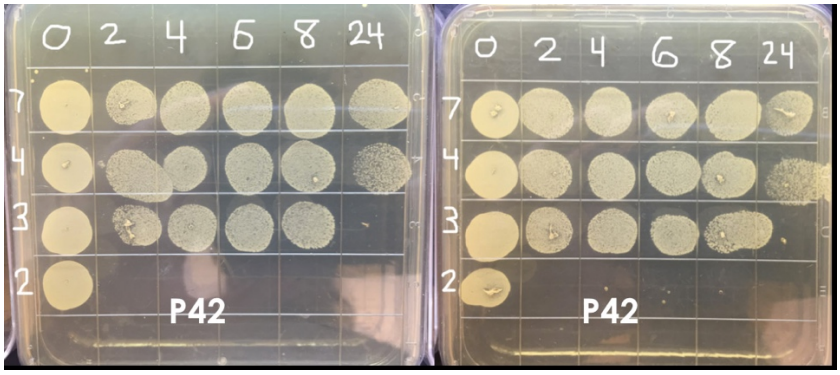
[a] MRS media was used for *Lactobacillus* strains and MMRS for *E. coli*. All strains were inoculated from an overnight culture and incubated (static) anaerobically at 37˚C overnight. The cells were centrifuged then resuspended in PBS and the OD_{600} was adjusted to yield ~ $1.0x10^8$ CFU/mL before 1mL was transferred to 9mL of PBS pH adjusted tubes at pH 7.0, 4.0, 3.0, and 2.0. The samples were acid neutralized before 10µL volumes were spotted for each pH at 0, 2, 4, 6, 8, and 24 hours. Data presented was from one independent experiment performed in duplicate. Horizontal numbers represent incubation time (hours) in acid; vertical numbers represent the pH at which the cells were exposed.

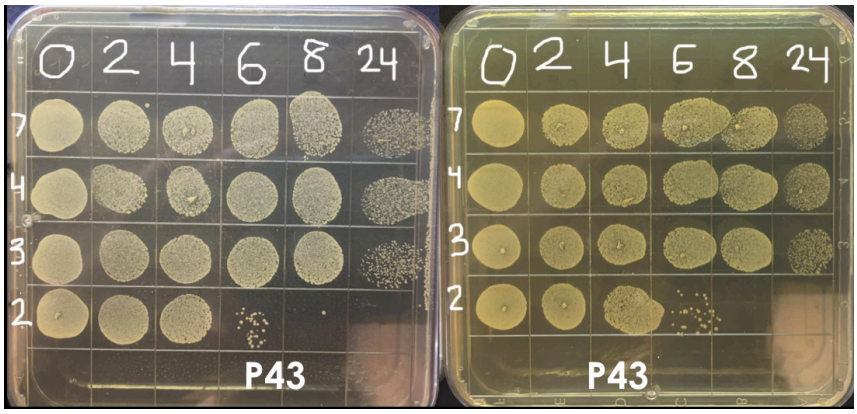

0 2 4 6 8 24
7
4
3
2
E. coli (control)
0 2 4 6 8 24
7
4
3
2
E. coli (control)


0 2 4 6 8 24
7
4
3
2
C25
0 2 4 6 8 24
7
4
3
2
C25


0 2 4 6 8 24
7
4
3
2
P17
0 2 4 6 8 24
7
4
3
2
P17


0 2 4 6 8 24
7
4
3
2
P28
0 2 4 6 8 24
7
4
3
2
P28


0 2 4 6 8 24
7
4
3
2
P42
0 2 4 6 8 24
7
4
3
2
P42


0 2 4 6 8 24
7
4
3
2
P43
0 2 4 6 8 24
7
4
3
2
P43

The data (Fig. 5) showed that P43 (*L. reuteri*) was the most acid-tolerant; it survived exposure to pH 2.0 for 4 hours. P17 (*L. animalis*), P38 (*L. animalis*), P42 (*L. acidophilus*), and *E. coli* exhibited a similar degree of sensitivity to acidic pHs; they all survived exposure to pH 3.0 for 8 hours. Conversely, P28 (*L. animalis*) survived exposure to pH 3.0 for 2 hours, while C25 was the most susceptible as it did not survive beyond 2 hours at pH 3.0. In conclusion the relative degree of acid tolerance among the *Lactobacillus* isolates tested was P43 > P42 > P38=P17 > P28 > C25. Previous studies showed that *L. reuteri* possess several mechanisms that contribute to its' acid tolerance. The amino acid glutamate can be decarboxylated to γ-aminobutyrate, a pathway known to increase acid tolerance in microorganisms, including strains of *Escherichia coli* [25]. Su *et al.* (2011) studied *L. reuteri* 100-23, a strain known to possess glutamate decarboxylase genes. The group also deleted the glutamate decarboxylase gene and tested the survival of the mutant and the wild type strains at pH 2.5 in the absence and presence of glutamate. The survival ability of the strain with the glutamate decarboxylase gene (i.e., the Wild Type) was 2 logs more as compared to the isogenic mutated strain. It was concluded that glutamate decarboxylase contributes to acid tolerance at pH 2.5 for *L. reuteri* [26]. Another study performed by Wall *et al.* (2007) looked at the survival of strain *L. reuteri* ATCC 55730 after acid shocking from a pH of 5.1 to 2.7 for up to 2 hours. The results showed that greater than 80% of the cells treated with acid survived at pH 2.7 for 1 hour and 60% survival after 2 hours. Wall *et al.* (2007) concluded *L. reuteri* may possess one or more mechanisms that combat acid stress, such as genes that change the composition of the cell membrane or cell wall, thus blocking the diffusion of protons into the cell or induction of general stress response genes [27]. Jin *et al.* (1998)

studied the acid response in *L. acidophilus* and showed low survival at a pH between 0.5 to 2.0, moderate survival at a pH of 3.0, and good survival at a pH at or above 4.0 [28]. The results produced by Jin *et al.* (1998) coincided with the results and conclusions produced from our experiment. One study looked at *L. Crispatus* LT116, subjecting it to a pH of 2 for 90 minutes at 37˚C and found that between 30 and 60% of the cells survived [29]. The results from Taheri *et al.* (2009) challenge the results of our experiment, as there were no cells detected in PBS at pH 2 for 2 hours in our study. The team led by Ehrmann *et al.* (2002) studied two strains of *L. animalis* and found one of the strains to have a survival rate of 24.2% in pH 2.0 for 4 hours [30]. It is important to keep in mind that this high acid tolerance performance was observed in one strain of many possible *L. animalis* strains' in existence. The three strains tested in our study did not perform as well in high acid environments; there are few studies on acid tolerance in *L. animalis*, so the results obtained by Ehrmann *et al*. (2002) may be the exception rather than the rule. The average pH of the human stomach is 1.5 with complete digestion taking place between 4-5 hours [31]. In the chicken GIT, the stomach is comprised of two organs; the proventriculous and the gizzard, of which the pH can be as low as 2.6, and complete digestion can take roughly two hours [29]. Strains C25 and P28 would likely struggle, P17, P38, and P42 may possibly tolerate exposure, and P43 would likely withstand and survive the exposure to acid in either of the GIT environments.

II.5.8. Bile Salt Tolerance and Survival:

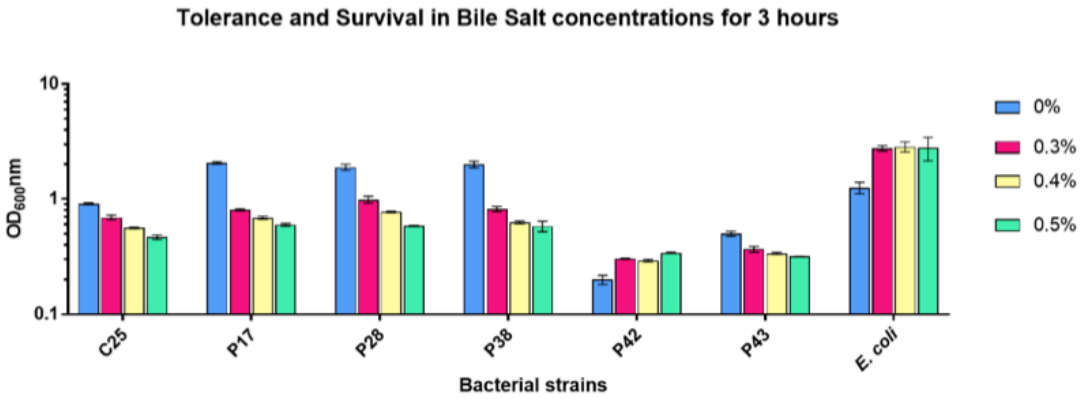


Figure 7: Growth and Tolerance to varying concentrations of Bile Salts for *Lactobacillus* and *E. coli*[a].

[a] MRS media was used for *Lactobacillus* strains and MMRS for *E. coli.* All strains were inoculated from an overnight culture and incubated (static) anaerobically at 37˚C overnight. The cells were centrifuged then resuspended in PBS and the OD_{600} was adjusted to 0.5. A 1 in 10 dilution was prepared in duplicate for tubes containing 0%, 0.3%, 0.4%, and 0.5% Oxgall (Sigma-Aldrich, MO) bile salts for a starting OD_{600} of 0.05. The tubes were incubated anaerobically at 37˚C (static) and the OD_{600} measured at 0 hours and 3 hours. Viable cell counts (Appendix C) were also performed in duplicate by implementing 10-fold serial dilutions in PBS and plating (100µL) on MRS or MMRS agar (1.5%) plates, depending on the strain. Values were determined from the OD_{600} readings from one independent experiment and duplicates averaged with standard deviation.

Bile salts are often encountered in the small intestine during transit through the gastrointestinal tract. These bile salts serve to break down fats, but often inflict damage to bacterial cells in the process [32], [33]. Upon exposure to varying concentrations of bile salts, growth was reduced, but not terminated in all strains except for P42 (*L. acidophilus*) and the

E. coli K12 control. According to Figure 7, P42 and *E. coli* grew to the highest OD_{600} values when grown in the presence of bile salts; approximately a 0.1 OD increase in P42 and 1.6 OD increase in *E. coli*. P43 followed by C25 exhibited a decline in growth concentration as the concentration of bile salts increased, though the decline was not as severe as in the strains P17, P28, and P38, when compared to the 0% bile salt control. At most, there was a decrease in growth concentration of roughly 0.180 ODs in P43 and 0.445 in C25. P17, P28, and P38 experienced the largest spread in growth concentration decline with a maximum decrease in concentration of approximately 1.4ODs. These results were further supported with the total plate counts found in Appendix C. *E. coli* are Gram negative bacteria commonly cultured on MacConkey Agar, of which, bile salts are a principle ingredient. *E. coli* was expected to grow well in the presence of bile salts. P42 (*L. acidophilus*) behaved in the same way as *E. coli*, growing to a higher concentration in the presence of bile salts. Several studies, such as those conducted by Jin *et al.* (1998) and Ahn *et al.* (2003) have examined bile salt sensitivity in *L. acidophilus*. Using methods such as broth inoculation with and without bile salts for growth curve generation to measure the delay in growth over time and bile salt- MRS agar plate assays measuring precipitate halo formation, the deconjugation of bile salts by *L. acidophilus* strains was found to be a common occurrence [28], [34]. The deconjugation of bile salts is accomplished by bile salt hydrolases; According to Begley *et al.* (2006) *L. acidophilus* has two bile salt hydrolase genes in its genome which contribute to its bile salt tolerance [35]. P43 (*L. reuteri*) and C25 (*L. crispatus*) were able to sustain their growth processes in environments with bile salts, but grew to the highest concentration in its absence. Moser and Savage (2001), characterized *L. reuteri* and *L. crispatus* as also

possessing two bile salt hydrolase enzymes. They looked at 49 *Lactobacillus* strains to determine specific bile salt hydrolase activity (taurodeoxycholic acid hydrolase and taurocholic acid hydrolase) in the presence of a conjugated bile salt (taurodeoxycholic acid) as well as the strain's tolerance to the bile salt. Of the 49 strains, 26 expressed both enzymes, but only 15 tolerated taurodeoxycholic acid, 6 were sensitive to it, and 5 were inconclusive. Of these 49 strains, 3 were *L. crispatus* of which all three possessed both enzymes, but only one was tolerant to the bile salt, and 2 were *L. reuteri*, both of which contained the two hydrolase enzymes and both were tolerant to the bile salt. Moser and Savage concluded that possession of these types of enzymes was unrelated to the tolerance of toxic effects from bile salts [36]. They did not offer an alternative theory. It should be noted that neither Jin *et al.*, nor Moser and Savage, conducted their studies using Oxgall. There have been few studies and insufficient data on bile salt tolerance in *L. animalis*. This data showed *L. animalis* to be most susceptible, *L. reuteri* and *L. crispatus* somewhat tolerant, and *L. acidophilus* preferring growth in bile salts.

II.5.9. Shotgun Whole Genome Sequencing:

Three of the six *Lactobacillus* isolates were sequenced using next-generation sequencing (Mi-Seq platform). The results have been deposited in Genbank and manuscripts accepted for publication in the Journal of Genome Announcements (See Appendix D and E). The general genome features of the sequences for each strain can be found in Table 7.

Table 7: Summary of the Genome Features for Shotgun Sequence Selected *Lactobacillus* Strains

	L. crispatus- C25	*L. animalis*-P38	*L. reuteri*- P43
Isolated from	cecal contents of chicken	cecal contents of chicken	cecal contents of chicken
Genome size	2.3mb	1.9mb	2.1mb
Total number of genes	2,377	1,959	2,097
Total number of coding genes	2,094	1,724	1,895
Total number of hypothetical proteins	626	455	472
rRNA copies	24	45	39
tRNA copies	65	69	70
CRISPR arrays	4	2	0
G/C %**	36.8%	41.1%	38.7%

** This information was found in the manuscripts accepted for publication.

It appears that *L. animalis* has the smallest genome size, while *L. reuteri* has the largest genome size (i.e., *L. reuteri* > *L. crispatus* > *L. animalis*). Additionally, *L. crispatus* and *L. animalis* have CRISPR arrays, which suggests the ability to activate the CRISPR/Cas system, a prokaryotic immune system, to prevent damage from foreign invaders. The whole genome sequences for three strains of *L. acidophilus* were compared by Stahl and Barrangou (2013), and CRISPR arrays were found in the three strains [37].

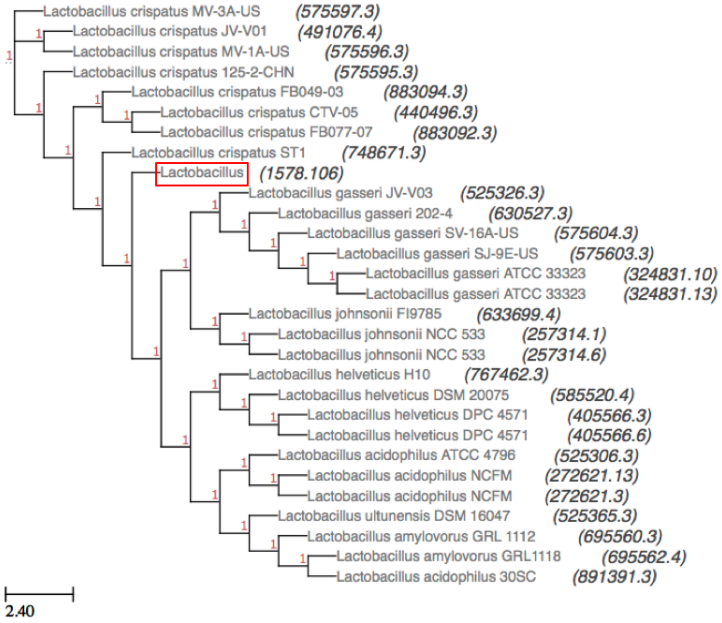
II.5.10. Distance Tree Comparison:

Distance trees represent the evolutionary closeness of species to one another. These distance trees were created for each of the three whole genome sequenced strains in order to validate their original identification based on their rRNA gene sequences. Data in Figure 8 confirmed

the correct classification of C25, P38, and P43 as *L. crispatus*, *L. animalis*, and *L. reuteri*, respectively. Indeed, L. crispatus (C25) is most closely related to *L. crispatus* ST1; *L. animalis* (P38) is most closely related to *L. animalis* KCTC 3501; and *L. reuteri* (P43) is most closely related to *L. reuteri* lpuph.

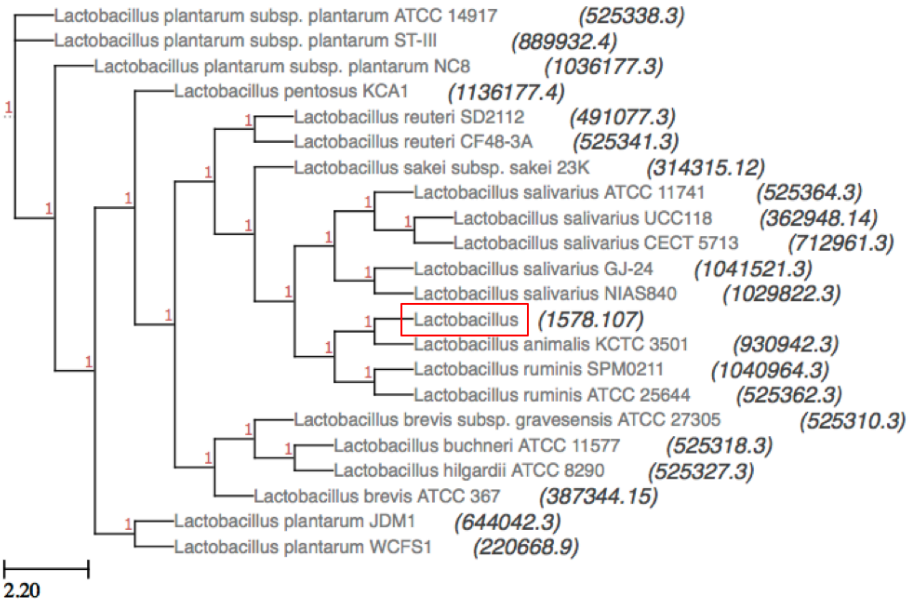
Figure 8: Distance Trees for the Three *Lactobacillus* Strains Sequenced[a].
[a] *Lactobacillus* strains highlighted in red are the strains analyzed in this study. Phylogenetic trees were built for each strain using other *Lactobacillus* genomes for comparison. The results placed each strain closest to its presumed species: C25 with *L. crispatus*, P38 with *L. animalis*, and P43 with *L. reuteri*. In addition, we assessed assembly quality by comparing known metabolism of each strain to both hand- and RAST-annotated functionality. The draft genomes were annotated using the NCBI Prokaryotic Genome Annotation Pipeline (www.ncbi.nlm.nih.gov/genome/annotation_prok/).

C25 Distance tree with other lactobacilli in system



- C25 outlined in red

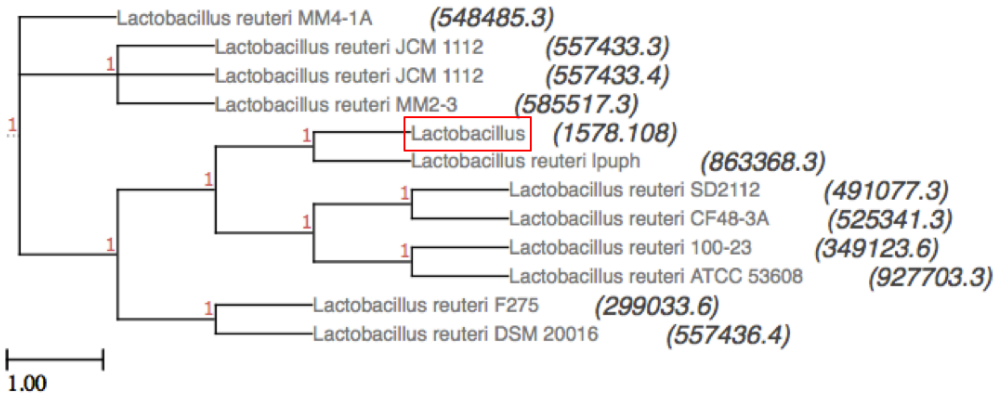
P38 Distance tree with other lactobacilli in system



- P38 outlined in red

P43 Distance tree with other *L. reuteri* in system

- P43 outlined in red – actually closest to strain lpuph



II.5.11. Identification of Potential Presence of Genes That Support the Physiological Properties Reported in this Study:

In-silico analysis of the annotated genes of the three sequenced genomes was conducted in an attempt to identify genes for oxidative stress, sugar utilization, acid tolerance, bile salt tolerance, bacteriocins, etc. In our analysis, we also included data from the literature for *L. acidophilus* that we did not sequence.

A) Oxidative Stress:

When grown in the presence of oxygen, some strains experienced reduced overall concentration, possibly due to uncoupled growth that resulted from interactions with reactive oxygen species. The annotated genomes of *L. animalis* (P38) and *L. reuteri* (P43) show that they possess genes encoding for enzymes capable of generating and eliminating hydrogen peroxide. *L. animalis* has the genes encoding for NADH Flavin reductase and peroxidase, while *L. reuteri* has the enzymes NADH oxidase and peroxidase. On the other hand, *L. crispatus* possesses the gene for pyruvate oxidase, which produces hydrogen peroxide, but no apparent enzymes to neutralize H_2O_2. I suspect this is not truly the case, as the genome sequence is incomplete, there are many hypothetical proteins whose function has not been determined, and the growth curve results show virtually the same maximal growth concentration when grown in the presence and absence of oxygen. According to the literature, *L. acidophilus* spp. possess NADH oxidase and NADH peroxidase, which contributes to their ability to

combat oxidative stress [38]. Additionally, none of the strains contained the enzyme catalase.

B. Sugar Utilization:

For the growth on semi-defined media with specified sugars, we tested four sugars: glucose, galactose, lactose, and galactooligosaccharide (GOS). Ten enzymes are required for microbes to engage in anaerobic glycolysis for the metabolism of glucose: Glucokinase, Glucose-6-phosphate isomerase, Phosphofructokinase, Aldolase, Triose-phosphate isomerase, Glyceraldehyde 3-phosphate dehydrogenase, Phosphoglycerate kinase, Phosphoglycerate mutase, Enolase (Phosphopyruvate hydratase), and Pyruvate kinase [39]. A genome search of potential genes that code for these enzymes turned up the following results: *L. animalis* contains the complete set of genes, however; *L. crispatus* and *L. reuteri* do not. *L. crispatus* is missing the gene for Glucokinase and *L. reuteri*, Phosphofructokinase. Since these three strains grew in commercial MRS whose sugar supplement is dextrose (D-glucose) as well as the semi-defined media with glucose, they probably possess all of the enzymes needed to metabolize this carbohydrate. Since the strain of *L. acidophilus* used in this study also performed similarly when grown in commercial MRS, it probably has all of the genes encoding for the enzymes required for anaerobic glycolysis of glucose as well. In order to metabolize galactose, the enzymes Galactose mutarotase, Galactokinase, Galactose-1-phosphate uridyltransferase, and UPD-galactose 4-epimerase (UDP-glucose 4-epimerase GalE) are required [40]. A genome search of

these enzymes shows all three strains possess all genes for making the enzymes required for galactose metabolism. This supports the growth concentration results observed in the study examining growth in galactose and semi-defined media. Since *L. acidophilus* grew in the presence of galactose, they probably contain all of the enzymes required to metabolize galactose as well, although the whole genome was not sequenced for this strain. In order to metabolize lactose into glucose and galactose, bacteria must possess the enzyme β-galactosidase [41]. All three strains contain at least one copy of the β-galactosidase gene. Since *L. acidophilus* grew in the presence of lactose, they probably contain the enzyme required to metabolize lactose as well. The mechanisms utilized by lactobacilli for metabolizing the prebiotic GOS are not as well understood as the previous three sugars; the current understanding is, "limited to in silico predictions based on genome sequencing projects" [42]. The Andersen *et al.* (2011) group studied *L. acidophilus* NCFM to try and identify proteins and enzymes upregulated in the presence of GOS. The group observed upregulation of the following proteins and enzymes, many of which are utilized for the other sugars metabolisms described: β-galactosidase, Lactose permease, Galactokinase, UPD-galactose 4-epimerase (UDP-glucose 4-epimerase GalE), Phosphonate transport system ATP-binding protein, Galactose-1-phosphate uridyltransferase, S-adenosylmethionine synthetase, Putative xanthine-uracil permease, and 30S ribosomal protein (specific protein not specified) [42]. After a search of the whole genome, all three strains returned the same results; they are missing Lactose permease, S-adenosylmethionine synthetase, and Putative xanthine-

uracil permease. While *L. acidophilus* in this study was not sequenced, they probably are missing the same, if not fewer of the proteins and enzymes highlighted by the Andersen group. All of the strains in this study were able to utilize GOS.

C. Acid Tolerance:

In response to being subjected to low pH, bacteria must possess one or several mechanisms to combat acid stress. According to Cotter and Hill (2003), lactic acid bacteria may utilize three different pathways: H^+-ATPase proton pump, Arginine deiminase pathway, and/or the glutamate decarboxylase system [43]. The following enzymes are utilized in each pathway: H^+-ATPase proton pump; ATPase, Arginine deiminase pathway; Arginine deiminase, Ornithine transcarbamylase, and Carbamate kinase, and the Glutamate decarboxylase system; Lysine, Arginine, and/or Glutamate decarboxylase [43]. A search of the genome showed that *L. reuteri* (P43) possesses all genes encoding the enzymes for all three pathways, while *L. crispatus* and *L. animalis* only possess the gene encoding for the ATPase enzyme for use in the H^+-ATPase proton pump pathway. This supports the results observed in the acid tolerance study, as *L. reuteri* was the most tolerant of all the strains tested. *L. animalis* showed moderate tolerance, while *L. crispatus* was the least tolerant to low pH environments (Figure 6). While *L. acidophilus* was not sequenced, this strain is likely to possess enzymes for at least one of the acid tolerance pathways, likely the H^+-ATPase proton pump pathway, as this strain's acid tolerance profile matched closely with *L. animalis* strain P38. However; there is not much in the literature to

corroborate this. There is some evidence to suggest a two component regulatory system may be utilized, but the consensus is that multiple molecular mechanisms are most likely involved [44], [45].

D. Bile Salt Tolerance:

It was previously thought that bile salt hydrolase enzymes were responsible for conveying bile salt tolerance in bacterial species, however; recent studies have turned away from this conclusion in favor of a broad list of proteins and enzymes that work together to contribute to bile tolerance. In fact, a search of the genome for each strain showed the absence of bile salt hydrolase enzymes. According to Ruiz *et al.* (2013), multidrug transporters, S-layer proteins, and oxidoreductase all contribute to the bile salt tolerance common to lactobacilli [33]. In addition, the group led by Hamon *et al.* (2012) found that enzymes involved in membrane modification- 3,5-epimerase, dTDP-glucose pyrophosphorylase, dTDP-4-dehydrorhamnose reductase, N-acetylglucosamine-6-phosphate deacetylase, and glucosamine-6-phosphate isomerase; detoxification- ATP-dependent Clp protease ATP-binding subunit, DnaK and Dna J chaperones; and central metabolism- 6-phosphogluconate dehydrogenase and threonyl-tRNA synthetase, were upregulated when *L. casei* strains were exposed to bile [46]. When the entire genome was searched for all of the listed enzymes, the results were such that: all three strains contain multiple multidrug transporters (nonspecific), oxidoreductase, ATP-dependent Clp protease ATP-binding subunit, and DnaK/ DnaJ chaperones. Only *L. crispatus* and *L. animalis* contain N-

acetylglucosamine-6-phosphate deacetylase. Only *L. crispatus* contain S-layer proteins and only *L. reuteri* contain dTDP-4-dehydrorhamnose reductase. None of the strains contained 3,5-epimerase, dTDP-glucose pyrophosphorylase, glucosamine-6-phosphate isomerase, 6-phosphogluconate dehydrogenase, and threonyl-tRNA synthetase. As seen in Figure 7, *L. animalis* was weakly tolerant to bile salts while *L. crispatus* and *L. reuteri* responded similarly and were found to be tolerant, when compared with the 0% bile salt control. It is possible, but unlikely, that the absence of S-layer proteins or dTDP-4-dehydrorhamnose reductase in *L. animalis* contributed to this strain's susceptibility to bile salts. In the case of *L. acidophilus*, the literature states a gene operon encoding a two-component regulatory system, multidrug transporters, S-layer proteins, and oxidoreductase tend to be upregulated in the presence of bile [33], [47].

E. Bacteriocins:

Lastly, the whole genome was searched for antimicrobial bacteriocin proteins and enzymes; all three shotgun sequenced strains do have the capacity to produce bacteriocins. *L. acidophilus* was not included in this sequencing, however; the literature states this strain also has the capability of producing bacteriocins, specifically, Lactacin B [48].

Conclusion:

Six *Lactobacillus* strains isolated from the gut of a chicken in a previous study were characterized and examined for their probiotic potential. The strains tested were *L. crispatus*, *L. animalis*, *L. acidophilus,* and *L. reuteri*. All *Lactobacillus* strains tested were found to be Gram positive, rod shaped bacteria with no catalase activity. Of the three *L. animalis* strains, P17, P28, and P38, P17 and P38 performed identically on each test; only P28 showed differentiation in the acid tolerance and bile tolerance studies. Generally, the growth of *L. animalis* was influenced by the presence of oxygen, whereas *L. crispatus*, *L. acidophilus,* and *L. reuteri* were not. *L. reuteri* and *L. crispatus* were able to utilize the most carbohydrates, between 19 and 22 of the 49 tested, respectively. When growth was measured anaerobically in the presence of certain carbohydrates (glucose, galactose, lactose, and GOS), all of the strains tested grew able to utilize GOS. Differentiation with regard to probiotic potential came during acid and bile tolerance testing. *L. animalis, L. acidophilus,* and *L. crispatus* were susceptible to high acid conditions, whereas *L. reuteri* was tolerant. When grown in the presence of bile, the growth of *L. acidophilus* and *L. reuteri* were least inhibited by the conditions, with the growth of *L. acidophilus* actually thriving in bile. Additionally, the whole genome was sequenced for three of the six strains, and after gene analysis, the same conclusions were reached. In the end, *L. animalis, L. acidophilus,* and *L. crispatus* were found to be unfit for use as potential probiotics, whereas *L. reuteri* was found to be fit for use as a potential probiotic, due to its high acid and bile tolerance.

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APPENDIX

Appendix A- *16sRNA sequences for the six Lactobacillus isolates*

>C25-crispatus
TGCAGGCNAGCGAANGGAACTAACANAGTTACTTCAGTAATGACGTTGGGAAAG
CGAGCGGCGGATGGGTGAGTAACACGTGGGGAACCTGCCCCATAGTCTGGGATA
CCACTTGGAAACAGGTGCTAATACCGGATAAGAAAGCAGATCGCATGATCAGCT
TTTAAAAGGCGGCGTAAGCTGTCGCTATGGGATGGCCCCGCGGTGCATTAGCTA
GTTGGTAAGGTAAAGGCTTACCAAGGCGATGATGCATAGCCGAGTTGAGAGACT
GATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGT
AGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAA
GAAGGTTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGGTAGTA
ACTGGCCTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAG
CGAGCGCAGGCGGAAGAATAAGTCTGATGTGAAAGCCCTCGGCTTAACCGAGGA
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Appendix B- *Relationship between OD_{600} and viable count (CFU/mL)*

Relationship between OD_{600} and viable count (CFU/mL) for *Lactobacillus* and *E. coli* strains tested[a]

[a] MRS media was used for the growth of *Lactobacillus* strains while MMRS was used for *E. coli*. All strains were inoculated from an overnight culture and incubated (static) anaerobically at 37˚C overnight. The OD_{600} was read for all strains. Cultures were serially diluted 10-fold in PBS and 100ul aliquots were plated in duplicate. Values were determined from the OD_{600} readings and plate counts from three independent experiments and averaged with standard deviation.

Strain	Average 1 OD standard with standard deviation
C25 (*L. crispatus*)	$(1.32 \pm 0.32) \times 10^8$ CFU/mL
P17 (*L. animalis*)	$(1.25 \pm 0.30) \times 10^8$ CFU/mL
P28 (*L. animalis*)	$(1.36 \pm 0.41) \times 10^8$ CFU/mL
P38 (*L. animalis*)	$(9.70 \pm 4.1) \times 10^7$ CFU/mL
P42 (*L. acidophilus*)	$(6.43 \pm 0.95) \times 10^7$ CFU/mL
P43 (*L. reuteri*)	$(1.19 \pm 0.007) \times 10^8$ CFU/mL
E.coli K-12 strain NC4468	$(5.29 \pm 0.63) \times 10^8$ CFU/mL

Appendix C- *Viable counts of strains exposed at varying lengths to different bile salt concentrations*

Viable cell counts of bacterial strains exposed to several bile salt concentrations at varying lengths of time averaged with standard deviation in CFU/mL.

CFU/ml / Strains					
	0hr | 0%	3hr | 0%	3hr | 0.3%	3hr | 0.4%	3hr | 0.5%
C25	$(8.9 \pm 0.71) \times 10^6$	$(2.56 \pm 0.03) \times 10^8$	$(1.90 \pm 0.10) \times 10^8$	$(1.20 \pm 0.007) \times 10^8$	$(8.6 \pm 0.42) \times 10^7$
P17	$(1.11 \pm 0.15) \times 10^7$	$(4.6 \pm 0.50) \times 10^8$	$(2.28 \pm 0.12) \times 10^8$	$(1.88 \pm 0.02) \times 10^8$	$(1.10 \pm 0.02) \times 10^8$
P28	$(1.16 \pm 0.16) \times 10^7$	$(4.7 \pm 0.14) \times 10^8$	$(2.31 \pm 0.08) \times 10^8$	$(1.87 \pm 0.04) \times 10^8$	$(1.19 \pm 0.007) \times 10^8$
P38	$(1.42 \pm 0.03) \times 10^7$	$(6.0 \pm 0.78) \times 10^8$	$(3.63 \pm 0.02) \times 10^8$	$(1.86 \pm 0.28) \times 10^8$	$(1.89 \pm 0.33) \times 10^8$
P42	$(8.3 \pm 0.07) \times 10^6$	$(2.12 \pm 0.15) \times 10^7$	$(3.2 \pm 0.21) \times 10^7$	$(4.0 \pm 0.28) \times 10^7$	$(5.5 \pm 1.1) \times 10^7$
P43	$(1.29 \pm 0.07) \times 10^7$	$(1.03 \pm 0.04) \times 10^8$	$(3.8 \pm 0.42) \times 10^7$	$(3.4 \pm 0.17) \times 10^7$	$(3.7 \pm 0.14) \times 10^7$
E. coli	$(3.06 \pm 0.30) \times 10^7$	$(1.26 \pm 0.00) \times 10^9$	$(1.53 \pm 0.02) \times 10^9$	$(1.75 \pm 0.02) \times 10^9$	$(1.77 \pm 0.08) \times 10^9$

Appendix D- *Genome announcements draft genome sequences of P38 (L. animalis) and P43 (L. reuteri)*

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Draft Genome Sequences of *Lactobacillus animalis* Strain P38 and *Lactobacillus reuteri* Strain P43 Isolated from Chicken Cecum

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Here, we present the genome sequence of *Lactobacillus animalis* strain P38 and *Lactobacillus reuteri* strain P43, both isolated from the cecum content of a 4-week old chicken fed a diet supplemented with the prebiotic β(1-4)galacto-oligosaccharide (GOS). These indigenous *Lactobacillus* isolates are potential probiotic organisms for poultry.

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Members of the genus *Lactobacillus* belong to the lactic acid bacteria (LAB) group. The genus *Lactobacillus* is one of the largest in the LAB group (1). *Lactobacillus animalis* and *Lactobacillus reuteri* have previously been isolated from food and animals (2–6).

Here, we report the genome sequences of *L. animalis* P38 and *L. reuteri* P43 isolated from the cecal microbiota of 4-week old female commercial white leghorn (W-36, Hy-line North America, Mansfield, GA). The birds were housed in climate-controlled HEPA-filtered isolation units (934-1 WP from Federal Designs, Inc., Comer, GA). Water and feed were provided *ad libitum*. Feed consisted of a standard corn-soybean starter diet (NC State Feed Mill) containing 1% commercial galacto-oligosaccharide powder (GOS-55%) (Yakult Pharmaceutical, Tokyo, Japan). The birds were maintained and euthanized according to a protocol approved by the Institutional Animal Care and Use Committee (OLAW#A3331-01). The cecal content from bird #365 was enriched anaerobically in a Coy anaerobic chamber (H_2 10%, CO_2 5%, and N_2 85%) (Coy Lab Products, Grass Lake, MI). The inoculum was enriched two-times using a modified glucose-free MRS (mMRS) medium containing 1.5% agar and 0.5% purified (90%) GOS (gift from Jose Barcena-Bruno). The isolates were selected based on their morphology and physiological properties. They were Gram-positive, rod-shaped, and non-spore formers; catalase negative; grown on glucose and lactose and produced acid and acid-clotted skim-milk. 16S rRNA gene sequencing showed that they were >97% and 95% close to *L. animalis* and *L. reuteri*, respectively. DNA was extracted from cells grown anaerobically in MRS media, using the Promega Wizard genomic DNA purification kit (Promega Corporation, Madison, WI).

Paired-end libraries were created for strains P38 and P43 with an average insert size of 251 bp. Libraries were sequenced on an Illumina MiSeq (Illumina, San Diego, CA) at Argonne National Laboratory (Lemont, IL). Modal k-mer coverage was 1100× for strain P38 and 2200× for strain P43. After error correction, reads were assembled using MIRA v4.9.5 (open source: http://genome.cshlp.org/content/14/6/1147.full). The final reported coverage was 90× for strain P38 and 60× for strain P43. After assembly, contigs with less than 20× coverage or length of less than 200 bp were discarded. The length of the draft genomes of *L. animalis* strain P38 and *L. reuteri* strain P43 are 2,151,063 bp and 1,940,664 bp with G+C contents of 41.1% and 38.7%, respectively.

Phylogenetic trees were built for each strain using other *lactobacillus* genomes for comparison (7). The results placed each strain closest to its presumed species: strain P38 with *L. animalis* and strain P43 with *L. reuteri*, giving evidence that the two strains were indeed the expected species and that the assembly was high quality. In addition, we assessed assembly quality by comparing known metabolisms of each strain to both hand- and RAST-annotated functionality (7).

The draft genomes were annotated using the NCBI Prokaryotic Genome Annotation Pipeline (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/).

Accession number(s). The genome sequences of *L. animalis* P38 and *L. reuteri* P43 were deposited in GenBank with accession numbers MCNR00000000 and MCNS00000000, respectively.

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Appendix E- *Genome announcements draft genome sequence of C25 (L. crispatus)*

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Draft Genome Sequence of *Lactobacillus crispatus* C25 Isolated from Chicken Cecum

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Lactic acid bacteria are important members of the gut microbiota of humans and animals. Here, we present the genome sequence of *Lactobacillus crispatus* strain C25, originally isolated from the cecum of 4-week-old chicken fed a standard diet. This isolate represents a potential probiotic strain for poultry.

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Lactobacilli are members of the lactic acid bacteria (LAB); they are found in diverse environments, including plants, fermented foods, oral cavities, and the gastrointestinal tracts of humans and animals. The genus *Lactobacillus* is one of the largest in the LAB group, and the genome sequence of a large number (~174 species) are available (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi).

Lactobacillus crispatus has previously been identified among the vaginal microbiota (1, 2) and the intestine of chickens (3, 4). *L. crispatus* strain C25 was isolated from the cecal microbiota of 4-week-old female commercial white leghorn laying-type chicks (W-36, Hy-line North America, LLC, Mansfield, GA, USA). The birds were housed in climate-controlled HEPA-filtered isolation units (934-1 WP from Federal Designs, Inc., Comer, GA, USA) and were fed *ad libitum* a standard corn-soybean starter diet (NC State Feed Mill). The birds were maintained and euthanized according to a protocol approved by the Institutional Animal Care and Use Committee (OLAW no. A3331-01). The cecal content were collected from bird number 337 and enriched on solid (1.5% agar) modified glucose-free MRS media containing 0.5% purified β (1–4) galacto-oligosaccharide (from Jose Barcena-Bruno) under anaerobic conditions (10% H_2, 5% CO_2, and 85% N_2) using a Coy anaerobic chamber (Coy Lab Products, Grass Lake, MI, USA). The isolate was selected based on its physiological and morphological properties (i.e., Gram-positive stains, non-sporeformer, rod-shaped, production of acid from glucose/lactose, acid coagulation of skim milk, inability to break down hydrogen peroxide, and 16S rRNA gene sequencing). The purified isolate was maintained at −80°C in MRS with 25% glycerol. DNA was extracted from cells, which were grown anaerobically for 20 h in MRS media, using the Promega Wizard Genomic DNA purification kit (Promega Corporation, Madison, WI, USA).

A paired-end library was created for *L. crispatus* C25 with an average insert size of 251 bp. The library was sequenced on an Illumina MiSeq (Illumina, San Diego, CA, USA) at Argonne National Laboratory (Lemont, IL, USA). Modal *k*-mer coverage was 1,060×. After error correction, reads were assembled using MIRA version 4.9.5 (open source: http://genome.cshlp.org/content/14/6/1147.full). The final reported coverage was 79×. After assembly, contigs with less than 20× coverage or length of less than 200 bp were discarded. The length of the draft genome is 2,341,728 bp with a G+C content of 36.8%.

A phylogenetic tree was built using other *Lactobacillus* genomes for comparison (5). The results placed C25 with *L. crispatus*, giving evidence that this isolate was indeed the expected species and that the assembly was of high quality. In addition, we assessed assembly quality by comparing the known metabolism of this strain to both hand- and RAST-annotated functionality (5). The combination of the correct phylogenetic results and correctly predicted metabolism gives sufficient confidence in the subsequent use of these assemblies for biological discovery.

The draft genome was annotated using the NCBI Prokaryotic Genome Annotation Pipeline (http://www.ncbi.nlm.nih.gov/genome/annotation_prok).

Accession number(s). The genome sequence of *L. crispatus* C25 was deposited in GenBank under the accession number MCJG00000000.

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