NEXT-GENERATIONGENOME SEQUENCING OF LACTOBACILLI ISOLATED FROM PRAIRIE VOLES

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Abstract: The gastrointestinal microbiome plays a critical role in aiding the host in maintaining homeostasis. Probiotic bacteria can aid the microbiota in maintenance of homeostasis by performing a multitude of functions such as modulating the immune system, maintaining the intestinal epithelium, and inhibiting pathogens. A probiotic organism is "-a live microorganism that, when administered in adequate amounts, confers a health benefit to the host." Several species from the genus of *Lactobacillus* are known probiotics.Lactobacilli have been used to manufacture fermented food products, have been found to be involved in the decay of plant matter, and are members of the oral, gastrointestinal and vaginal microbiomes. A body of evidence that probiotics, including certain strains of *Lactobacillus*, may be able to positively influence the gut-brain axis is emerging.*Microtusochrogaster*, the prairie vole, is a highly social animal and an excellent model for the studying the effect of environmental factors on behavior. Males that have not pair-bonded exhibit a high degree of interest in unfamiliar voles,but when exposed to mercury a shift in this behavior occurs and the animals develop an aversion to strangers. It is possible that administration of probiotics, such as lactobacilli, may be able to reverse this altered behavior. The probiotic potential of lactobacilli has been shown to be strain specific and there is a need to characterize the molecular mechanisms involved in probiosis. To understand the underlying mechanics by which probiotic strains of *Lactobacillus* could potentially reverse the effectof mercury exposure on prairie vole behavior,it is necessary to understand the genes involved and their function at a molecularlevel. To lay the foundation for future studies regarding these mechanisms, the genomes of three*Lactobacillus*strains previously isolated fromthe gastrointestinal tract of prairie voles, and tested *in vitro* forprobiotic characteristics,were sequenced using the Ion Torrent PGM®. Potential homologues of genes involved in probiotic action were identified and described in this study.

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CHAPTER I

INTRODUCTION

The gastrointestinal microbiota plays an important role in the normal functioning of the host and has been shown to be involved in key processes such as break down of dietary components,immune system modulation, regulation of fat storage, intestinal epithelial integrity, and protection of the host by exclusion of pathogens [1]. Some members of the human gut microbiota have been selected as probiotics. Selection is based on their health benefits, safety, stability and ability to survive within the human host [2, 3]. A probiotic is "-a live microorganism that, when administered in adequate amounts, confers a health benefit to the host" [4]. The probiotic effects of lactobacilli have been studied extensively and many of these studies demonstrated that probiotics can be used to promote health while somestudies have had mixed results. These conflicting results point to the factthat a better understanding of the mechanisms by which probiotics function at the molecular level is needed[2].Application of comparative genomics to genomes of probiotic strains could provide deeper insight into these mechanisms of probiosis. In recent years, the emergence of new sequencing technologies has dropped the price and amount of time required to sequence a bacterial genome, making it feasible to identify the genes that confer probiotic qualities and investigate their molecular functions.

The genus *Lactobacillus*was first proposed in 1901 by Beijerinck based on physiology and morphology[5],close to the time whenMetchnikoff suggested that consumption of lactic acid

bacteria benefits one's health[6].The genus *Lactobacillus* is a groupof gram-positive bacteria belonging to the family *Lactobacillaceae*, order *Lactobacillales*, class *Bacillus* and phylum *Firmicutes*. Currently, over 100 species of *Lactobacillus* have been identified[7], some of which are used in food production, others are involved in the decay of plant material, and others are members of the microbiotas of the gastrointestinal tract, vaginal flora, or the oral cavity. Lactobacilli are non-sporeforming rods with low G+C content genomes, most are microaerophilic and catalase negative[5]. Being nutritionally fastidious they require a rich growth medium and typically ferment carbohydrates to produce lactic acid as the major end product. The probiotic characteristics of lactobacilli include immunomodulation, inhibition of pathogens and microbe-microbe interactions, as well asstrengthening of the gut epithelium[2].

Microtusochrogaster, the prairie vole, is a highly social animal with many communal behaviors that are similar to humans making this animal an excellent model for the study of social behavior[8]. Curtis et al. have developed *M. ochrogaster* as an animal model for social behavior that could also serve as a model for the behavioral aspects of Autism Spectrum Disorders (ASD)[8]. When exposed to mercury chloride in drinking water, non-pair-bonded males develop a strong aversion to unfamiliaranimals and prefer to be with familiar animals. This behavioral change was sex specific, affecting only non-pair bonded males and not females or pair bonded males. Additionally, an increase in locomotor activity in response to amphetamine exposure was not seen in the non-pair-bonded voles exposed to mercury chloride but was seen in the control group and female animals. Response to amphetamines is mediated via the central dopamine pathways implying that exposure to mercury may alter this pathway.

The gut-brain-axis is the biochemical connection between the gut and the central nervous system[9]. There is a growing body of research on the communication between the gut and brain and the influence the gut microbiota may have on this communication[10]. It has been demonstrated that probiotics can have a positive effect on the gut-brain axis. Probiotics may be

able tonegate the altered social behavior seen in male prairie voles exposed to mercury. To beginthe process of testing this hypothesis Köhleret al.(pers. communication) isolated thirty *Lactobacillus*strains from the gastrointestinal tract of *M. ochrogaster*.*Lactobacillus* strains native to the prairie vole intestinewere selected because adaptation to the hostis an important prerequisite for an effective probiotic.According to 16SrRNAgene sequencing and Random Amplification of Polymorphic (RAPD) DNA fingerprinting all isolated strains were most closely related to *Lactobacillusjohnsonii.* All strains were tested for probiotic characteristics including bile resistance, acid tolerance, adherence to intestinal cells (Caco-2 cell line), hydrogen peroxide production, and antimicrobial effects, i.e. the inhibition of *Candida albicans, Pseudomonas aeruginosa*, *Staphylococcus aureus,* and non-pathogenic *Escherichia coli*K-12. Furthermore, the ability to survive in the presence of mercury chloride was tested. Based on the results of these *in vitro* tests,the genomes ofthree of the isolated vole *Lactobacillus* strains were selected to be sequenced using the Ion Torrent PGM^{TM}next-generation sequencer (Life Technologies, Carlsbad, CA).

Sequencing of the first human genome was completed in 2003 at a cost of 2.7 billion dollars[11]. Today next-generation sequencing allows for massively parallel sequencing, which has made it possible to sequence the genome of a person within only a few daysat a dramatically reduced cost. Sanger sequencing is considered first-generation sequencing and next-generation sequencing is sometimes broken down into second-generation and third-generation[12]. Second-generation sequencing requires a clonal amplification step prior to sequencing while third-generation sequencing is based on single molecule sequencing. The Ion Torrent PGM was launched in 2011 and utilizes emulsion PCR to clonally amplifya prepared DNA fragment library onto beads that are then loaded into individual sensor wells in a semiconductor chip[13]. During the sequencing reaction the chip is flooded with one deoxynucleotidetriphosphate at a time, and as they are incorporated a hydrogen ion is released that is detected by an ion-sensitive field-effect

transistor. When compared to Roche 454 GS Junior (Roche 454 Life Sciences,Branford, CT) andIlluminaMiSeq (Illumina Inc. San Diego, CA) next-generation sequencing systems, the Ion Torrent PGM was found to be the least expensive, with the highest throughputand shortest run time, but produced the highest number of homopolymers errors[14]. Ion Torrent technology was successfully utilized during the early stages of the 2011 German outbreak of enterohemorrhagic*Escherichia coli* O104:H4 to sequence the strain's genome within three days and allow for timely identification of virulence factors as well as tracking of the source [15]. As another example, Ion Torrent sequencing has also been used successfully to profile the intestinal microbiome in patients who received fecal transplants after *Clostridium difficile* infections[16]. In the present study the Ion Torrent PGM™ was utilized to sequence the genomes of three *Lactobacillus* strains isolated from the intestinal tract of *M. ochrogaster*. The genomes were annotated using Rapid Annotation using Subsystem Technology (RAST)[17] and genes that may confer probiotic capabilities were identified.

CHAPTER II

REVIEW OF LITERATURE

Immunomodulation

The volume of research concerning thepotential mechanisms by which lactobacilli can modulate the immune system is rapidly growing. A review by Kemang et. al. outlines the mechanisms how lactobacilli can alter immune functionssuch as increasing secretory IgA release, stimulating antimicrobial compound and mucinproduction,as well as the inhibition of pathogens at the gastrointestinal mucosa[18]. The ability of certain strains to promote the release of anti-inflammatory cytokines is also discussed, as well as the ability of other strains to stimulate the proliferation of lymphocytes and increase macrophage activity. Many studies have reported positive effects of lactobacilli on the immune system. *Lactobacillus casei*decreased the severity and death rate of mice infected with *Salmonella typhimurium*by improving the immune response [19, 20]. *Bifidobacteriumlactis* BB12 initiated the NF-κB and p38 MAPK pathways upon initial colonization triggering IL-6 gene expression [21] suggesting this strain could potentially be utilized to stimulate the immune system. It is not known,however, if this strain would induce inflammation in immunosuppressed animals or in animals in which antibiotics have altered the gut microbiota [22]. *Lactobacillus plantarum*was able to stimulate lymphocyte responses in immunocompromised and immunocompetent mice despite the fact that the bacteriumwas shown to be unable to persist in the gut [23].

Many probiotic characteristics such as stimulation of anti-inflammatory responses, improvement of allergic reactions and resistance to pathogens can be attributed to the ability to modulate immune responses [24, 25]. One way lactobacilli may influence immune responses is through dendritic cells. Dendritic cells are antigen presenting cells which play a central role in regulating the immune response both systemically and at mucosal sites. Dendritic cells are found in locations that are exposed to the environment including the intestine where they can be found interdigitating epithelial cells. As dendritic cells encounter microbial antigens they undergo phenotypic and functional changes that lead to secretion of chemokines and cytokines that stimulate the cells of innate and adaptive immunity. Dendritic cells can stimulate $CD4^+$ T cells (T helper) cells to become T helper 1 or T helper 2 cells[26]. Excessive response of T helper 2 cells is implicated in atopic allergies[27].*Lactobacillus gasseri, L.johnsonii,* and *L.reuteri*exhibited the ability to shift cytokine expression in dendritic cells toward T helper 1 differentiation[28].How lactobacilli modulate immune responses is strain-specific as seen in a study in which *L. johnsonii*NCC533 caused a shift toward the expression of IgG1 isotype in immune cell, which is associated with IL4 induction of B cells and the T helper 2 response, while *L.paracasei*caused a shift toward the expression of the IgG2aisotype, which is associated with IFN-γ stimulation of B cells and a T helper 1immune response [29]. Additionally, exposure of mouse spleen tissue to *L. acidophilus* ATCC 4356, *L.gasseri* ATCC 33323, *L.rhamnosus* LC705, and *L.casei*Shirota resulted in differing levels of T and B cell proliferation that were unique to the individual strains[30].

Probiotics may also help to negate the negative impact of stress on the immune system. Mice that were stressed due to restriction of food and mobilitythat received *L.casei* CRL 431exhibited an increased level of $CD4^+$ cells in the lamina propria, an increase in IgA-producing cells, an increase in secretory IgA in the lumen, and lower levels of IFN-γ when compared to mice that did notreceive the *L. casei* CRL 431 but where subjected to the same stressors[31]. Broiler

chickenssubjected to heat stress and given a *Lactobacillus*-based probiotic showed an improved humoral immune response to vaccinations given prior to exposure to heat stress, as well as decreased cortisol levels when compared to the control group [32]. In another study regarding the ability of probiotics to negate the negative effects of stress on the immune system, 136 university students, who were undoubtedly stressed because the study occurred during the period prior to final examinations, were given either milk fermented with*L.casei* DN-114001 (Actimel®)or skimmed milk[33]. The study occurred over a six week period and the group that received the probiotic milkdrink exhibited an increase in the number of total lymphocytes and $CD56^+$cells. Students in the control group exhibited a decrease in lymphocytes and $CD56^+$cells(CD56 is a marker for natural killer cells).

Pathogen Inhibition

A favorable influence on the composition of the gut microbiome is an important aspect of probiosis in which actions such as inhibition of pathogens through competition for nutrients, production of antimicrobial compounds, and competitive exclusion are generally considered,but probiotics may also work through synergistic mechanisms with endogenous bacteria [2]. When *Bacteroidesthetaiotaomicron*, a prominent member of the adult microbiome, was introduced into germ-free micein the presence of *Bifidobacteriumlongum*, a minor member of the adult microbiome and a probiotic, an expansion in the diversity of polysaccharides degraded by *B.thetaiotaomicron*was observed[34].Comparison of host epithelial transcriptomes from mono-colonized and co-colonized mice in this study found that co-colonization resulted in an induction of host genes involved in innate immunity while mono-colonization failed to do so.*Bacteriodes*species produce a number of oligosaccharides from the breakdown of complex carbohydrates. These oligosaccharides are fermented by lactic acid bacteria which thermodynamically favors further metabolism of complex carbohydrates by *Bacteriodes*[2, 35]. While probiotic bacteria comprise a small portion of the gastrointestinal microbiota, they play a vital role in the support of larger populations within the microbiota helping to strengthen and maintain the normal intestinal microbiota and thus act as strong pathogen deterrent.

Enteric pathogens have been shown to attach to oligosaccharide receptor sites in the intestine. There is evidence that probiotic bacteria can utilize the same attachment sites thereby excluding pathogens[2]. HT29 cells, a human colorectal cell line, upregulated expression of mucin genes in the presence of *L.plantarum* 299v and when co-incubated with *Escherichia coli* E2348/69 decreased the ability of *E. coli* to adhere to the cell line[36]. A mutant version of *L.plantarum*299v deficient in a mannose-specific adhesin exhibited a marked reduction in the ability to decrease *E. coli* adhesion. Another study found that biosurfactants produced by *L.*

*reuteri*RC-14(formerly *L. acidophilus* RC14) and *L.fermentum* B54 decreased the ability of the uropathogen*Enterococcus faecalis*1131 to adhere to glass in a parallel-plate flow chamber [37]. Lactobacilli also synthesize antimicrobial compounds such as hydrogen peroxide, bacteriocins, and lactic acid, all ofwhich can inhibit pathogens [2].

Epithelial Barrier Enhancement

Another important mechanism by which probiotic bacteria benefit their host is through enhancement of the epithelial barrier. One mode in which lactobacilli may enhance the function of the gastrointestinal epithelium is via the production of lactic acid, which is then metabolized to butyrate by endogenous bacteria[2, 38]. Butyrate is the preferred energy source for colonic epithelial cells [39] and has been shown to play an important role in colonic health. At low butyrate concentrations a decrease in permeability occurs and permeability decreases as butyrate concentration increase, however, at very high butyrate concentrations an increase in permeability is seen [40]. Another mechanism by which lactobacilli may promote the health of the gastrointestinal epithelium is throughincreasing the production of mucin[41]. Mucin genes were induced in Caco-2 cells upon exposure to*L.salivarius*UCC118. Disruption of a sortase gene (*srtA)* reduced *L. salivarius*UCC118 adhesion to the Caco-2 cells and resulted in a reduction in the expression of mucin genes. Certain *Lactobacillus* strains may also protect epithelial tight junctions from damage.For example, *L.rhamnosus*GG reduced the damage to tight junctions caused by *Escherichia coli* O157:H7 in polarized MDCK-I (Madin-Darby canine kidney) and T84 (human colonic) cell lines[41]. Additionally,*L. rhamnosus*GG cells and culture supernatant were able to mitigate cytokine-induced apoptosis in cultured mouse colon explantsand in the mouse colonic cell line KSRI (kinase suppressor of Ras1 knockout), as well as in HT29 (human colonic) cells [42]. While the exact mechanism by which probiotics enhance epithelium barrier function is not fully understood, it appears to be an important function of probiotic bacteria such as lactobacilli.

Probiotics and the Gut-Brain Axis

The biochemical signaling occurring between the gastrointestinal tract and the nervous system is referred to as the gut-brain-axis and it is regulated by both the central and enteric nervous systems. The gut-brain-axis is essential in maintenance of bodily homeostasis and disturbances in this system result in alterations in behavior and response to stress [9]. There is growing acknowledgement of the bidirectional communication between the gut and brain as well as the central role the influence of the gut microbiota might play in this communication [10]. The underlying mechanisms of this relationship are not yet fully understood.

Changes in the composition of the gut microbiota are associated with changes in behavior. Bercik*et al*. suggest a direct connection between the gut microbiota and the brain in light of their study which found that alteration of the gut microbiota with antimicrobials caused a change in the behavior of mice as well as modification in the expression of brain-derived neurotropic factor (BDNF) in the hippocampus[43]. These changes reversed with the withdrawal of antimicrobials and no such changes were present in germ-free mice that were given antimicrobials. The central nervous system also has profound effects on the composition of the gut microbiome. In mice that were subjected to the social stressor, -'social disruption,'- a shift in the microbiota was noted, i.e. with a decrease in the genus *Bacteriodes*and an increase in the genus *Clostridium*[44]. This change was most notable immediately after stress exposure. An increase in the proinflammatory cytokine IL-6 and the chemokine MCP-1 (Monocyte Chemoattractant Protein 1) wasalso seen after stress exposure. This increase in IL-6 and MCP-1 was not seen in stressed mice which were treated with antibiotics.

There is also increasing evidence that probiotics including certain*Lactobacillus*strains may be able to influence the gut-brain axis in a positive manner. When mice were infected with *Citrobacterrodentium*, a Gram-negative bacterium known to cause colitis in mice, they showed

symptoms of memory dysfunction upon exposure to acute stress[45]. Administration of*L.rhamnosus*R0011 and *L.helveticus*R0052 prevented the memory dysfunction. A combination of *L.helveticus* R0052 and *Bifidobacteriumlongum* R0175 reduced anxiety-like behavior in rats and in human volunteers it produced beneficial psychological effects in addition to reducing serum cortisol levels [46]. Both viable and attenuated *L.reuteri*ATCC 23272 decreased the perception of pain caused by colorectal distensionin healthy Sprague-Dawley rats [47]. *L. acidophilus* was found to increase the expression of opioid and cannabinoid receptors in gut epithelial cell which may cause an analgesic affect [48]. Children with Irritable Bowel Syndrome (IBS), who were given *L.rhamnosus*GG showed moderate improvement in abdominal pain during an eight week randomized control trial [49]. Induced colitis in mice produced anxiety-like behavior and also altered the expression of BNDF in the hippocampus[50]. Administration of *B.longum,* a known probiotic, reversed the altered behavior and brain chemistry. Levels of proinflammatory cytokines remained high in the animals given *B. longum,*indicating that the reversal of the anxiety-like behavior in this case was not related to immunomodulation. In another study, administration of *L.rhamnosus*JB-1 reduced anxiety-like and depression-like behaviors, lowered the level ofcorticosterone and altered expression of GABA receptors in mice [51].

There is clear evidence that probiotics can influence the gut microbiome and the gut-brain-axis in a positive manner.Probiotics may influence the gut-brain axis via direct interaction with the enteric nervous system, by the production of molecules that influence the nervous system or by improving epithelial function and restricting toxic metabolites from entering the circulation. The molecular mechanisms by which the microbiome or probioticsinteract with the gut-brain axis, however,are not well understood. The relationship is undoubtedly complex, most likely involving multiple signaling pathways. There is a need to understand both how the central nervous system of the host can affect the gut microbiome and how the microbiome can in turn

affect the central nervous system. A fuller grasp on how the molecular mechanisms of probiosis function will aid in a clearer understanding of this complex relationship.

A History of Conflicting Clinical Trials

Since Metchnikoff first proposed that the long life of Bulgarian peasants was due to the large amount of lactic acid bacteria-containing yogurt they consumed, the use of probiotics to promote health has steadily increased and now is a multi-billion dollar industry. Currently probiotics are categorized as supplements and are not subject to rigorous evaluation by the US Food and Drug Administration (FDA). For a probiotic product to be marketed for therapeutic use the manufacturer must submit an Investigational New Drug Application. To date there are no probiotic products on the market thathave been approved for therapeutic use by the FDA[52]. Clinical trials on the effectiveness of probiotics to treat various diseases have conflicting results, but there is enough compelling evidence to support the use of probiotics to treat and prevent disease.

There is extensive literature regarding the treatment of Inflammatory Bowel Disease (IBD) with probiotics, which has yielded mixed results. IBD is a spectrum of disorders characterized by ulceration, inflammation and stenosis of the gastrointestinal tract. An inability to tolerate commensal organisms is thought to play a role in the pathogenesis of IBD. The immune system of the gut is unique in its tolerance of commensal organisms that if found in other parts of the body would cause a severe and damaging immune response[53]. Some studies have shown probiotic intervention to be highly effective in treatment of IBD patients with pouchitis, an inflammation of the pouch created from the small intestine to hold waste after surgical removal of the colon. In a randomized double-blind placebo-controlled trial of forty patients with pouchitis, half receivedVSL#3 (4 strains of lactobacilli, 3 strains of bifidobacteria, and 1 strain of *Streptococcus salivarius* subsp. *thermophilus*) of which only three were reported to show signs of relapse, while all twenty in the placebo group had a relapse of pouchitis[54]. Additionally all patients had a subsequent relapse after discontinuation of probiotic treatment. The same probiotic

mixture was again shown to be effective in treatment of recurrentpouchitis in a second group of patients who underwent surgery for IDB[55] and again in patients which chronic pouchitis[56]. Not all studies, however, have shown probiotics to be of benefit in the treatment of IBD. A different studyusing VSL#3 resulted in many of the patients discontinuing use due to a lack of improvement in their chronic pouchitis[57].Another study also found the use of *L.johnsonii*LA1 ineffective for prevention of Crohn's disease relapse in patients in remission after surgery[58].

Much literature regarding the use of probiotics to treat female urogenital disorders exists, albeit with mixed results.Lactobacilli are the predominant member of the vaginal microbiome and low levels of *Lactobacillus* has been associated with bacterial vaginosis[59]. In a randomized control trial of 125 premenopausal women diagnosed with bacterial vaginosis, treatment with metronidazole in combination with *L.rhamnosus* GR-1 and *L.reuteri*RC-14resulted in an 88% cure rate while treatment with metronidazole alone resulted in a cure rate of only 40% [60]. A randomized control trial comparing the effectiveness of *L. acidophilus*, acetic acid and a placebo in the treatment of bacterial vaginosis in pregnant women found an 88% cure rate in the *L. acidophilus* group, 38% in the acetic acid group, and only 15% in the placebo [61]. As with the clinical trials for probiotic treatment of IBD, not all of the studies on treatment of bacterial vaginosis with probiotics were successful. A double-blind placebo-controlled study with 187 women found that women who were treated intravaginallywith a mixture of freeze-dried *L. fermentum, L. casei, L. rhamnosus* and *L. gasseri* in addition to receiving clindamycin had an improvement rate of only 56% while the placebo group had a cure rate of 63%[62]. A study of *L. acidophilus* NCDO 1748 alone for treatment of bacterial vaginosisresulted in only 7% cure rate versus 93% for metronidazole alone[63].

It is possible that lactobacilli may provide a bacterial barrier to the pathogens that cause urinary tract infection as the number of lactobacilli in women with recurrent urinary tract infections are often depleted [64]. However, little success has been seen in clinical trials.In a randomized

double-blind placebo-controlled trial, there was no difference in the recurrence of urinary tract infections in patients who used *L. rhamnosus*suppositories compared to the control group [65]. Another study using *L. rhamnosus*GG found 39% of women utilizing the probiotic had recurrence of urinary tract infections. Women who were given cranberry-lingonberry juice concentrate had a recurrence rate of only 16% while the control group had a recurrence rate of 39%[66].In contrast, a recent pilot study of nine women found *Lactobacillus crispatus* GAI 98332 effective in reducing the number of recurrent urinary tract infections [67].

Lactobacilli have been shown to be effective in the treatment of gastrointestinal problems such as colitis associated with *Clostridium difficile* infection [68], diarrhea associated with antibiotics(often due to*C. difficile* overgrowth), diarrhea due to infectious diseases and travel [69], as well as prevention of necrotizing enterocolitis in preterm neonates [70]. Probiotics may also help prevent colorectal cancer. Administration of the prebiotic, oligofructose-enriched inulin (SYN1) with probiotic strains, *L.rhamnosus*GG (LGG) and *Bifidobacteriumlactis* Bb12 (BB12) increased the number of *Bifidobacterium*and *Lactobacillus* and decreased the number of *Clostridium perfringens* while altering tumor makers favorably in patients with a history of colorectal cancer or polyps [71].

Much of the evidence from clinical trials for the use of probiotics in the treatment and prevention of disease is conflicting, but it must be taken into account that clinical trials depend largely on the compliance of the individuals involved and that probiotic qualities are specific to individual strains. The difference among strains is demonstrated by the ability of *L.rhamnosus*GR-1, which was isolated from the female urogenital tract, to colonize the vagina more readily and protectagainst urinary tract infections when compared to *L.rhamnosus*GG, which was isolated from the gastrointestinal tract[72]. The genetic difference between strains needs to be taken into account when investigating probiotics for disease prevention and treatment.

The Genes behind Probiotic Outcomes

Probiotic bacteria benefit their host though multiple factors such as suppressing the overgrowth of harmful bacteria, enhancing epithelial barrier function, and modulation of the immune system [2].For a bacterial strain to be effective as a probiotic it must be able to survive the harsh conditions of the gastrointestinal tract, including pH levels below 3.0, the presence of bile salts, and a constantly moving environment. The ability to persist in the intestinal tract and provide health benefits varies greatly between bacterial strains and depends on the genetic makeup of the strain. Following is a description of genes and molecules that have been characterized in regard to their involvement in mechanisms of probiosis.

D-alanylation of Lipoteichoic Acids

Cell wall composition and the ability to incorporate D-alanine into teichoic acids play an important role in the ability of bacteria to survive in the gastrointestinal tract. The *dltABCD* operon, found in Gram-positive bacteria, encodes four proteins involved in the process of addition of D-alanine tolipoteichoic acids (LTA)[73-75].*DltA*encodes a D-alanyl carrier protein ligase, which activates D-alanine with ATP. *DltB*encodes a putative transmembrane protein that is potentially involved in movement of the activated complex of carrier protein and D-alanine across the glycerol phosphate backbone of LTA. *DltC* encodes the carrier protein and *dltD*a membrane protein involved in ligation of D-alanine to LTA[73-75]. D-ala ester mutants vary widely in phenotype and the relationship of genotype to phenotype is strain specific due to the complexity of this operon[73].

Inactivation of the *dlt*operon in *L. rhamnosus*GG resulted in a 2.4-fold-increased cell length, a lowered ability to survive in gastric juice, an increased susceptibility to human beta-defensin-2,

and an increased rate of autolysis[73]. This mutant strain also displayed a decreased ability to grow in the presence ofcationic peptides such as those produced by the innate immune system. This mutant, however, still had the ability to adhere to a human cell line and form a biofilm. The cytokine expression in cell lines challenged with the wild-type and the mutant remained similar. An *in vivo* study of another*dlt*operon mutant, generated from a*L. reuteri*strain exhibited an impaired ability to colonize the gastrointestinal tract of *Lactobacillus*-free mice and a reduced ability to form a biofilm layer in the foregut[76]. This mutant also showed evidence of cell wall damage when inspected with electron microscopy, as well as a decreased ability to survive under acidic conditions and in the presence of the lantibioticnisin. However,*ex vivo* testing showed no decrease in the mutant's ability to adhere to foregut epithelium . *L. plantarumdlt*mutants produced a decrease in the inflammatory response [77, 78], demonstrating the importance of LTA composition in immunomodulation. The variability of cell wall composition may offer an explanation to the conflicting results regarding the ability of *Lactobacillus* strains to regulateimmune responses [78].

Bile Resistance

Bile salts contribute to the harsh environment encountered by bacteria in the gastrointestinal tract and bile salt hydrolases are common in lactobacilli and bifidobacteria isolated from the intestinal tract while uncommon in members of these species isolated from other locations[2]. Conjugated bile salt hydrolases have an N-terminal cysteine residue, belong to the chologlycine hydrolase family, and are classified as N-terminal nucleophilic hydrolases [79].Several studies have shown that bile salt hydrolase genes are expressed in*Lactobacillus*spp.in the gastrointestinal tract as well as upon exposure to bile salts during *in vitro* testing[80-82]. The number of bile salt hydrolase genes varies between species of lactobacilli and the reason for variability in the gene copy

number remains unknown[83].A lower tolerance to glycine-conjugated bile salts was seen in a*bsh-1*mutant strainof *L.plantarum*WCFS1 [84]. Inactivation of two genes encoding for bile salt hydrolases in *L. acidophilus* NCFM, however,resulted in no reduction inbile salt resistance[85]. In addition no reduction in bile resistance was noted in a mutant strain of *L. johnsonii*NCC533 after a triple knock-out of bile salt hydrolase genes [86].

Another mechanism that lactobacilli may use to resist bile is multidrug resistance (MDR) transporters. While MDR transporters are well known for their role in antibiotic resistance, they also have been shown to play a role in bacterial bile resistance [2, 87].Three exporter proteins, one of which was a MDR transporter, were found to be activated by the presence of bile in *L.plantarum*WCFS1 [88]. Disruption of a multidrug resistance transporter associated with a two-component response regulator involved in bile tolerance in *L. acidophilus* NCFM resulted in increased sensitivity to bile[89].

While it is unclear if the hydrolysis of bile salts is necessary for lactobacilli to survive within the gastrointestinal tract, bile salt hydrolysis may benefit the host by lowering of cholesterol. Bile salts are formed from cholesterol and once bile salts are deconjugated by intestinal bacteria, they are less soluble and more likely to be excreted. Lactobacilli are the largest subset of intestinal bacteria responsible for bile salt hydrolysis in the murine and chicken intestinal tract [90, 91].*In vitro* testing demonstrates differences among strains in their ability to alter cholesterol levels.Ingestion of *L. acidophilus* RP32 helped to lower serum cholesterol in pigs fed a high cholesterol diet while *L. acidophilus* P47 did not[92]. Both strains were resistant to bile, but only strain RP32 was able to remove cholesterol from growth media and lower cholesterol in the animal model.*L.plantarum*was able to lower cholesterol levels and increase LDL receptor expression in rats fed a high fat diet[93]. *L.plantarum*LP27, isolated from Tibetan kefir, was able to lower serum levels of cholesterol, triglycerides, and LDL-C in rats fed a high-cholesterol diet[94]. In addition, expression of theNiemann-Pick C1-like 1 (NPC1L1) gene, which encodes a

protein involved in absorption of cholesterol, was lower in Caco-2 cells when exposed to *L. plantarum*LP27.It is unclear whether bile salt hydrolases do increase the ability of lactobacilli to survive in the gastrointestinal tract, but they may still benefit the host by aiding in the regulation of cholesterol levels.

Bacteriocins

Many lactobacilli produce bacteriocins, a group of anti-microbial peptides, which are targeted at closely related organisms and appear to be regulated by population density[2]. Class II bacteriocins or non-lantibioticbacteriocins are heat stable, non-modified proteins and are the most common type of bacteriocin produced by lactobacilli[95]. The ability to produce bacteriocins has traditionally been considered important in the selection of probiotic strains, although there are relatively few studies that have clearly demonstrated the role of bacteriocins in gastrointestinal tract colonization or an involvement in probiosis[96].

Bacteriocins have shown some potential to inhibit pathogens such as *Listeria monocytogenes.Lactobacillus salivarius* UCC118 produces the broad spectrum class II bacteriocin, Abp118, which can protect against *L.monocytogenes*infection in a mouse model[97]. Mutant strains unable to produce Abp118 were incapable of stopping a*L.monocytogenes*infection in mice. Strains of *L.monoctyogenes*with an immunity gene for Abp118 were still able to cause an infection despite administration of *L.salivarius* UCC118 . Production of bacteriocins appears to play a role in aiding bacteria to carve out a niche in an intensely colonized environment such as the gastrointestinal tract [96]. In weaned pigs fed a probiotic mixture of *L.murinus* DPC6002, *L. murinus* DPC6003, *L.pentosus* DPC6004, *L.salivarius* DPC6005, and *Pediococcuspentosaceus* DPC6006, the strain producing a bacteriocin, *L.salivarius*DPC6005 was found in the highest amounts in ileum digesta and bound to the ileal mucosa[98]. Modification of *Streptococcus*

mutans to produce higher levels of bacteriocin allowed this strain to survive in the oral cavity for up to fourteen years with only a single application [96, 99, 100]. It is also possible that bacteriocins may act as signaling peptides in Gram-positive bacteria when concentrations are low, while acting in an inhibitory manner at high concentrations [101]. Additionally certain bacteriocins may also act as signaling peptides in an interspecies manner as well as cross-kingdom by communication with the host [96].

N-acyl Homoserine Lactone Hydrolases

Lactobacilli may alsohave the ability to degrade N-acyl homoserine lactones, a class of Gram-negative quorum sensing molecules involved in the induction of virulence factors in certainpathogenic bacteria.*Bacillus* spp. have shown the ability to degrade these signaling molecules through the expression of a N-acyl homoserine lactone hydrolase and inhibit the growth of Gram-negative plant pathogens [102, 103].*Bacillus* spp. N-acyl homoserine lactone hydrolases are classified as metallo-β-lactamases and contain the conserved motif HXHXDH and a zinc-binding motif [104]. *Bacillus* strain AI96 which was isolated from pond sediment was found to express *aiiA*, a gene encoding for an N-acyl homoserine lactone hydrolase. When used as an aquatic food additive in zebrafish tanks it was found to attenuate the virulence of *Aeromonashydrophila*, a Gram-negative pathogen[105]. *L.plantarum*has shown the capacity to inhibit the activity of N-acyl homoserine lactones produced by *Pseudomonas aeruginosa*and improve the healing process of burn wounds in mice with *P. aeruginosa* infections[106]. The ability of lactobacilli to produce N-acyl homoserinelactonases could be a key to the probiotic quality of pathogen inhibition which has been noted.

Adhesins: Mucin-binding &Fibronectin-binding Proteins

Adhesion to the gastrointestinal mucosal layer is an important trait of lactobacilli that facilitates probiosisbyincreasingthe time of persistence in the gut, pathogen exclusion, and interactions with the host that may modulate the immune response [2, 107]. Adherence to the gastrointestinal tract is a complex process and involves multiple factors. There are many proposed mechanisms for adhesion to the gastrointestinal tract mucosa,such as those instigated bysortase-dependent proteins, mannose-specific adhesins, extracellular matrix-binding proteins, mucus/mucin-binding proteins, and proteins with moonlighting functions such as elongation factor Tu, glyceraldehyde-3-phosphate dehydrogenase, and heat shock protein GroEL[2]. The two proteins that will be focused on in this paper arefibronectin-binding proteinsand proteins that potentially bind to the mucus layer of the gastrointestinal tract.

Intestinal epithelium is covered by a protective mucus layer comprised of a complex mixture of glycoproteins, antimicrobial compounds, immunoglobulins, lipids, and electrolytes.The thickness of this layer varies throughout the gastrointestinal tract; it is thickest in the colon and rectum. There are two layers, aloose outer layer that can easily be removed and an inner layer that is firmly attached to the underlying epithelium[108]. The presence of bacteria has been shown to be restricted to the outer layer[109]. Production of mucus-binding proteins in lactobacilli plays an important role in adhesion to the mucus layer and colonization of the intestinal tract.Mucus-binding proteins are cell surface proteins with a typical signal peptide and aLPxTG anchoring motif in the C terminus for covalent attachment to the cell surface[108]. Mucins are large glycoproteins that constitute a major component of the mucus layer.An *in silico* study found that 9*Lactobacillus* species harbored 48 proteins with mucus-binding domains[110]. The size of the protein and number of repeats varied greatly and were most common in, but not exclusive to bacteria that reside in the gastrointestinal tract.

Mucus-binding proteins that were able to bind pig gastric mucin as well as hen intestinal mucus have been described in *L.reuteri*1063 [111]. *L.fermentum* BCS87 expressed both mucus-binding and mucin-binding proteins and was able to bind pig mucus as well as partially purified pig gastric mucin[112]. *L.plantarum*WCFS1 was found to produce a mannose-specific adhesin, and when the gene encoding for this protein was inactivatedthe result was a mutant that was no longer able to agglutinate the yeast *Saccharomyces cerevisiae,* whose cell wall contains mannose [113].A study done on a*L.salivarus* UCC118 mutant deficient in a sortase-dependent mucus-binding protein,revealed that the strain had a significantly decreased ability to adhere to Caco-2 cells [114].*L. rhamnosus*is able to produce a mucus-binding pili as well as a mucus-specific adhesin that may work together synergistically in binding to the mucus layer [115].In a study conducted by Buck et al., several*L. acidophilus* NCFM mutants deficient in genes associated with adherence were created[116]. A mutant deficient in a mucin-binding gene showed a 65% decrease in ability to bind to Caco-2 cells and a fibronectin-binding protein mutant showed a decrease of 76%.No single mutant became unable to bind to the cell line implying that adhesion to gastrointestinal epithelium is complex and multigenic[116].

Fibronectin is an adhesive glycoprotein and a major component of the extracellular matrix in vertebrates.Fibronectin plays important roles in cell adhesion, migration, growth and differentiation[117]. Fibronectin binding has been connected to the ability of pathogens such as *Streptococcus pyogenes*to cause infection[118]. Fibronectin as well as other extracellular matrix components such as fibrinogen and collagen are shed into the mucus layer or may be exposed if there is tissue damage. It is possible that the ability of lactobacilli to bind extracellular matrix components may provide protection from pathogens in the event of tissue injury[119].In a survey of nineteen strains of *Lactobacillus* used in fermented dairy products all were able to bind fibronectin, as well as fibrinogen and collagen[119]. *L. acidophilus* and *Lactobacillus agilis*were both found to bind the fibronectin of human intestinal 407 cells [120]. *L. acidophilus* CRL 639

bound fibronectin that was immobilized but not soluble forms, in addition this strain was able to bind immobilized collagen[120].

Mercury Resistance

The ability to survive in the presence of heavy metals and to protect their host from the toxic effects of heavy metals is not traditionally considered in probiotic studies, but will be touched upon briefly here due to the planned use of the probiotic strains being developed in animals experimentally exposed to mercury. Heavy metal exposure has been associated with a wide variety of diseases and lactobacilli may offer protection from heavy metals. Certain strains of lactobacilli are able to bind heavy metals and may prevent them from entering the hosts system via the gastrointestinal tract[121]. *L.reuteri*strains isolated from mud and sludge were able to bind cadmium and lead and to remove these heavy metals from growth media. These strains also demonstrated the ability to tolerate bile and acidic conditions as well as adhere to mucus[122]. Lactobacilli have been shown to bind a wide variety of heavy metals[123-125]. In a test of 103 lactic acid bacteria most strains were able to bind cadmium, lead, arsenic and mercury[126]. The amount of mercury removed from media was so high that the growth of the bacteria was hindered; possibly indicating that mercury was being taken into the cell. A potential cell surface protein involved in mercury binding was also described.*In vitro* experiments done with HT29 cells have shown that certain *Lactobacillus*strains can reduce oxidative stress induced by heavy metals while otherstrains of this genus can be a source of oxidative stress[127]. In a mouse model*L.plantarum*CCFM8610 reduced absorption of cadmium from the intestinal tract, reduced tissue accumulation of cadmium, and reduced oxidative tissue damage[128].

In summary, the mechanisms by which lactobacilli and other probiotic bacteria provide health benefits to their hostsare multiple and multifaceted, involvingthe interplay of a wide range of

genes and molecules. It is clear that probiotic characteristics are strain dependent and in order to understand the probiotic characteristics of a specific strain it must be analyzed on a molecular level. Following the annotation of the genomes sequenced in this study, putative homologues of the genes and gene families discussed above were identified by comparison to closely related genes found in the NCBI data bases in order to assess their putative functions and potential to impart probiotic characteristics.

CHAPTER III

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Thirty *Lactobacillus* strains were previously isolated from the intestine of *Microtusochrogaster,* the prairie vole, using enrichment by growth on Difco Lactobacilli MRS (de Mann, Rogosa and Sharpe medium for lactobacilli; BD Diagnostics, Franklin Lakes, NJ) at 37°C for 48 h within a GasPak^{TM} 100 container and EZ Anaerobe Pouch system (BD Diagnostics) to generate anaerobic conditions (Assefa *et al*., in preparation). The strains were assessed *in vitro* for probiotic characteristics and phylogenetic relationships (based on 16S rRNAgene sequences) to known probiotics (Assefa et al., in preparation). For this study, three strains, PV012, PV021,and PV034,were selected for genome sequencing based on the results of these *in vitro* tests.The strains were cultured from frozen stocks and grown at 37^0C on agar plates with Difco Lactobacilli MRS (de Mann, Rogosa and Sharpe medium for lactobacilli; BD Diagnostics, Franklin Lakes, NJ). Individual colonies were then selected and subcultured in Difco Lactobacilli MRS broth and incubated at 37^0C for approximately 48 hours without shaking.

DNA Extraction

DNA was extracted using the following phenol chloroform method. The cultures were spun down for 10 minutes at 4,500xg and the MRS broth was removed. The cells were then

resuspended in TE (10mM Tris; 1 mM EDTA, pH 8.0) and transferred to a clean tube. Lysozyme was added and the cells were incubated for 30 min at 37°C. Proteinase K and 10% SDS were added and the cells were incubated up to 3 hours. One tenth volume of 5M NaCl was added and a phenol chloroform extraction was performed three times. Isopropanol (1:1 vol.) was used to precipitate the DNA which was then washed with cold 70% ethanol. The DNA was resuspended in TE containing RNase (15mM Tris-HCl, pH 8.0, 10mM EDTA; 100µg/ml RNAse;Teknova, Cat. Nr. T4579) and an additionalphenol chloroform extraction was performed. One tenth volume 3 M Na-Acetate and 2.5 volumes ethanol were used to precipitate the DNA. After centrifugation, the DNA pellets were washed with 70% ethanol, air dried and subsequentlyresuspended in 200µL of 10 mMTris pH 7.5. DNA concentrations were determined using aQubit® fluorometer (Catalog no. Q32866, Invitrogen, Carlsbad, CA) in conjunction with the Qubit® dsDNA BR Assay Kit (Q32850, Invitrogen) according to the manufacturer's protocol for determining the concentration of double stranded DNA. Due to the fact that the extractions produced variable levels of DNA concentration and quality, additional DNA was extracted using the ZR Fungal/Bacterial DNA Mini Prep kit (Zymo Research, Irvine, CA) according to the manufacturer's protocol.

Library Building and Sequencing

Isolated genomic DNA was fragmented to an approximate length of either 200 or 400 base pairs (depending on the sequencing protocol used) using the DiagenodeBioRuptor® sonicator (Diagenode, Denville, NJ). The settings used for the sonicator were chosen as described in the *Ion Xpress™ Plus gDNA Fragment Library User Guide* (Publication Part Number 4471989, Life Technologies, Grand Island, NY) under the section "Fragment gDNA with the BioRuptor® Sonication."For the 200 base pair libraries, the samples were sonicatedfornine 10minute periods, for a total of 90 minutes with rest periods of 10 minutes between sonications. For the 400 base

pair library, samples were sonicated for four 10 minute intervals and one 4 minute interval, for atotal of 24 minutes, without rest periods.

Ion Torrent adapters were ligated to the DNA fragments and the nicks were repaired using the Ion Plus Fragment Library Kit according to the protocol in the *Ion Xpress™ Plus gDNA Fragment Library User Guide* (Publication Part Number 4471989) under the section "Ligate adapters, nick-repair, and purify the ligated DNA." Fragments approximately 330 base pairs in length were selected for the 200 base pair library and fragments 480 base pairs were selected for the 400 base pair library, using Life Technologies E-Gel®SizeSelect™Agarose Gels according to the guidelines under the section "Size-select the library with the E-Gel® SizeSelect™Agarose Gel" (Publication Part Number 4471989). To determine the proper dilution factor for emulsion PCR, real time PCR was performed using the Ion Library Quantitation Kitfollowing the protocol in the user guide (Publication Part Number 4468986 Rev. C). The samples were then diluted and emulsion PCR was performed using the Ion PGM™ Template OT2 200 Kit V2 or the Ion PGM™ Template OT2 400 Kit, according to the protocol in the accompanying user guide (Ion Torrent Publication Number 4478372). After emulsion PCR, the percent of templatedion sphere particles (ISPs) was measured using the Qubitflourometer with the Ion Sphere Quality Control Kit according to the protocol (Ion Torrent Publication Part Number 4478372).The samples were then sequenced using the Ion PGM™ Sequencing 200 Kit v2 or the Ion PGM™ Sequencing 400 Kit according to the protocol in the section "Sequencing protocol—Ion 314™ Chip" (Ion Torrent Publication Number MAN0007273). The Ion Torrent PGM 3.4.1 and 3.4.2 platforms were utilized with the 314 chip. On the Ion Torrent Server, the Whole Genome setting was utilized with default settings.

Bioinformatics

Sequences were assembled both with reference genomes and *de novo* using the Ion Torrent MIRA assembler plug-in (v3.4.1.1 and v3.4.2.0) available on the Ion Torrent Server.

Additionally, all three genomes were also aligned to known *Lactobacillus* genomes using the Ion Torrent alignment plug-in (v3.4.48996). Assembled sequences were then uploaded toRAST for annotation[17, 129]. PSI-BLAST was used to query the NCBI database[130] for proteins of similar function. The amino acid sequences were also queried through the NCBI Conserved Domain Database[131-133]. Amino acid sequences of the genes of interest were also compared to one another using CLUSTAL[134].

CHAPTER IV

RESULTS

The genomes of PV012 and PV021 were sequenced using a 200 base pair read length sequencing kit (Ion PGM™ Sequencing 200 Kit v2) and the genome of PV034 was sequenced using a 400 base pair read length kit (Ion PGM™ Sequencing 400 Kit). With an average of 268 base pairs,the average read lengths from the 400 base pair kit were much shorter than expected. The read length, however, was long enough to increase the length of the contigs after assembly (see Table 1). All three genomes aligned poorly with published lactobacilli whole genomes and *de novo* assembly produced the best assemblies.

Table 1: Sequencing and assembly summary

Sequencing and Assembly Summary			
Strain	PV021	PV012	PV034
Sequencing kit	200 bp	200 bp	400 bp
Average read length	213	178	286
Viable reads	502,739	432,448	466,717
Assembled reads	465,498	391,484	426,433
Contigs	162	38	39
Longest contig	214,706	235,324	380,249
N50[a)]	51,611	183,313	216,286
N90[b)]	3,732	44,233	24,772
N95[c)]	1,993	11,827	13,120
Coverage[d)]	65.64X	49.00X	97.12X
Genome length	1,717,565 bp	1,549,227 bp	1,590,902 bp

a) N50 is a weighted statistical measure of the median contig length in a set of sequences. The N50 value is the length L (in base pairs) such that 50% of the bases are in contigs the size ofL or greater. Larger N50 values correlate to more complete assemblies.
b) N90: 90% of assembled reads are this length or greater.
c) N95: 95% of assembled reads are this length or greater.
d) The average coverage per base considering only contigs that are at least 5kb in length.

***Lactobacillus* PV021 sequencing overview: 200 bp kit**

Qubit quality control estimated 58.12% of Ion Sphere Particles (ISPs) weretemplatedprior to enrichment. Sequencing produced a total of 1,024,696 reads, 47% of which were filtered due to polyclonality, 4% were filtered due to low quality and less than 1% of reads were filtered due to primer dimers resulting in a total of 502,739 viable reads. The average read length produced was 213 base pairs and the longest read produced was 372 base pairs (Fig. 1).

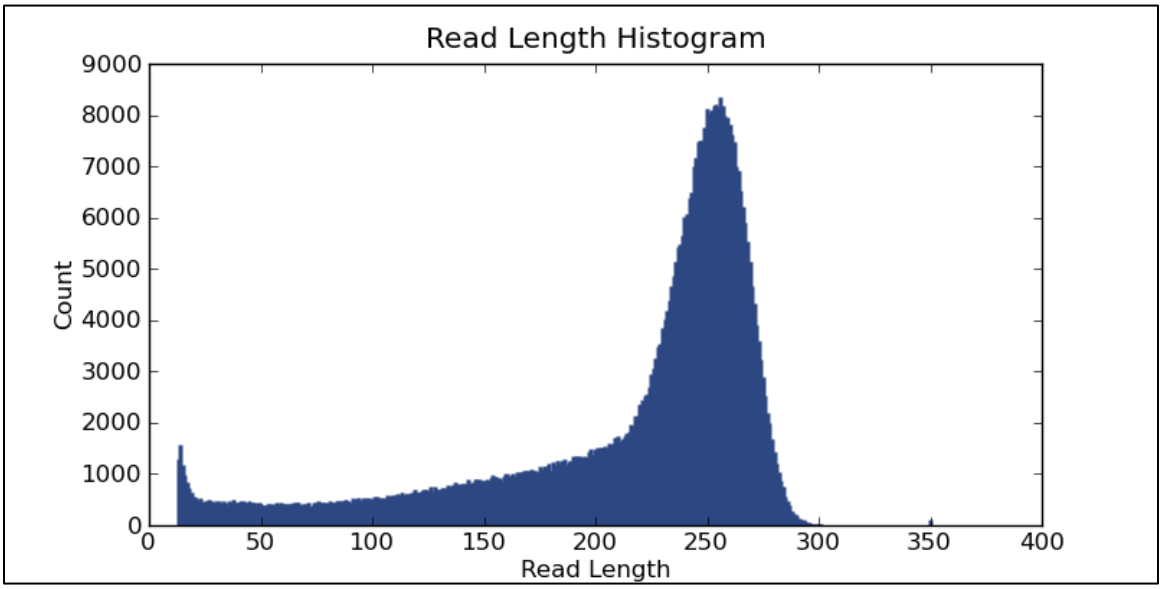

Fig. 1: Strain PV021 read length histogram

Strain PV021 assembly with MIRA and annotation with RAST

De novo assembly with MIRA yielded the highestnumber of contigs in all strains sequenced. Assembly of strain PV021 with MIRA produced 162 contigs with the largest being 214,706 base pairs long. A total of 465,498 reads were assembled with 65.64X coverage (Table 1).RAST discovered 220 subsystems, 1,658 protein coding sequences, 74 RNAs and 39 potential missing genes for strain PV021. The genome size was 1,717,565 base pairs and60% of the genes identified by RAST were allocated to subsystems shown in Figure 2 and Table2. The closest relatives determined by RAST were first*L. johnsonii*NC533 and second*L. gasseri*ATCC 33323 (Fig. 7).

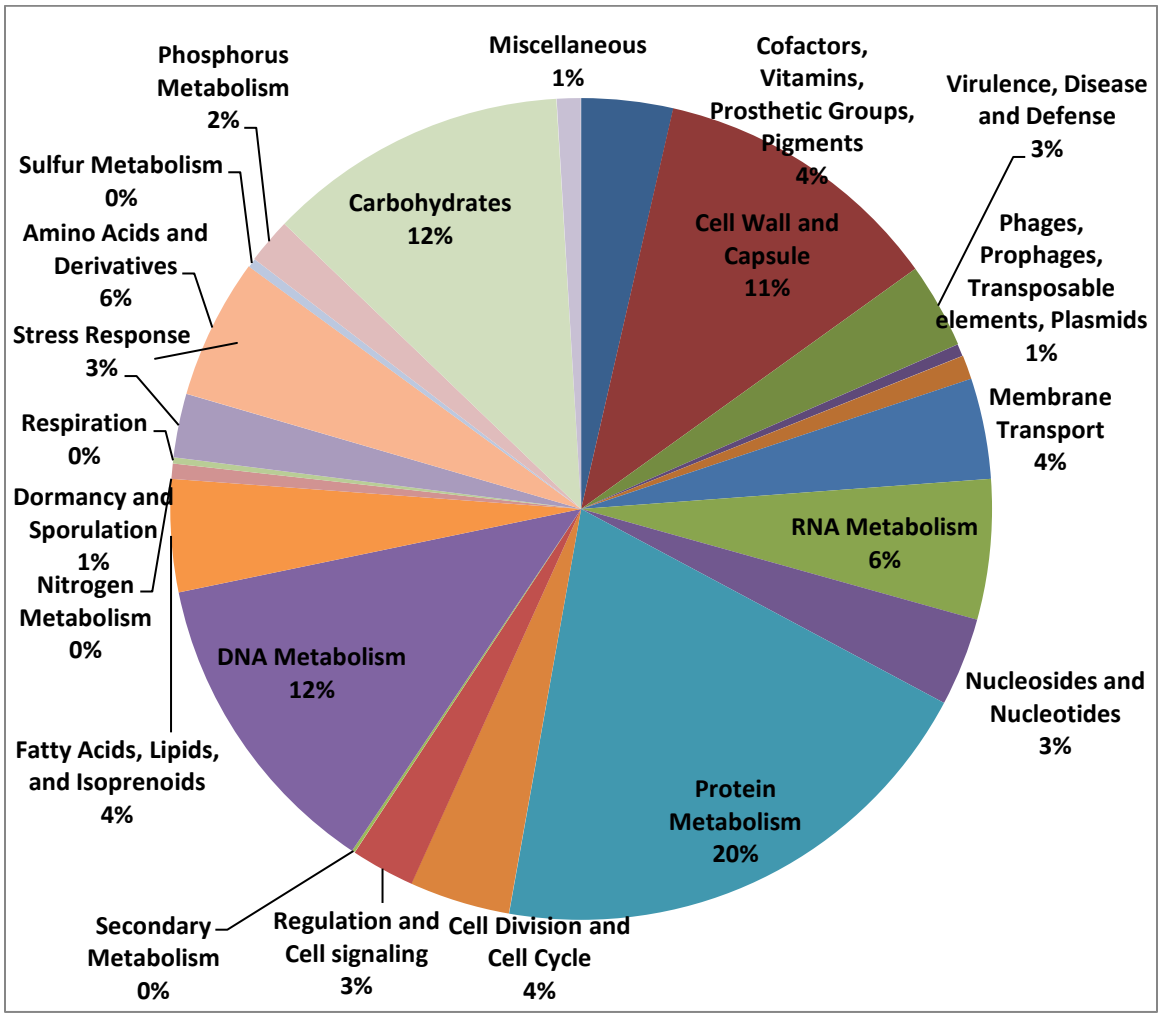

Fig. 2: PV021 distribution of genes into subsystems

***Lactobacillus*strain PV012 sequencing overview: 200 bp kit**

Qubit quality control estimated 52.88% of ISPs were templated prior to enrichment. Sequencing produced a total of 916,599 reads, 46% of which were filtered due to polyclonality, 7% were filtered due to low quality and less than 1% of reads were filtered due to primer dimers resulting in 432,448 viable reads. The average read length produced was 178 base pairs and the longest read produced was 367 base pairs (Fig. 3).

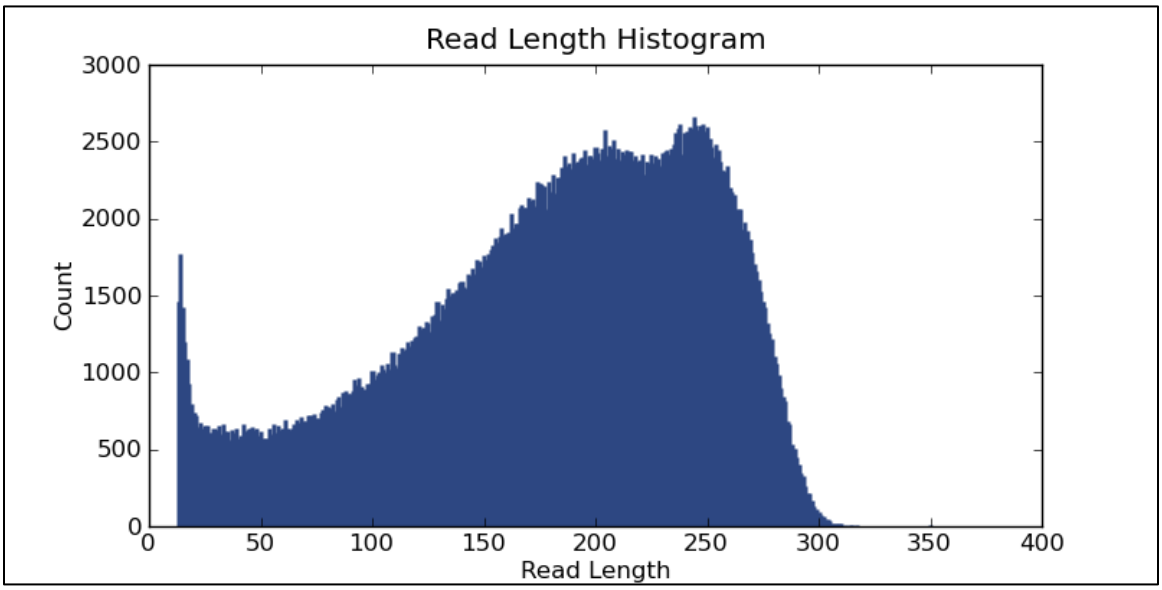

Fig. 3: Strain PV012 read length histogram

Strain PV012 assembly with MIRA and annotation with RAST

Assembly of sequence data for strain PV012 with MIRA produced 38 contigs with the largest being 235,324 base pairs long. A total of 391,484 reads were assembled with 49.00X coverage (Table 1).In strain PV012 RAST discovered a total of 214 subsystems, with 1,486 protein coding sequences, 101 RNAs and 16 potentially missing genes. The genome sizewas 1,549,227 base pairs and 57% of the features discovered using RAST were allocated to the subsystemsshown in Figure 4 andTable 2. The closest relatives determined by RAST were first*L. johnsonii*NC533 and second*L. gasseri*ATCC 33323 (Fig. 7).

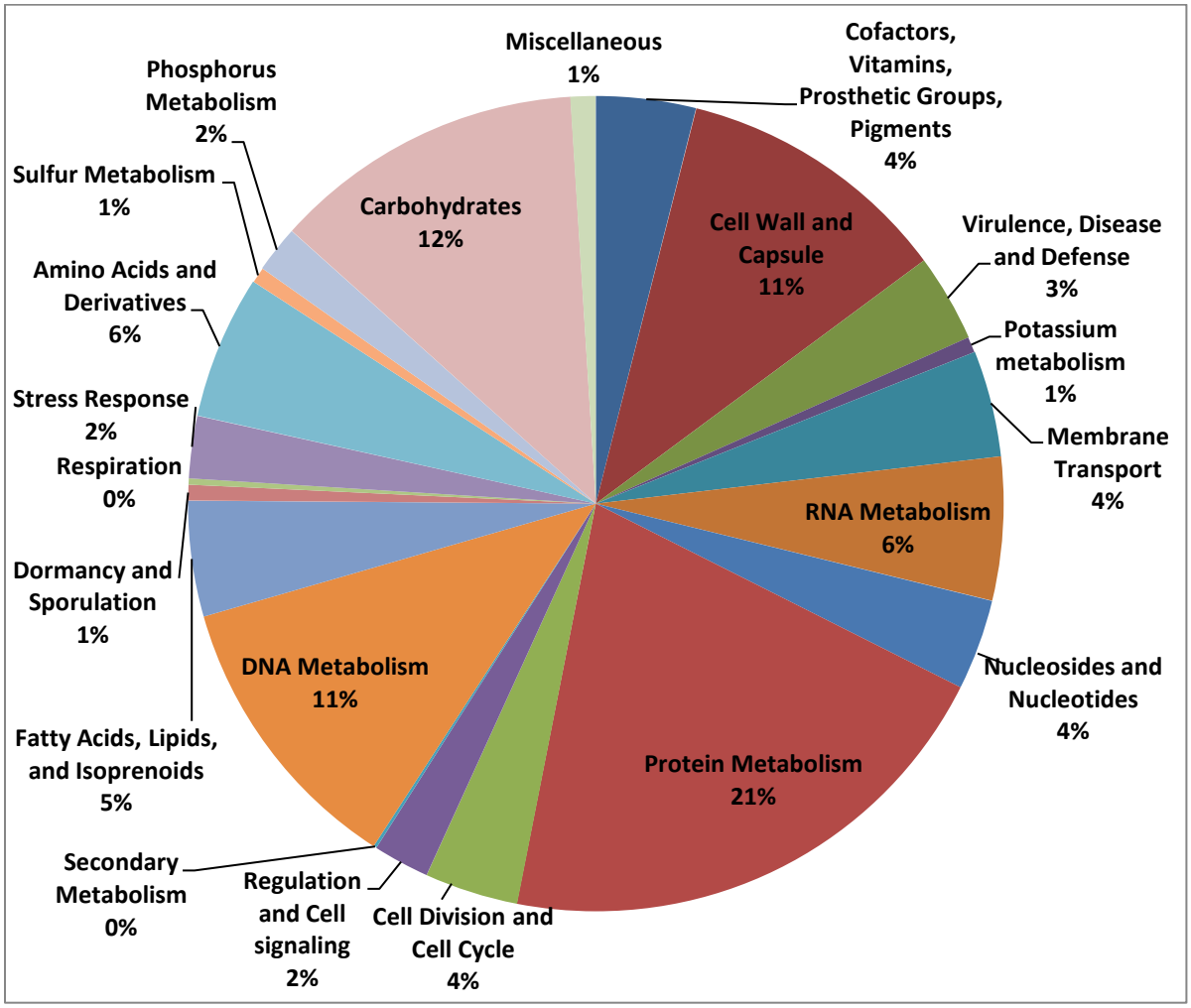

Fig. 4: Distribution of genes into subsystems for PV021

Strain PV034 sequencing overview: 400bp kit

Strain PV034 had a Qubit quality control estimation of 24.50% templated ISPs prior to enrichment. Sequencing produced a total of 1,017,403 reads, 45% of which were filtered due to polyclonality, 9% were filtered due to low quality and less than 1% of reads were filtered due to primer dimers resulting in 466,717 viable reads. The average read length produced was 286 base pairs and the longest read produced was 635 base pairs (Fig 5).

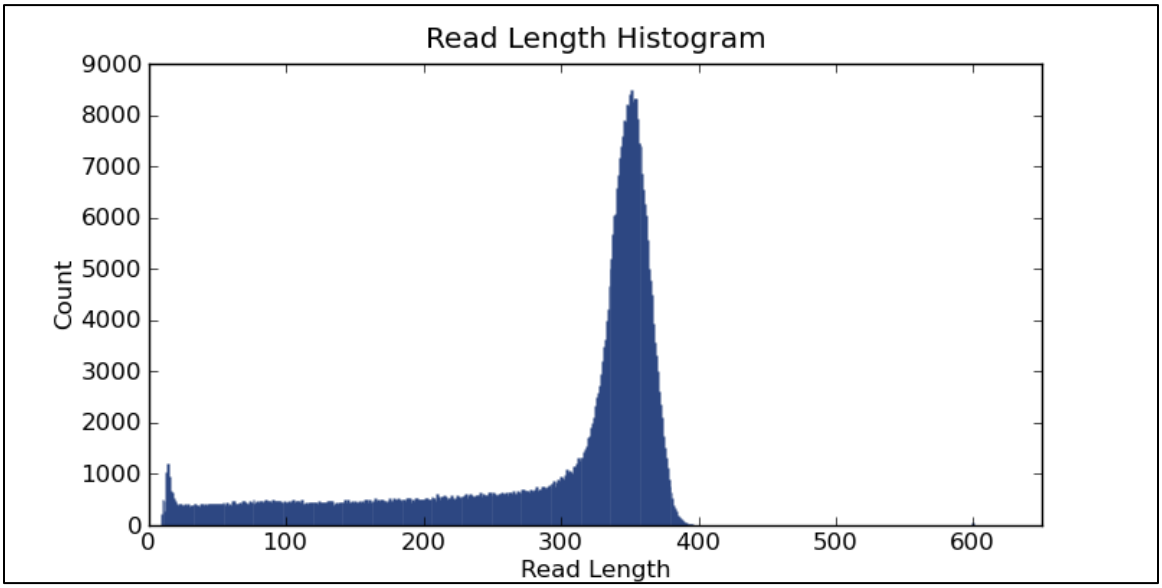

Fig. 5: Strain PV034 read length histogram

Strain PV034 assembly with MIRA and annotation with RAST

Assembly of sequence data for strain PV034 with MIRA produced 39 contigs with the largest being 380,249 base pairs long. A total of 426,433 reads were assembled with 97.12X coverage (Table 1).In strain PV034 RAST discovered a total of 227 subsystems,1,531 protein coding sequences, 68 RNAs and 22 potentially missing genes. The genome size was 1,590,902 base pairs and 59% of the features discovered using RAST were allocated to the subsystems shown in Figure 6 andTable 2. The closest relatives determined by RAST were first*L. gasseri*ATCC 33323 and second*L. johnsonii*NCC 533 (Fig. 7).

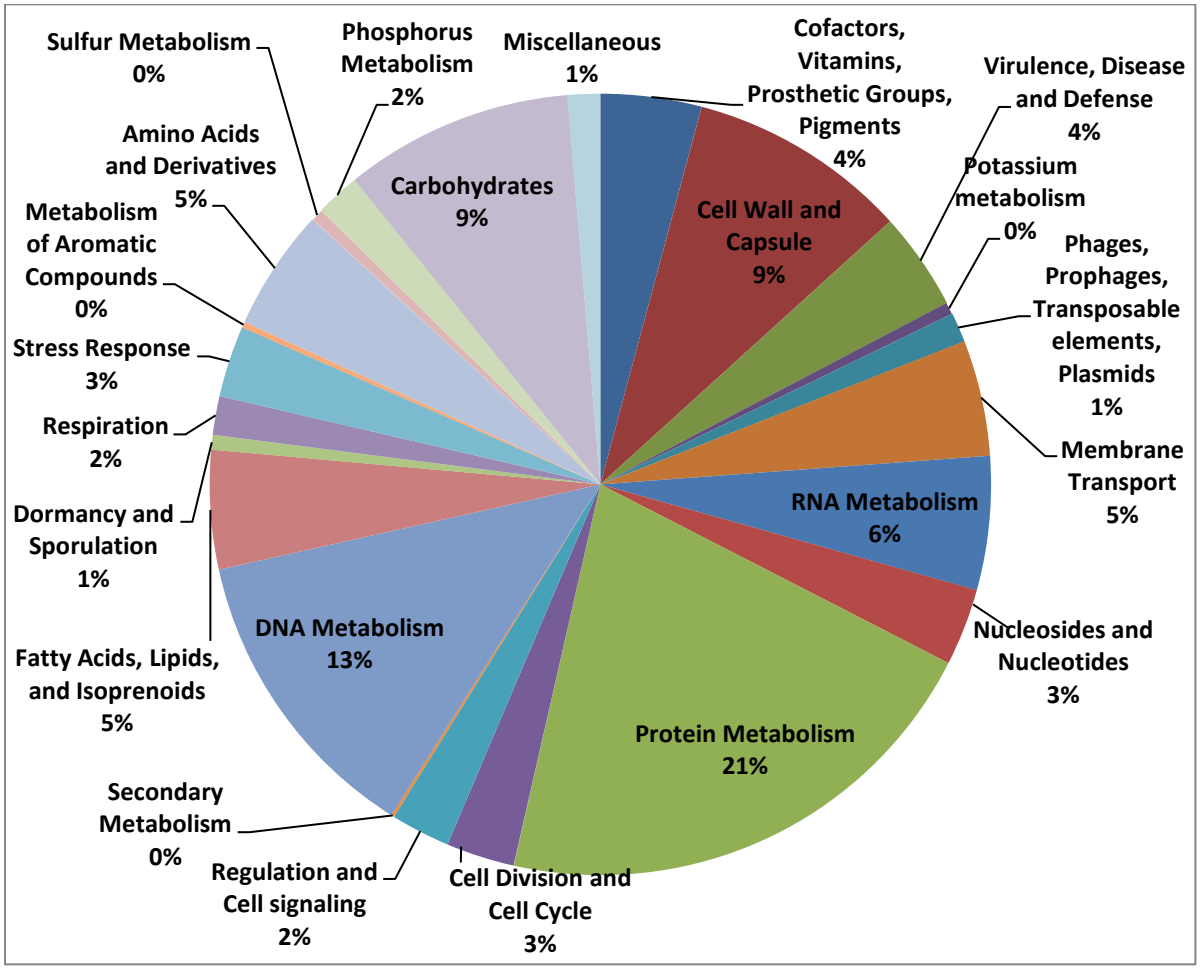

Fig. 6: Distribution of genes into subsystems for PV034

Table 2. RAST gene allocation to subsystems in genome sequences of strains PV021, PV012, and PV034

Subsystems assigned by RAST	Number of features		
	PV021	**PV012**	**PV034**
Cofactors, Vitamins, Prosthetic Groups, Pigments	30	32	34
Cell Wall and Capsule	96	88	74
Virulence, Disease and Defense	28	28	33
Potassium metabolism	4	5	4
Photosynthesis	0	0	0
Phages, Prophages, Transposable elements, Plasmids	8	0	10
Membrane Transport	33	34	39
Iron acquisition and metabolism	0	0	0
RNA Metabolism	46	46	45
Nucleosides and Nucleotides	29	29	26
Protein Metabolism	167	167	171
Cell Division and Cell Cycle	33	30	23
Motility and Chemotaxis	0	0	0
Regulation and Cell signaling	21	18	20
Secondary Metabolism	1	1	1
DNA Metabolism	103	92	102
Regulons	0	0	0
Fatty Acids, Lipids, and Isoprenoids	37	37	40
Nitrogen Metabolism	0	0	0
Dormancy and Sporulation	5	5	5
Respiration	2	2	13
Stress Response	21	20	24
Metabolism of Aromatic Compounds	0	0	2
Amino Acids and Derivatives	46	46	41
Sulfur Metabolism	3	5	4
Phosphorus Metabolism	15	15	15
Carbohydrates	99	100	77
Miscellaneous	8	8	11
Total	**835**	**808**	**814**

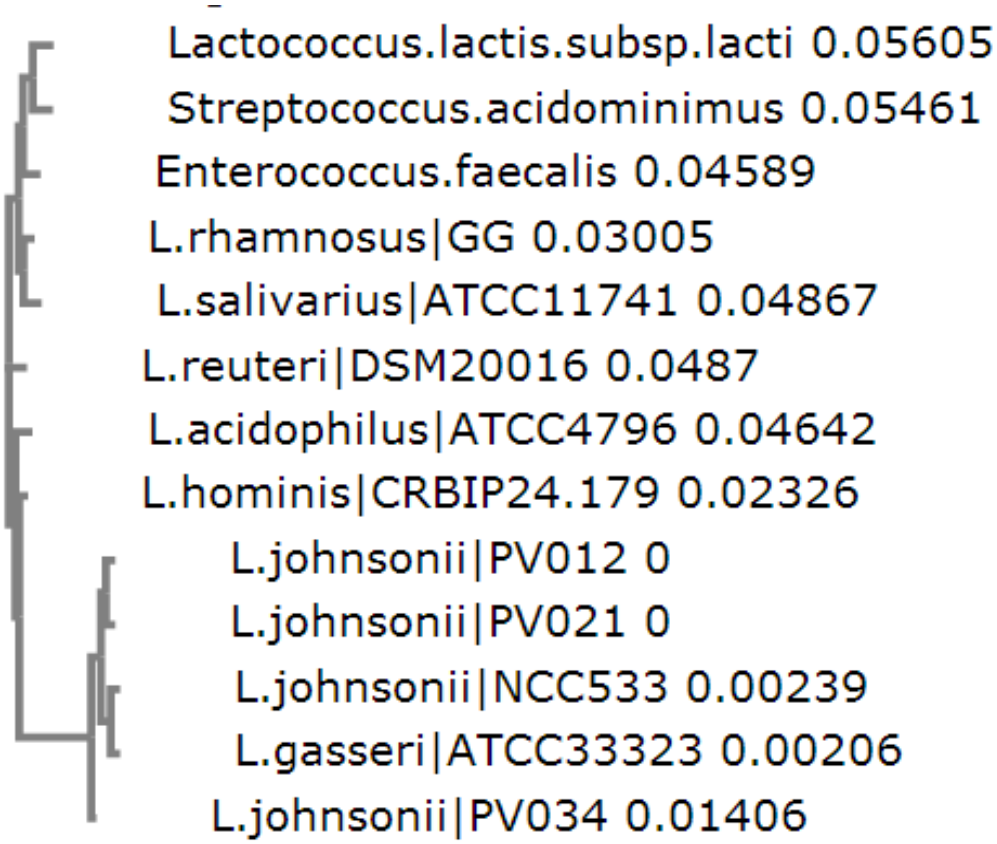

Fig. 7: Phylogenetic tree created using CLUSTAL with16SrRNA genes

Genes conferring potential probiotic functions discovered via RAST

Aligning the genomes of all three strains with published genomes of other *Lactobacillus* spp. produced only low levels of alignment (all under 30%). OverallPSI-BLAST protein queries resulted in matches with similar proteins identified in *Lactobacillus* spp. When compared to one another using CLUSTAL, amino acid sequences from strains PV012 and PV021 shared a greater identity with one another than with PV034 (Table 3). Many genes were found in all three strains while in some instances the genome of PV034 did not contain some of the potentially beneficial genes present in the PV021 and PV012 genomes. For example, asecond heavy metal ATPase was lacking in PV034. Additionally, the genome of PV034 only contained a single bacteriocin gene. The amino acid sequences of proteins with mucin-binding domains in strain PV034 were significantly different from those found in PV021 and PV012. The only strain without phage elements detected by RAST was strain PV012.

D-Alanylation of Lipotechoic Acids

The genes of the *dltABCD* operon as well as the *DltR* gene, the two component response regulator associated with the *dltABCD*operon, were indentified in all three genomes by RAST. The number of genes within theoperon varied between strains, however all operons shared the same conserved domains (Fig. 8a, 8b, 8c). In strains PV021 and PV012the *dltA*gene encoded for a protein 507 amino acids in length and in PV034 the *dltA*gene encoded for 504 amino acids in length.For all three strains a PSI-BLAST search revealed that all three amino acids sequences shared an identity of 74% with aD-alanine--poly(phosphoribitol) ligase identified in*L. crispatus.* A query of the NCBI Conserved Domain Database resulted in a specific hit for a D-alanine:D-alanyl carrier protein ligasedomain for all three strains.The *dltB*gene encoded for a protein 406 amino acids in length in all three strains. In strains PV021 and PV012, the amino acids sequence

of this protein shared 81% identity with a D-alanyl transfer protein described in*L. johnsonii.* In strain PV034, the amino acid sequence shared 83% identity with the same protein.A query of the NCBI Conserved Domain Database resulted in a specific hit fora D-alanyl-lipoteichoic acid biosynthesis protein, DltB. *DltC*encoded for a protein 80 amino acids in length in all three strains. In PV021 and PV012a PSI-BLAST query revealed a shared identity of 75%with a D-alanine--poly(phosphoribitol) ligase identified in*Lactobacillus hominis*and a shared identity of 90% with the same protein for PV034. A conserved domain for D-alanine--poly(phosphoribitol) ligase subunit 2 was discovered in all three strains. *DltD*was identified as two separate genes in strain PV021 by RAST, the first of which encoded for a protein 101 amino acids in length that shared a 77% homology with aD-alanyl transfer protein described*L. gasseri* and a query of the amino acids resulted in specific hit for DltD N-terminal region domain. The second *dltD*gene identified in strain PV021 encoded a protein647 amino acids in length which shared 66% identity with a D-alanyl transfer protein described in*L. gasseri.* The conserveddomain hits include a specific hit for aDltD C-terminal region, a specific hit for a beta-lactamase domainand a non-specific hit for aDltD central region (Fig 8a). In strain PV012, the *dltD*was reported as a single gene encoding for 755 amino acids that shared 70% identity with a D-alanyl transfer protein described in*L. gasseri.* The conserved domains identified were the same as those found in the two individual genes identified in PV021(Fig. 8b). In strain PV034,one*dltD*gene was found that encoded for a protein 429 amino acids in length that shared 72% identity with a D-alanyl carrier protein described in*L. gasseri.* A gene containing the same beta-lactamase domain followed the *dltD*gene, this section of sequence data was considered to be part of the *dltD*gene in both of the other genomes. This gene encoded for a protein 294 amino acids in length that shared 35% identity with a serine-type D-Ala-D-Alacarboxypeptidase protein described in*L. plantarum.* Again the same conserved protein domains were found in both genes(Fig 8c). It is likely that an assembly or annotation error occurred in the *dltD*gene for strain PV012 and PV021 as the most

common arrangement of conserved domains for the dltD protein seen in the NCBI data base is similar to the arrangement in PV034.

The *dltR*gene associated with regulation of *dltABCD* and the regulation of D-alanyl-lipoteichoic acid biosynthesis, sensor-histidine kinase genes were present in all three genomes. The *dltR*gene encoded for a protein 222 amino acids in length in all three genomes. A PSI-BLAST query resulted in a matched identity of 56% in PV021, 57% in PV012 and 40% in PV034 with a protein involved in regulation of D-alanyl-lipoteichoic acid biosynthesis identified in *Lactobacillus sucicola* JCM 15457. In all three amino acids sequences, a specific hit for a signal receiver and a transcriptional regulatory protein with DNA binding sites were revealed in a conserved domain query. The genesensor histidine-kinase gene encoded for a protein 428 amino acids long in PV021 and PV012, which shared an identity of 61% with a signal transduction histidine kinase identified in *L. gasseri.* In PV034 this gene encoded for a protein 425 amino acids long that shared an identity of 56% with a signal transduction histidine kinase identified in*L. gasseri* . All three amino acids sequences contained specific hits for histidine kinase A and histidine kinase-like ATPase conserved domains.

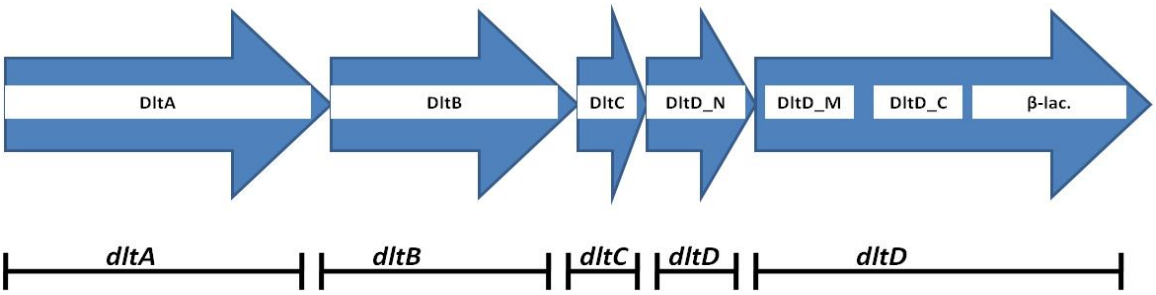

Fig. 8a: PV021*Dlt*operon arrangement in RAST

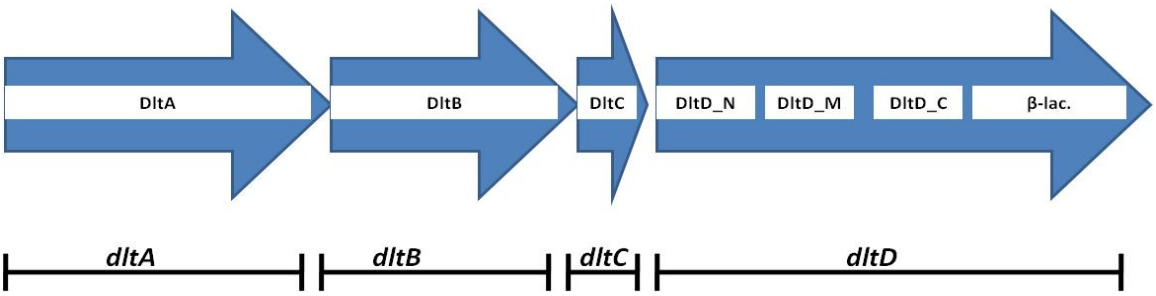

Fig. 8b: PV012*Dlt*operon arrangement in RAST

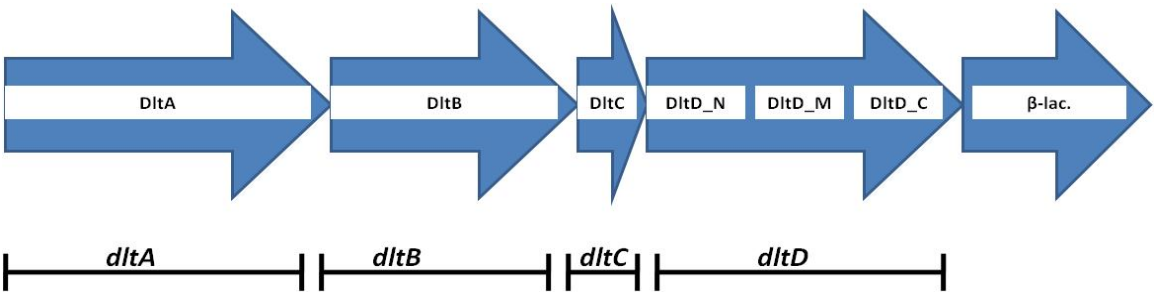

Fig. 8c: PV034*Dlt*operon arrangement in RAST

Fig. 8: Arrangement of the *dltABCD*operon and location of conserved domains. The schematic depicts dltABCD operon structure and conserved protein domains in strains PV021 (a), PV012 (b), and PV034 (c): D-alanine:D-alanyl carrier protein ligase subunit 1(DltA), D-alanyl-lipoteichoic acid biosynthesis protein (DltB), D-alanine--poly(phosphoribitol) ligase subunit 2 (DltC), DltD N-terminal region domain (DltD_N), DltD central region (DltD_M) a DltD C-terminal region (DltD_C) a beta-lactamase domain (β-lac.)

Bile resistance

All three genomes contained a gene encoding for a choloylglycine hydrolase. The protein was326 amino acids in length and shared a 60% identity with a choloylglycine hydrolase described in*L. reuteri.* Query of the NCBI Conserved Domain Database resulted in a specific hit for a conjugated bile salt acid hydrolase (CBAH) for all three amino acid sequences. All three genomes were also found to have several multidrug transporters. RAST discovered two multidrug-efflux transporters of the major facilitator superfamily in PV021 and PV012 as well as a single ABC-type multidrug transport system, a singlepermease of the drug/metabolite transporter (DMT) superfamily and one nonspecific multidrug transporter. The genome of strain PV034 contained one ABC-type multidrug transport system, two genes for a permease of the drug/metabolite transporter (DMT) superfamily and one nonspecific multidrug transporter. It is not known if these transporters play a role in bile resistance but theyare mentioned here because multidrug transporters have been associated with bile resistance.

Bacteriocins

RAST discovered a total of five genes encoding forthe bacterocinhelveticinin the genome of strain PV012, four of which were identical to genes found in strain PV021. In strain PV012, four of the five genes were arranged within close proximity to one another (Fig 9), while in strain PV021 only two were near one another. In PV021 many ofthe genes encoding for bacterocinswhere found near the end of contigs.Of the genes encoding bacterocins in PV021 and PV012, the geneencoding for a protein of342 amino acids in length, shared 46% identity with a bacteriocin identified in*L. helveticus.* The gene encoding for a protein of 65 amino acids in length shared 56% of identity with a bacteriocin identified in*L. helveticus.* The gene encoding for a protein of 326 amino acids in length shared a 65% identity with bacteriocinhelveticin-J identified

in*L. helveticus*and the gene encoding for a protein of 328 amino acids in length shared65% identity with bacteriocinhelveticin-J identified in*Lactobacillus amylolyticus*. The gene encoding for a protein of 38 amino acids in length, which was only found in strain PV012 shared 63% identity with a bacterocin identified in*L. helveticus.* Only one gene encoding for a bacteriocin was discovered by RAST in the genome of strain PV034. It encoded for a protein 324 amino acids in length that shared a 54% identity withbacteriocinhelveticin-J from *L. hominis*.No conserved domains were identified.

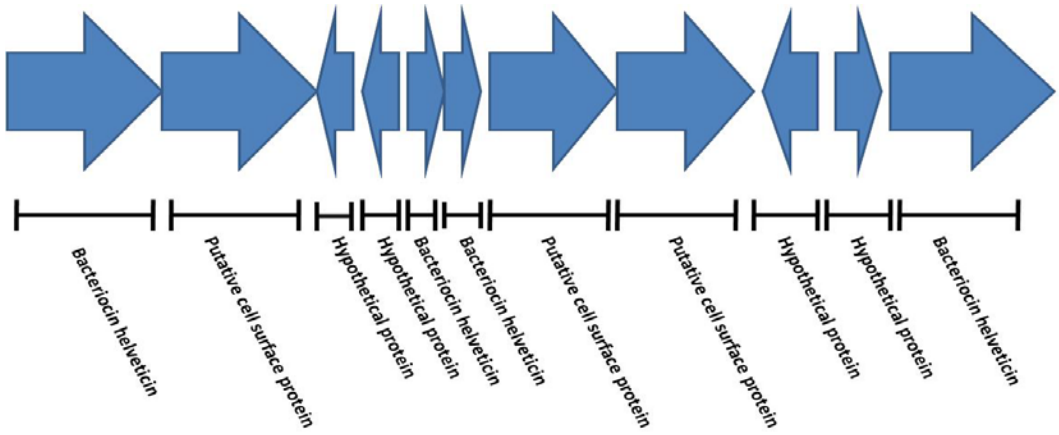

Fig. 9: Schematic of bacterocin genes found in proximity to one another in PV012

N-acyl homoserine lactone hydrolase

RAST identified a gene encoding a putative N-acyl homoserine lactone hydrolase in PV021 and PV012 that encoded for a protein 283 amino acids in length. In PV021 and PV012, the gene encoded for an amino acids sequence which shared an identity of 67% and 65% respectively, with a metallo-beta-lactamase identified*L. gasseri* and in PV012 the amino acids sequence shared a 65% identity with the same protein. A conserved domain query resulted in a specific hit for the metallo-beta-lactamase superfamilydomain and a nonspecific hit for a Zn-dependent hydrolase. No N-acyl homoserine lactone hydrolase was identified in strain PV034 although a gene

identified as a hypothetical protein shared 87% to 88% identity with the N-acyl homoserine lactone hyrolase in the other two genomes. A query of conserved domains resulted in a specific hit for a metallo-beta-lactamase superfamily but no nonspecific hits for Zn-dependent hydrolase for the amino acid sequence of the hypothetical protein in strain PV034.

Adhesins: Mucin-binding & fibronectin-binding proteins

Several potential mucin-binding proteins were identified in all three genomes. In strains PV021 and PV012 RAST identified a gene encoding for a hypothetical protein 1663 amino acids in length. In both instances the gene was located alone on a single contig. Both amino acids sequences shared a 31% identity with an adhesin described in*L. gasseri.* The conserved domains included five specific hits formucin-binding domains and a non-specific hit for a Rib/alpha-like repeat domain (Fig. 10a). In strain PV021 RAST also identified a gene encoding for a protein 1117 amino acids in lengththat shared 32% identity with an adhesin described in*L. gasseri.* Conserved domains included a specific hit for mucin-binding domain, a Gram-positive anchor and rib/alpha-like repeat (Fig. 10b). A similar gene was identified for strain PV012 that encoded for a protein 692 amino acids in length that shared a 32% identity with an adhesin identified in *L. gasseri.* Again the conserved domains located include amucin-binding domain, a Gram-positive anchor and a rib/alpha-like repeat (Fig. 10c). In strain PV034 RAST identified a gene encoding for a protein 229 amino acids in length that shared a 33% identity with a mucus binding protein identified in *L. hominis.* The conserved domain hits included a specific hit for a mucin-binding domain and a Gram-positive anchor (Fig. 10d). Another gene identified in strain PV034 encoded for a protein 1381 amino acids in length that shared a 26% identity with a mucus binding domain described in *L. johnsonii.* Conserved domains revealed included a specific hit for a mucin-binding domain and Gram-positive signal peptide in the YSIRK family (Fig. 10e).

In strain PV021 RAST identified a gene which encoded for fibronectin-binding protein which was 564 amino acids in length and shared a 69% identity with a putative fibronectin-binding protein identified in *Lactobacillus delbrueckii* subsp. lactis. The conserved domain hits included a specific hit for a domain of unknown function (DUF814) which occurs in proteins that have been annotated as fibronectin or fibrinogen binding protein[135]. Additionally there was a multi-domain hit for fibronectin-binding protein A. In strain PV012 it appears that RAST split this sequence into two genes, the portion of the gene containing the DUF814 domain encoded for 139 amino acids thatshared a 79% identity with a fibronectin-binding protein identified in *L. gasseri*.The portion of the gene resulting in the multi-domain hit for a fibronectin-binding A encoded for a protein 379 amino acids long and shared a 70% identity with a fibronectin-binding protein identified in *L. gasseri*. Splitting of this gene may be due to a sequencing, assembly, or annotation error.In strain PV034 the putative fibronectin-binding protein gene encoded for a protein 564 amino acids in length which shared 80% identity with a fibronectin-binding protein identified in *L. gasseri.* Both the multi-domain hits for fibronectin-binding and DUF814 were present.

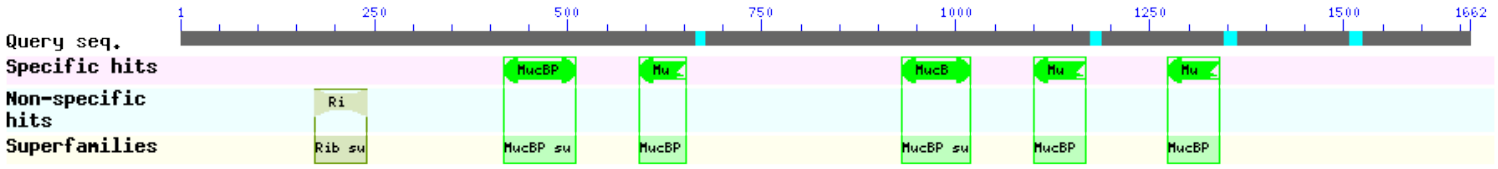

Fig. 10a: PV012 and PV021MucBPa

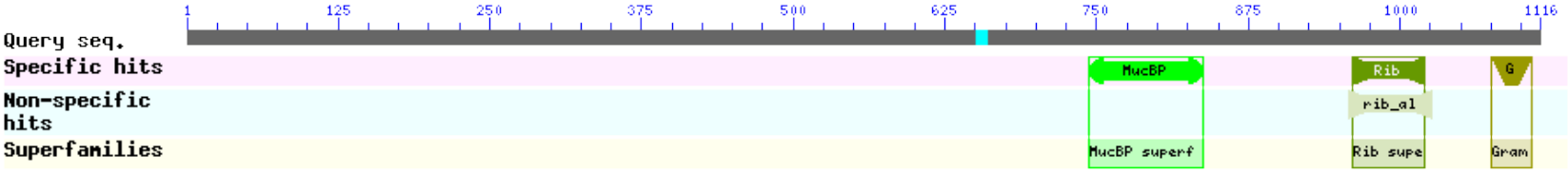

Fig. 10b: PV021MucBPb

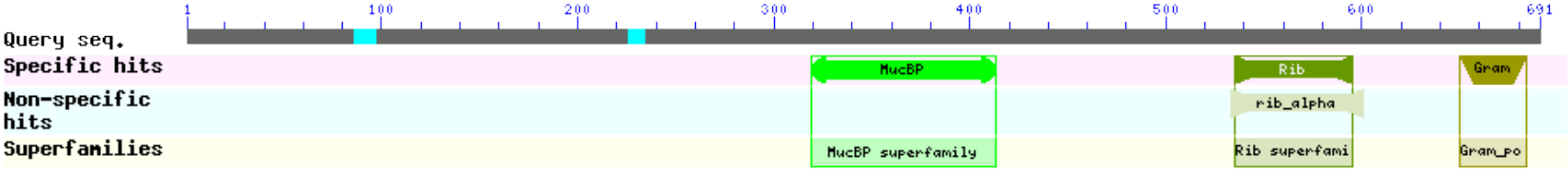

Fig. 10c: PV012MucBPc

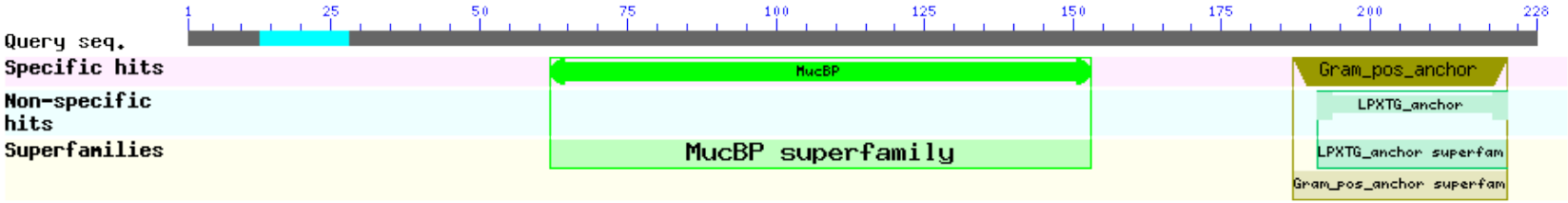

Fig. 10d: PV034MucBPd

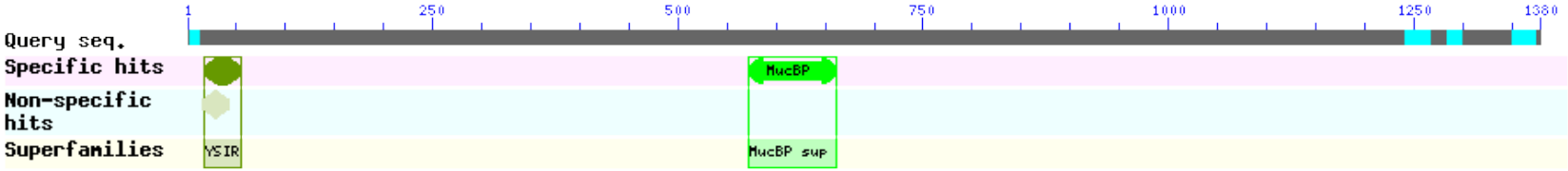

Fig. 10e: PV034MucBPe

Fig. 10a-e: Conserved domains identified in potential mucin-binding proteins by query of the NCBI Conserved Domain Database

Mercury Resistance

RAST identified a putative mercuric ion reductase gene which encoded for a protein 444 amino acids in length in all genomes. According to PSI-BLAST results the amino acid sequence for the gene in strain PV021 shared 69% identity with a pyridine mercuric reductase described in *L. helveticus.* In PV012 the amino acid sequence shared a 68%with the same pyridine mercuric reductase and for PV034 there was a 72% shared identity with the same protein. In all three amino acid sequences, conserved domain hits included specific hits for a pyridine nucleotide-disulphideoxidoreductase and a pyridine nucleotide-disulphideoxidoreductasedimerization domain. A gene encoding for a lead, cadmium, zinc and mercury transporting ATPase was identified in all three genomes. The gene encoded for a protein 618 amino acids long in PV021 and PV012 and 629 amino acids long in PV034. The amino acid sequences encoded by the genes in PV021 and PV012 shared an 83% identity with a metal ABC transporter ATPase described in*L. reuteri.* Forstrain PV034 the amino acid sequence shared a 71% identity with a heavy metal translocating P-type ATPase described in *Lactobacillus* sp. ASF360. Conserved domains in all three protein sequences include anE1-E2 ATPase domain and haloaciddehalogenase-like hydrolase domain. Strains PV021 and PV012 had an additional gene for a lead, cadmium, zinc and mercury transporting ATPase identified by RAST which encoded for a protein 627 amino acids in length. Both amino acid sequences shared a 73% identity with a heavy metal translocating P-type ATPase described in*Lactobacillus* sp. ASF360 and contained specific hit for an E1-E2 ATPase domain and a predicted ATPase Soluble P-type ATPase domain, as well as a multi-domain hit for a zinc/cadmium/mercury/lead-transporting ATPase.

Table 3: CLUSTAL comparison of amino acid sequences of genes of interest

Gene	PV012 : PV021	PV012 : PV034	PV021 : PV034
DltA	99.60%	81.31%	80.91%
DltB	99.75%	77.48%	77.04%
DltC	100.00%	74.68%	74.68%
DltD	99.87%	69.86%	70.09%
DltR	93.67%	72.40%	69.23%
Sensor histidine kinase (*dltR)*	98.83%	58.25%	57.78%
Bile salt hydrolase	99.69%	89.54%	89.23%
N-acyl homoserine lactone hydrolase	96.10%	N/A	N/A
Bacterocinhelveticin (326 aa)	98.17%	51.70%	52.01%
Bacteriocinhelveticin (65 aa)	100.00%	N/A	N/A
Bacteriocinhelveticin (342 aa)	100.00%	N/A	N/A
Bacteriocinhelveticin (326 aa)	100.00%	N/A	N/A
Mercury reductase	99.32%	73.14%	73.14%
Lead, cadmium, zinc mercury ATPase pump (a)	99.84%	78.43%	78.59%
Lead, cadmium, zinc mercury ATPase pump (b)	98.70%	N/A	N/A
Fibronectin binding protein	96.12%	72.29%	76.55%
MucBPa	100.00%	N/A	N/A
MucBPb : MucBPc	95.33%	N/A	N/A

CHAPTER V

CONCLUSION

Host adaptation is an important factor to consider in the selection of probiotic strains, and somany of the genes investigated in this study are involved in the ability to survive in the gastrointestinal tract. Bacterial host adaptation can come about in many ways and may occur through large scale gene acquisitions or losses; change can also occur in more subtle ways such as the modification of individual genes or molecular pathways [136-138]. These evolutionary changes occur over time and shape bacteria so that they attain optimal fitness in a certain environment. In a study conducted by Frese et. al. *L. reuteri*F275, a human derived strain, was unable to colonize the gastrointestinal tract of *Lactobacillus*-free mice; however, *L. reuteri*100-23, a mouse derived strain was able to colonize *Lactobacillus*-free mice[136]. A comparison of the two genomes revealed that *L. reuteri*100-23 contained 633 genes with noorthologs in strain F275. The genes with designated functions unique to *L. reuteri*100-23 included: transport proteins, regulatory proteins, enzymes, glycosyltransferases, cell wall and membrane bound proteins, an auxiliary protein secretion system, and a urease gene cluster. The only unique set of genes in *L. reuteri*F275 with an identified function were in a *pdu-cbi-cob-hem* cluster. This gene cluster is involved in the production of coenzyme B_{12} [136].This study illustrates the significance of host adaptation and its importance when considering probiotic candidates. How the gut microbiota forms is not fully understood, but there is evidence that host genetics play a role in shaping the composition of the gut microbiota [139]. The relationship between

gut microbiota and the host is complex. Theco-evolution of the microbiota and hostmay be the basis for the strain-specificresults seen in probiotics. The genes involved in host adaptation described in this study were closely related to genes described in other *Lactobacillus* spp.,but they appeared to be more closely related to one another. These three strains should be better able to colonize and produce a positive effect in the prairie vole animal model.

Because lactobacilli tend to live in nutritionally rich environments such as the gastrointestinal tract some species have lost genes involved in the metabolism of certain nutrients.In fact *Lactobacillus* spp. are one of the few organisms that have no requirement for iron [140, 141].Certain strains of *Lactobacillus* do appear to benefit from the presence of iron depending on the availability of particular nucleotides [140]. The ability to survive without the presence of iron give lactobacilli a competitive edge over pathogenic bacteria [142]. No genes involved in the acquisition and metabolism of iron were found in the three strains sequenced. Strain PV034 was also the only strain in which RAST identified genes that were involved in the metabolism of aromatic compounds. Additionally, no genes involved in nitrogen metabolism were found. Interestingly, RAST identified only two genes involved in respiration in the genomes of strains PV021 and PV012 while it identified thirteen genes involved in respiration in strain PV034. PV034 was the only strain able to produce hydrogen peroxide during the *in vitro* testing and the additional respiratory genes may be involved in this strain's ability to do so.

Bacteriophages, plasmids, and transposons are sources of genetic diversity in bacteria[143]. It has been proposed that prophages carry genes which are of selective benefit to their host in a specific ecological niche [144]. Certain bacteriophagescarry virulence factors and can integrate into a bacterium's genome creating pathogenicity islands; often over time these phages lose the genes that allow them to become lysogenic again. One such example is the diphtheria toxin which is carried by a bacteriophage that allows *Corynebacteriumdiphtheriae*,a common non-pathogenic resident of the upper respiratory tract, to produce diphtheria toxin and cause disease.

Pathogenicity can make up as much as 10 to 20% of a bacterial genome[143]. No *Lactobacillus* phages have ever been reported to carry virulence genes.While little research exists on the relationship of phages and probiotics, in dairy fermentation, phage infection can have a deleterious effect leading to food spoilage [145]. When selecting probiotic candidate strains, those without prophages would likely be more desirable, because these strains would have more genetic consistency and would likely have a higher degree of fitness. Of the three sequenced genomes in this study, RAST identified phage elements in strains PV021 and PV034. Whether these phages can become lysogenic and what characteristics they impart remains to be determined. Strain PV012 was the only strain without a phage, potentially making it a better probiotic candidate.

While *Lactobacillus* spp. are generally recognized as safe (GRAS) and have only been associated with infection in severely immunocompromised individuals[146],genes homologous to ones that enable pathogenic bacteria to cause disease, have been found inthe genomes of lactobacilli.A study assessing the virulence of*Lactobacillus*strains isolated from the fecal material of healthy adults, blood isolates from patients with bacteremia, and commerical probiotic strains found that the blood isolates showed a trend toward a higher ability to adhere to mucus than the probiotic strains or strains from fecal isolates (P=0.07)[147]. In addition, probiotic strains induced lower levels of respiratory bursts in peripheral blood mononucleocytes (P=0.05) and showed a trend toward lower sensitivity to human serum (P=0.07). The condition of the patients from whom the blood isolates originated is not stated in this paper, but it is likely that these individuals were immunocompromised. Adhesins in pathogenic bacteria contribute to their virulence, but in lactobacilli adhesion to mucus is thought of as a desirable quality and a mechanism by which lactobacilli compete with and exclude pathogens[22]. There are numerous studies which have tied the ability of lactobacilli to adhere to mucus with pathogen inhibition.Fibronectin-binding proteins have been identified in lactobacilli; however, their function is not understood.

Fibronectin-binding proteins have been shown to play a role in invasion of host cells by *Streptococcus pyogenes*[148]. Additionally, certain types of fibronectin-binding proteins allow *S. pyogenes*to evade phagocytosis by inactivating the complement pathway.A study which induced peptic ulcers in mice found that promotion of *Lactobacillus* colonization supported wound healing and lowered the presence of Gram-negative bacteria [149]. It is possible that ability of lactobacilli to bind extracellular matrix components and deter pathogens could contribute to the increased wound healing seen in this study. While lactobacilli are generally considered safe the presence of potential virulence factors should be taken into consideration, especially in the case of immunocompromised persons.

Another aspect to consider in the selection of probiotics is antibiotic resistance. Many lactobacilli are resistant to a wide variety of antibiotics, but the most commonly reported antibiotic resistance genes are*tet*(M), which confers tetracycline resistance and *erm*(B), which confers erythromycin resistance. The antibiotic resistance genes, described in lactobacilli, are in some cases mobile.A growing body of whole genome sequences has made assessment of antibiotic resistance genes easier[150]. Genes conferring potential antibiotic resistance were identified by RAST in all of the three genomes sequenced in this study. It is not known if they are functional and which antibiotics they are effective against. Antibiotic resistance is generally thought of as an undesirable characteristic of bacteria and in probiotics,but there could be circumstances in which antibiotic resistance in lactobacilli would make them more effective, such as cases of antibiotic-induced diarrhea.

Whether these three strains of *Lactobacillus* can be classified as an already known species or if they are a newly identified species must still be determined.The use of the 16S rRNA gene to classify bacteria has been the standard since discovered by Carl Woese and his colleagues in the 1970s [151-153].Using the 16S rRNA gene to perform taxonomic classification can be problematic. For instance *Bacillus globisporus* and *Bacillus psychrophilus* which share 99.8%

sequence identity when comparing 16S rRNA genes, but only a 23-50% identity when their entire genomes are compared using DNA-DNA hybridization[154]. One issue with using 16S rRNA for taxonomic classification is intra-genomic heterogeneity. Bacteria often contain more than one copy of the 16S rRNA gene that are usually identical, but not always, which can lead to difficulties using 16S rRNA for classification[155]. Additionally, while it was originally thought that 16S rRNA genes did not participate in horizontal gene transfer events such events have been reported [155].

RAST identified *L. johnsonii*NC533 and *L. gasseri*ATCC 33323 as the two most closely related bacteria to all three strains based on a comparison of universal protein families and/or large highly conserved protein families. PV012 and PV021 were more closely related to *L. johnsonii*NC533 and PV034 to *L. gasseri*ATCC 33323. Results were similar when comparing the 16SrRNA genes (Figure 7). In this instance, the results arrived at using the 16S rRNA gene to classify these lactobacilli, were supported by the results from RAST's comparison of conserved proteins. The use of the 16S rRNA gene may have several disadvantages, but because it is so widely used and well documented that it is logical to continue to implement its use. One important point made by Yarza et. al. is that only full 16S rRNA genes of good quality should be used[156]. The need for a well delineated set of thresholds for cultured and uncultured species such as the one devised by the author and his colleagues is also stressed.

Next-generation sequencing platforms such as the Ion Torrent PGM™, which was used in this study,have made it less expensive to sequence bacterial genomes. Unfortunately read lengths are still too short, making it difficult to complete a bacterial genome sequence using next-generation sequencing alone.When assessing 454 GS Junior, MiSeq, and Ion Torrent PGM™ during the German *E. coli* O104:H4 outbreak none of the three bench top sequencers were able to produce a genome that could be assembled into one contig[14]. *De novo* genome assembly of second-generation sequence data has been proven a difficult task for which there is no current

computational solution [157]. Repetitive sequences are problematic when the read length is shorter than the repetitive region.One problems is that these repetitive reads can be collapsed together on top of one another, when they should be two separate sequence areas[158]. For strains PV012 and PV034 the number of contigsafter assembly was fairly low, but because of repetitive areas such as the rRNA operon the genomes were not closable. Further assessment of genes identified by RAST may also be required, as the information in public data bases utilized by automated annotation pipelines such as RAST does contain errors and as a result genes may be annotated incorrectly [159]. In the genomes of the three strains sequenced for this study several genes were split into two when they were most likely single genes, whether these were truly errors and if the error was due to the annotation or to sequencing it not known. Next-generation sequencing is a valuable tool that has lowered the price of sequencing and lead to a dramatic rise in the number of published genomes. The need for longer read lengths is apparent, butfortunately the level of competition between leading manufacturers of next-generation platforms is high and new advances in technology are occurring rapidly.

The genomes of all three *Lactobacillus* spp. isolated did contain genes which were similar to genes described prior studies on probiotic mechanisms. Probiosis is a multifactorial processthat involves a large number of genes (known and unknown); for brevity's sake only the genes of particular interest to this study were included. These genes were chosen based on their relevance in the vole gastrointestinal tract and are only a small percentage of the genes identified by RAST. Many putative genes were identified by RAST for which the function is unknown. Sequencing of a genome produces a vast amount of data that must be sifted through and organized to be useful. Fortunately there are many publically available tools with which to accomplish this task. The sequencing of these three genomes will lay the foundation for future studies on the mechanisms of probiosis.

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APPENDIX

Table A1. RAST Annotation Details

Strain	RAST feature identification	Contig	Start	Stop	Feature Function
PV012	fig|6666666.64077.peg.502	13	67812	69332	D-alanine--poly(phosphoribitol) ligase subunit 1 (EC 6.1.1.13)
PV012	fig|6666666.64077.peg.503	13	69329	70546	D-alanyl transfer protein DltB
PV012	fig|6666666.64077.peg.504	13	70568	70807	D-alanine--poly(phosphoribitol) ligase subunit 2 (EC 6.1.1.13)
PV012	fig|6666666.64077.peg.505	13	70800	73064	Poly(glycerophosphate chain) D-alanine transfer protein DltD
PV012	fig|6666666.64077.peg.856	3	56524	55241	Regulation of D-alanyl-lipoteichoic acid biosynthesis, sensor histidine kinase
PV012	fig|6666666.64077.peg.857	3	57199	56534	Regulation of D-alanyl-lipoteichoic acid biosynthesis, DltR
PV012	fig|6666666.64077.peg.844	3	41512	40535	Choloylglycine hydrolase (EC 3.5.1.24)
PV012	fig|6666666.64077.peg.416	12	29259	30242	Bacteriocinhelveticin
PV012	fig|6666666.64077.peg.1374	8	23129	24154	Bacteriocinhelveticin
PV012	fig|6666666.64077.peg.1378	8	25992	26105	Bacteriocinhelveticin
PV012	fig|6666666.64077.peg.1379	8	26069	26263	Bacteriocinhelveticin
PV012	fig|6666666.64077.peg.1384	8	29638	30615	Bacteriocinhelveticin
PV012	fig|6666666.64077.peg.88	10	21230	22078	N-acyl homoserine lactone hydrolase
PV012	fig|6666666.64077.peg.790	21	403	5391	hypothetical protein
PV012	fig|6666666.64077.peg.1263	6	136936	139011	hypothetical protein
PV012	fig|6666666.64077.peg.168	10	95709	96845	Fibronectin-binding protein
PV012	fig|6666666.64077.peg.169	10	96985	97401	Fibronectin-binding protein
PV012	fig|6666666.64077.peg.115	10	50152	48821	Putative Dihydrolipoamide dehydrogenase (EC 1.8.1.4); Mercuric ion reductase (EC 1.16.1.1); PF00070 family, FAD-dependent NAD(P)-disulphideoxidoreductase

PV012	fig|6666666.64077.peg.469	13	37834	35981	Lead, cadmium, zinc and mercury transporting ATPase (EC 3.6.3.3) (EC 3.6.3.5); Copper-translocating P-type ATPase (EC 3.6.3.4)
PV012	fig|6666666.64077.peg.498	13	65190	63310	Lead, cadmium, zinc and mercury transporting ATPase (EC 3.6.3.3) (EC 3.6.3.5); Copper-translocating P-type ATPase (EC 3.6.3.4)
PV021	fig|6666666.64078.peg.459	15	9901	8381	D-alanine--poly(phosphoribitol) ligase subunit 1 (EC 6.1.1.13)
Strain	**RAST feature identification**	**Contig**	**Start**	**Stop**	**Feature Function**
PV021	fig|6666666.64078.peg.458	15	8384	7167	D-alanyl transfer protein DltB
PV021	fig|6666666.64078.peg.457	15	7145	6906	D-alanine--poly(phosphoribitol) ligase subunit 2 (EC 6.1.1.13)
PV021	fig|6666666.64078.peg.456	15	6913	6611	Poly(glycerophosphate chain) D-alanine transfer protein DltD
PV021	fig|6666666.64078.peg.455	15	6591	4651	Poly(glycerophosphate chain) D-alanine transfer protein DltD
PV021	fig|6666666.64078.peg.229	11	105141	104476	Regulation of D-alanyl-lipoteichoic acid biosynthesis, DltR
PV021	fig|6666666.64078.peg.228	11	104466	103183	Regulation of D-alanyl-lipoteichoic acid biosynthesis, sensor histidine kinase
PV021	fig|6666666.64078.peg.727	22	49663	48686	Choloylglycine hydrolase (EC 3.5.1.24)
PV021	fig|6666666.64078.peg.582	19	1351	326	Bacteriocinhelveticin
PV021	fig|6666666.64078.peg.659	20	16194	17177	Bacteriocinhelveticin
PV021	fig|6666666.64078.peg.895	29	1387	1581	Bacteriocinhelveticin
PV021	fig|6666666.64078.peg.901	29	4957	5934	Bacteriocinhelveticin
PV021	fig|6666666.64078.peg.500	16	31774	30926	N-acyl homoserine lactone hydrolase
PV021	fig|6666666.71045.peg.1246	55	5760	772	hypothetical protein
PV021	fig|6666666.64078.peg.1045	4	61920	58570	hypothetical protein
PV021	fig|6666666.64078.peg.315	12	11153	12844	Fibronectin-binding protein
PV021	fig|6666666.64078.peg.471	16	2382	3713	Putative Dihydrolipoamide dehydrogenase (EC 1.8.1.4); Mercuric ion reductase (EC 1.16.1.1); PF00070 family, FAD-dependent NAD(P)-disulphideoxidoreductase
PV021	fig|6666666.64078.peg.1304	73	280	771	Lead, cadmium, zinc and mercury transporting ATPase (EC 3.6.3.3) (EC 3.6.3.5); Copper-translocating P-type ATPase (EC 3.6.3.4)

PV021	fig|6666666.64078.peg.464	15	12521	14401	Lead, cadmium, zinc and mercury transporting ATPase (EC 3.6.3.3) (EC 3.6.3.5); Copper-translocating P-type ATPase (EC 3.6.3.4)
PV034	fig|6666666.71150.peg.1099	4	14200	12689	D-alanine--poly(phosphoribitol) ligase subunit 1 (EC 6.1.1.13)
PV034	fig|6666666.71150.peg.1098	4	12692	11475	D-alanyl transfer protein DltB
PV034	fig|6666666.71150.peg.1097	4	11415	11176	D-alanine--poly(phosphoribitol) ligase subunit 2 (EC 6.1.1.13)
Strain	**RAST feature identification**	**Contig**	**Start**	**Stop**	**Feature Function**
PV034	fig|6666666.71150.peg.1096	4	11183	9897	Poly(glycerophosphate chain) D-alanine transfer protein DltD
PV034	fig|6666666.71150.peg.1095	4	9778	8897	Beta-lactamase class C and other penicillin binding proteins
PV034	fig|6666666.71150.peg.1006	3	297541	298205	Regulation of D-alanyl-lipoteichoic acid biosynthesis, DltR
PV034	fig|6666666.71150.peg.1007	3	298217	299491	Regulation of D-alanyl-lipoteichoic acid biosynthesis, sensor histidine kinase
PV034	fig|6666666.71150.peg.288	10	136075	135098	Choloylglycine hydrolase (EC 3.5.1.24)
PV034	fig|6666666.71150.peg.195	10	36947	37918	Bacteriocinhelveticin J
PV034	fig|6666666.71150.peg.20	1	27144	27830	Putative mucus binding protein
PV034	fig|6666666.71150.peg.404	15	6750	7556	Adhesin of unknown specificity SdrC
PV034	fig|6666666.71150.peg.425	16	19582	21273	Fibronectin-binding protein
PV034	fig|6666666.71150.peg.11	1	18300	19631	Putative Dihydrolipoamide dehydrogenase (EC 1.8.1.4); Mercuric ion reductase (EC 1.16.1.1); PF00070 family, FAD-dependent NAD(P)-disulphideoxidoreductase
PV034	fig|6666666.71150.peg.1103	4	16665	18551	Lead, cadmium, zinc and mercury transporting ATPase (EC 3.6.3.3) (EC 3.6.3.5); Copper-translocating P-type ATPase (EC 3.6.3.4)

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