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RESEARCH ARTICLE

Genetic and Biochemical Characterization of Six *Lactobacillus* Isolates from American Quarter Horses

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ABSTRACT
The internal cavity of equine contains symbiotic microorganisms that are collectively referred to as the gut microbiota, which interact with the host immune system from birth. The microorganisms in the gut microbiota are shaped by their interactions with the gut environment throughout the life of the host, i.e., exposure to antibiotics and diet. Lactobacilli are one of the major groups found in the gastrointestinal tracts of humans and animals. Lactobacilli are members of the lactic acid bacteria, and they help to maintain a balanced gut microbiome and stimulate the host's immune system. In this study six equine *Lactobacillus* spp. were isolated from three American Quarter horses of different ages (i.e., 1-day post-weaning, 1.5-month post-weaning, and 10-year-old mature gelding). The metabolic properties that allowed the isolates to survive in the harsh environment of the gut were characterized. Thus, we evaluated their abilities to metabolize different carbohydrates and to withstand acidic pH, bile salts, antibiotics, and to inhibit pathogenic bacteria which may be encountered during their passage to the small/large intestine. We also identified the genetic elements that allow the isolates to survive and persist in the host's gut environment by using data generated from whole genome sequencing. The data indicated that the isolates were metabolically adapted to the age of the host and the type of feed consumed. The characterized isolates are potential probiotic candidates for enhancing the gut health of equines.

Key Words: Horse Lactobacilli, Gut microbiome, Probiotics, Bile tolerance, Acid tolerance, *Salmonella*, Genomic

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Introduction

Probiotic research began in 1907 and has continued since its discovery. [1] Probiotics are defined as "live microorganisms which, when administered in adequate amount confer a health benefit on the host. [2,3] Within the host, probiotic bacteria play an important role in maintaining a balanced gut microbiome and help to prevent intestinal disorders such as Inflammatory Bowel Disease (IBD). [4] Current research in this area shows that there are trillions of microorganisms living within the gut. Using genomic sequencing, scientists have demonstrated that bacteria present in the gut are selected and promoted by the host to aid in the breakdown of nutrients and provide protection against harmful organisms.[5] Variations in diet and lifestyle cause changes in the gut microbiome. [6] Thus, the diet and the gut micro-environment determine the diversity of the microbiota from birth and how it evolves based on age, sex, and preexisting conditions. Intake of beneficial foods that contain inulin, inositol, and complex carbohydrates which are considered as prebiotics may benefit the microbiota. [7] Galacto-oligosaccharides (GOS) is a complex polysaccharide that is not utilized by the host but is metabolized by probiotic organisms. It has been shown that GOS is present in mothers' breast milk and is stable enough to make it to the lower gastrointestinal (GI) tract and be utilized by the probiotic bacteria. [8] The production of GOS by *Sporobolomyces singularis* allows for a large-scale production that has potential to be beneficial in promoting the growth of probiotic bacteria. [9]

In young horses, the rapidly changing gut pH and frequency of nursing affects the small and large intestine which are functioning differently than that of adult horses. At the start of foals' life, lactase levels are extremely high and decrease as they enter adulthood, whereas maltase levels are very low but increase with age. These changes offer insight into the type of probiotic bacteria residing in the GI tract during development. [10]

In addition to changes between foals and mature horses, the differences in the microbial communities between the small and large intestines vary greatly. These changes in microbial composition along the GI tract of equine result from the chemical changes food undergoes during its journey down the GI tract. The changes in pH and the type of carbohydrate present affect bacterial Genera/Species that can make it to the intestine. Overall, the presence of bacteria that can withstand the GI elements determines what survives and persists within the small and large intestine. [11]

Probiotics are "generally regarded as safe" and have become readily available. Indeed, the increased interest in using probiotics resulted in numerous feed products being developed where the bacterial origin and concentration of the probiotic organisms are unknown. [4] However, the efficacy of these commercial products has been questioned, [12] most likely due to the fact they were not selected from the appropriate host, age, or environment. In our view, to develop and select an effective probiotic consortium, it is important to use isolates that have been adapted to the host of interest, i.e., host-adapted (or species/specific) probiotic organisms. [13,14]

The goal of this study is to isolate equine-specific lactobacilli from American Quarter horses at different stages of development and characterize their biochemical and genetic traits that allow them to survive and persist in the host. The American Quarter horses are the most popular breed in the United States of America (USA). It originated in the early 1600s by crosses between local horses and horses brought to Colonial America from England and Ireland. Quarter horses are more muscular and stockier than the Thoroughbred horses and they are the fastest breed in races of one quarter mile or less. We used this breed in our studies because of its availability at our institution and popularity in the USA and around the world. In this study, we assessed the abilities of the isolates to resist acidic pH, bile salts, antibiotics, and inhibit pathogenic bacteria, as well as the genetic traits that afford their activities and survival in their host.

Materials and Methods

Media and Growth Conditions

DeMan-Rogosa-Sharp (MRS) media[15] (Becton Dickinson, NJ) adjusted to a pH of 6.5±.0.2 was used throughout this study. Also, where indicated, a Modified MRS (M-MRS) media lacking added sugars was used for testing growth on specific sugars. The M-MRS contained: 10 g Tryptone, 2 g Yeast extract, 3 g Sodium acetate, 2.6 g Dipotassium phosphate, 2 g Ammonium citrate, 0.1 g Magnesium sulfate, 0.05 g Manganese sulfate, 0.75 g Citric acid, and 1 mL of Tween 80 in 1 Liter at a pH of 6.5± 0.2. Solid MRS medium was prepared by adding 1.5% agar. Media were autoclaved at 120°C for 20 minutes. Freshly prepared media were incubated for 24 hours in a Coy Anaerobic Chamber (Coy Lab Products, Grass Lake, MI) using anaerobic gas mixture (H_2 10%, CO_2 5%, and N_2 85%), to become anaerobic, before use.

To start a culture from frozen stocks, the isolates were inoculated in MRS media or plates, transferred into the anaerobic chamber, and allowed to grow at 37°C overnight. After ~16-24 hours, the isolate was transferred for a second time in MRS media and grown anaerobically before it

was used to inoculate experimental media. This protocol was used throughout the study to ensure that the cells in the inoculum were grown under true anaerobic conditions. Bacterial growth and cell densities were measured as Optical Density at 600 nm (OD_{600}) using a Bio-Rad Smartspec 3000 with a 1 cm light path (BIO-RAD, Smart Spec 3000, PA). Standard plate count was used to determine viable cell counts, as colony forming units (CFUs). [16]

Bacterial Strains

Salmonella Typhimurium (*S.* Typhimurium) (ATCC strain 14028s) was used for the antimicrobial assay. It was grown anaerobically overnight in Brain Heart Infusion (BHI) media (Becton Dickinson, NJ) at 37°C without shaking. Also, six Lactobacilli isolated from Equines were used.

Isolation of Equine Lactobacilli

Fresh equine manure was collected from the Equine Educational Unit at North Carolina State University. The age and feed of the horses was known at the time of collection. The manure samples were suspended in PBS at a concentration of 100 mg/mL and vortexed well. The large solid particles were allowed to settle out of suspensions for 30 minutes at room temperature. After settlement, the samples were subjected to an enrichment step in the presence or absence of 1% Galactooliogosaccharide (GOS) 90% pure (a gift from Dr. Jose Bruno-Barcena, Department of Plant and Microbial Biology, NC State University, Raleigh, NC 27695); 1 mL was transferred for a final volume of 10 mL of MRS (A) and 10 mL of MRS+1% GOS (B).The cultures (A and B) were grown anaerobically at 37°C for 24 hours before they were serially diluted in PBS and plated in 100 µL aliquots on solid MRS plates. The plates were incubated anaerobically at 37°C for 24-48 hrs. From each sample, approximately 12 colonies were selected and t-streaked 3 times to ensure purity. A total of 35 isolates were selected representing three horses at different ages/development and on different types of diet. These isolates were observed under the microscope, tested using preliminary biochemical tests, and were sequenced using 16s rRNA sequencing. Based on colony morphology, preliminary biochemical tests, and 16s rRNA sequencing, we selected six independent (non-siblings) isolates representing the different stages of horse development for further in-depth studies. Only one isolate came from the GOS-enrichment, group (B).

Gram Stain and Cell Morphology

Isolates were inoculated onto MRS plates, and after 24 hours, single colonies were picked up and suspended in 15 µL of sterile diH_2O on a glass slide. Gram stain was performed using standard procedure. [17] Cell morphology was observed microscopically at 100X magnification using a Nikon Alphaphot-2 YS2 compound microscope (Nikon, Japan).

Colony Morphology

To analyze colony morphology isolates were grown in MRS media for 24 hours before they were streaked onto MRS plates and allowed to incubate for 24-48 hours. The colony morphology including shape, texture, size, color, and elevation were recorded after incubation.

Litmus Milk Solution

The litmus milk[17] solution was used for preliminary identification of the isolates. The isolates were grown anaerobically in MRS and10 µL of each was used to inoculate previously acclimated anaerobic litmus milk tubes and incubated anaerobically for 24-48 hours. After incubation, the tubes were evaluated and data recorded for gas formation (bubbles), curdling of the milk, color changes, liquification (casein hydrolysis). [17]

Auto-aggregation Assay

Anaerobically grown cultures were used. The cells were spun down at 5,000xg for 15 minutes and washed twice with 5 mL of PBS. After the wash, the cell pellets were resuspended in 10 mL of PBS and the density adjusted to an OD_{600} of 1.10-1.50. The cultures were allowed to stand undisturbed for 4 hours before a sample was taken off the top of the culture tubes to measure the OD_{600}. Auto-aggregation was determined by using the formula: (%) auto-aggregation = [1-(OD_{600}@4h/OD_{600}@0h)] x 100; where OD_{600}@4h represents the density at 4 hours and OD_{600}@0h represents the starting OD. [18]

Growth on Lactose, Glucose, Galactose, and GOS using M- MRS Media.

The specified sugars were added to the M-MRS media at a final concentration of 1%. Anaerobically grown isolates were inoculated into the M-MRS + the different sugars to a starting OD_{600} of 0.02. Tubes were grown anaerobically for 24 hours, after which pH and OD_{600} of each culture was recorded.

Carbohydrate Metabolism

The API 50 CH test strips (bioMérieux, NC) were used to determine the ability of the isolates to utilize the 49 different sugars included in the test strips. This test was done using CHL media to inoculate the isolates according to the manufacturer instructions.

Acid Tolerance

The acid tolerance studies were performed in PBS (no energy source) and in MRS. The pH of PBS and MRS was adjusted using 1N HCl (i.e., same acid found in the gastric juice) to final pH of 7.0, 6.0, 5.0, 4.0, 3.0, and 2.0. The acidified PBS and MRS were dispensed into screw cap tubes (10 mL/tube) and sterilized by autoclaving. The isolates were

grown anaerobically in MRS overnight and spun down at 8,000xg and rinsed twice with PBS (pH 7.4). The washed cells were added to the acidified PBS/MRS at ~5X10^7 cells/mL and incubated anaerobically. At the specified time intervals (0, 2, and 4 hours), 100 µL of the samples were transferred to 900 µL of PBS (pH 7.4) to neutralize the acidity and then 7 µL (equal to ~3.5X10^4 @ time zero) was spot plated onto MRS plates. The plates were incubated anaerobically overnight and photographed.

Bile Salt Tolerance

A 10% stock solution of Bovine Bile Salt (Sigma-Aldrich, St. Louis, MO) was prepared in MRS media and filter sterilized using 0.22 µm Millipore filter. MRS tubes containing different concentrations of bile salts (i.e., 0%, 0.25%, 0.5%, and 1%) were prepared from the 10% filter sterile Bile Salt stock solution. The isolates were inoculated into MRS media from freezer stocks and grown anaerobically for 24 hours at 37°C. After 24 hours, the OD_{600} of the cultures was taken and adjusted so that each tube was inoculated to an OD_{600} of ~ 0.05. After inoculation, the tubes were incubated anaerobically and the OD_{600} of each isolate was taken after 4 hours of anaerobic growth at 37°C.

To determine whether the bile salts are bactericidal or bacteriostatic, we examined the effects of 0.25 and 0.50% on the viability of the cells in M-MRS (i.e., without added energy source). Anaerobically grown isolates were inoculated into M-MRS +/- the bile salts to an initial concentration of ~10^7 cells per mL. After 4-hours of anaerobic exposure to bile salts the viable counts (CFU/mL) were determined.

Antibiotic Resistance

Cultures were grown anaerobically in MRS media for 48 hours (i.e., to obtain cultures in their stationary phase). The cultures were adjusted to have an OD_{600} of 0.4 in 12 mL of MRS media and combined with 12 mL of 1.4% molten agar prepared in MRS (i.e., a 1:1 dilution is being made to yield a MRS containing 0.7% agar and an OD_{600} of 0.2). Five mL aliquots of the cell suspended in the 0.7% (Soft) agar were layered on top of regular MRS plates. Once the soft agar was solidified, a sterile paper disc (BD Blank Paper Discs, 6 mm, Cat # 231039) was set onto of the center of the soft agar plate. Each disc was inoculated with 5 µL of the test antibiotic at a concentration of 2 mg/mL. Plates were incubated anaerobically at 37°C with the disk side up for 24 hours after which the zone of inhibition was measured from the edge of the disc. [19]

Evaluation of the Isolates' Antimicrobial activity

Isolates were grown anaerobically overnight in MRS at 37°C. For each isolate, 1 µL spot of the overnight culture was pipetted onto the center of an MRS plate in two sets of triplicates and incubated anaerobically for 48 hours. After incubation, one set for each isolate was used without change, the second set of triplicates was made into two separate test plates. One plate contained the core (including the colony) placed in a fresh MRS plate. The plate from which the core (7 mm) was removed had a clean core replaced to make the second test plates. This results in 3 test plates in triplicate for each isolate: one with as is (supernatant + cells), one containing just the core with respective colony (cells alone), and one with the remaining supernatant and fresh core (supernatant alone). These plates were all overlaid with 5 mL of BHI soft (0.7%) agar contain *Salmonella* Typhimurium at an OD_{600} of 0.02 (cultured overnight at 37°C in BHI aerobically). After a 24-hour aerobic incubation zones of inhibition on each plate were measured from the edge of the respective colony or core.

Genomic DNA Preparation and Sequencing

Isolates were inoculated into MRS media and grown for 24 to 48 hours, i.e., until they reached their maximum OD_{600}. The Promega Wizard Genomic DNA purification kit (catalog number: A1120) was used with slight modifications. For each isolate, 2 mL of the culture was spun down at 16,000xg, and treated with lysozyme at 10 mg/mL. Throughout the procedure, the max incubation time was used for each step. After isolation, the DNA from each isolate was resuspended in 100 µL of rehydration solution. The DNA obtained was sent to Genewiz (Morrisville, NC) for sequencing the 16s rRNA gene. The sequences of the forward and reverse strands were run through BLAST[20] to identify the most likely speciation for each isolate. Based on this primary speciation, we selected six Lactobacilli for whole genome sequencing.

The procedures for whole genome sequencing (i.e., DNA preparation, sequencing, circularization, and annotation) have been previously described in Meinders et al. [21] The size of the chromosomes and plasmids for each of the isolate assemblies, along with the number of genes in each assembly, is listed in **Table 1**. The NCBI Prokaryotic Genome Annotation Pipeline (PGAP) was used for assembly annotation. [22,23] The accession numbers for the assemblies, as well as for the raw sequencing reads, can be found in the Data Availability section.

Table 1: Isolate genome assembly characteristics. Data adapted from Meinders et al. [21]

Isolate ID	Assigned Lactobacillus Species	Chromosome		Plasmids		Genes
		Size (bp)	GC%	Size (bp)	GC%	
1A	*L. saerimneri*	1,802,611	42.6	----	----	1,838
1B	*L. reuteri*	2,243,550	38.9	29,164	40.1	2,284
1D	*L. crispatus*	2,349,358	36.9	---	---	2,383
2D	*L. salivarius*	1,700,858	33.2	P1: 248,523	32.3	2,008
				P2: 33,778	39.3	
3B	*Lactobacillus sp.*	2,150,064	42.6	37,548	37	2,165
3DG	*L. johnsonii*	1,995,616	34.4	----	----	1,929

Statistical Analysis

Data were analyzed with Graph Pad Prism version 7.03 (Graphpad Software, CA, USA). Differences were determined with two-way analysis of variance (ANOVA) test with ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$ and NS, $p>0.05$.

Results

Isolation of the Lactobacilli

The isolates were collected from three different groups of horses. The whole genome sequence of each isolate was determined and circularized as previously described, [21] and the isolates were identified to the species level (**Table 2**).

Table 2: Source of the isolates and their age and type of feed.

Isolate ID	Species	Equine #	Source	Diet	Feed Type
1A	*L. saerimneri*	1	Weanling (1.5 months post weaning)	Grain/Grass	Mule City Super Complete Horse Feed
1B	*L. reuteri*	1	Weanling (1.5 months post weaning)	Grain/Grass	Mule City Super Complete Horse Feed
1D	*L. crispatus*	1	Weanling (1.5 months post weaning)	Grain/Grass	Mule City Super Complete Horse Feed
2D	*L. salivarius*	2	Foal (1 day post weaning)	Milk -> Grain/Grass	Mule City Super Complete Horse Feed
3B	*Lactobacillus sp.*	3	Mature gelding	Grass/Hay	Timothy Mix Hay
3DG	*L. johnsonii*	3	Mature gelding	Grass/Hay	Timothy Mix Hay

Isolate 2D (*L. salivarius*) came from a young foal that was transitioning from mother's milk to a grains/grass diet for one day. Isolates 1A, 1B, and 1D (*L. saerimneri, L. reuteri* and *L. crispatus*, respectively) were from a weaned horse feeding on grain and grass for 1.5 months after transitioning from mother's milk. The isolates from the mature gelding contained *Lactobacillus* sp. (3B) and *L. johnsonii* (3DG) – where the letter "G" designates that this isolate was from a GOS enrichment.

Characteristics of Isolates

Microscopic techniques were used to determine basic characteristics of the isolates, such as motility and morphology. Each isolate was also characterized using Gram Stain and Phase Contrast microscopy. Colony morphology from MRS+1.5% agar plates was examined for color, shape, size, moisture visibility, and edges (**Table 3**). All isolates were Gram positive (G+), rod shaped, non-motile, and did not form chains.

Table 3: Morphology of cells and colonies.

Isolate	Cell Morphology	Colony Morphology
L. saerimneri (1A)	small fat rods	small raised white colonies, rounded tops
L. reuteri (1B)	long skiny rods	clear, rough edges, not raised
L. crispatus (1D)	long thick rods	tiny white colonies, rough edges and tops
L. salivarius (2D)	short fat rods	white colonies, slight raised and shiny
Lactobacillus sp. (3B)	short fat rods	white colonies, slight raised and shiny
L. johnsonii (3DG)	medium fat rods	medium round, white colonies, and shiny

Reactions in Litmus Milk

Litmus milk is an undefined media that can evaluate for lactose fermentation, litmus reduction, gas (CO_2) production, casein coagulation, and protein hydrolysis. The results of these reactions appear in the form of bubbles, coagulation of the milk, and changes in color. The combination of these properties is used to aid in the identification of LAB. All isolates, except *L. reuteri* (1B) and *L. johnsonii* (3DG) reduced the litmus dye and fermented lactose. None of the isolates showed casein hydrolysis. Gas production (CO_2) was noticed for *L. saerimneri* (1A) and *L. reuteri* (1B).

Auto-Aggregation Assay

The percent auto-aggregation of the different isolates is shown in **Table 4**. The auto-aggregation percentage was calculated as described in the Materials and Methods and in. [18] As the bacteria auto-aggregate, clumps are formed that result in the cells precipitating to the bottom of the solution in the tube. All isolates were able to aggregate to a different degree: *L. crispatus* > *L. salivarius* > *L. saerimneri* > *L. johnsonii* > *L. reuteri* > *Lactobacillus* sp.

Table 4: Percentage of auto-aggregation of the different isolates.

Isolate	Starting OD	4 hour OD	% autoaggregation
***L. saerimneri* (1A)**	1.10	0.74	32.73
***L. reuteri* (1B)**	1.40	1.05	25.00
***L. crispatus* (1D)**	1.55	0.80	48.39
***L. salivarius* (2D)**	1.45	0.88	39.31
***Lactobacillus sp.* (3B)**	1.40	1.08	22.86
***L. johnsonii* (3DG)**	1.15	0.80	30.43

Growth in M-MRS with Glucose, Galactose, Lactose, or GOS

Growth in the M-MRS containing 1% of glucose, galactose, lactose, or GOS was determined by measuring OD_{600} after 24h. The M-MRS media seemed to have some energy source sufficient to support the growth of the inoculum for ~1-3 generations. In addition to measuring cell densities, a change in pH was also used to corroborate the fermentation of the sugars. As shown in **Table 6,** the controls have a pH between 5.90 and 6.2 (initial pH of the uninoculated media was 6.5± 0.2) which matches the low cell density seen in **Figure 1**. Isolates *L. reuteri* (1B) and *L. johnsonii* (3DG) did not grow on lactose; compared to the other isolates, they had much lower growth on galactose and GOS except for glucose. This is confirmed by the pH (**Table 5**) which is much higher when grown on lactose and galactose compared to the other isolates.

Figure 1: Growth of the Isolates in M-MRS media containing 1% of glucose, galactose, lactose, or GOS. Optical densities were measured after 24 hours of growth.

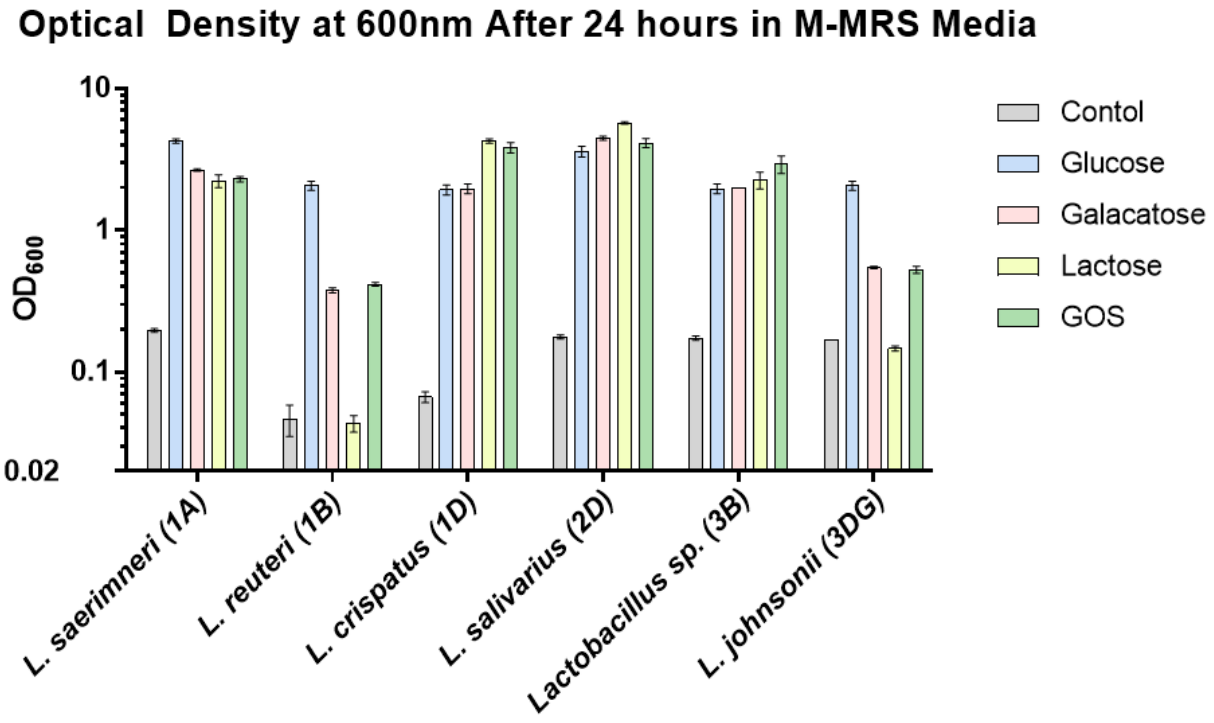


Table 5: Final pH after growth in M-MRS media with the Test Sugars. The pH of the initial media was 6.5± 0.2. The pH after 24 hours of growth of the isolates in M-MRS media with the test sugars was measured. The statistical analysis of average pH was preformed using the [H+] values of the pH (i.e., anti-log).

	pH at 24 hr				
Isolate	**Control**	**Glucose**	**Galactose**	**Lactose**	**GOS**
***L. saerimneri* (1A)**	6.33 (+/- 0.12)	4.29 (+/- 0.04)	4.42 (+/- 0.01)	4.68 (+/- 0.04)	4.79 (+/- 0.15)
***L. reuteri* (1B)**	6.34 (+/- 0.04)	4.34 (+/- 0.04)	5.78 (+/- 0.07)	6.28 (+/- 0.03)	5.67 (+/- 0.04)
***L. crispatus* (1D)**	6.12 (+/- 0.02)	4.39 (+/- 0.07)	4.73 (+/- 0.04)	4.17 (+/- 0.02)	4.25 (+/- 0.02)
***L. salivarius* (2D)**	6.03 (+/- 0.07)	4.33 (+/- 0.05)	4.16 (+/- 0.02)	4.11 (+/- 0.01)	4.28 (+/- 0.02)
***Lactobacillus sp.* (3B)**	6.31 (+/- 0.04)	4.95 (+/- 0.03)	5.27 (+/- 0.02)	5.16 (+/- 0.01)	4.91 (+/- 0.02)
***L. johnsonii* (3DG)**	6.08 (+/- 0.03)	4.40 (+/- 0.05)	5.37 (+/- 0.06)	6.06 (+/- 0.04)	5.53 (+/- 0.04)

Carbohydrate Utilization (API 50)

The API 50 carbohydrate test is used to determine carbohydrate utilization by the different isolates. The ability to utilize the carbohydrate by the isolates is visualized by a color change within each substrate in the test strip. The change in colors is a result of pH change due to the production of acid during the fermentation of the test substrate. The test strips were read after both 24 and 48 hours of incubation before the change in colors were recorded, as per the instruction of the manufacturer. A yellow color change indicates acid production except for Esculin, where the color changes to black. The results are recorded as a +/- for each substrate/isolate (**Table 6**).

Table 6: API 50 test Results. Each isolate was tested for the ability to use 49 different substrates; (+) indicates utilization of the tested carbohydrate, while (-) indicates lack of utilization. Carbohydrates not

utilized by any of the isolates and the controls are highlighted in yellow, while carbohydrates utilized are highlighted in greens.

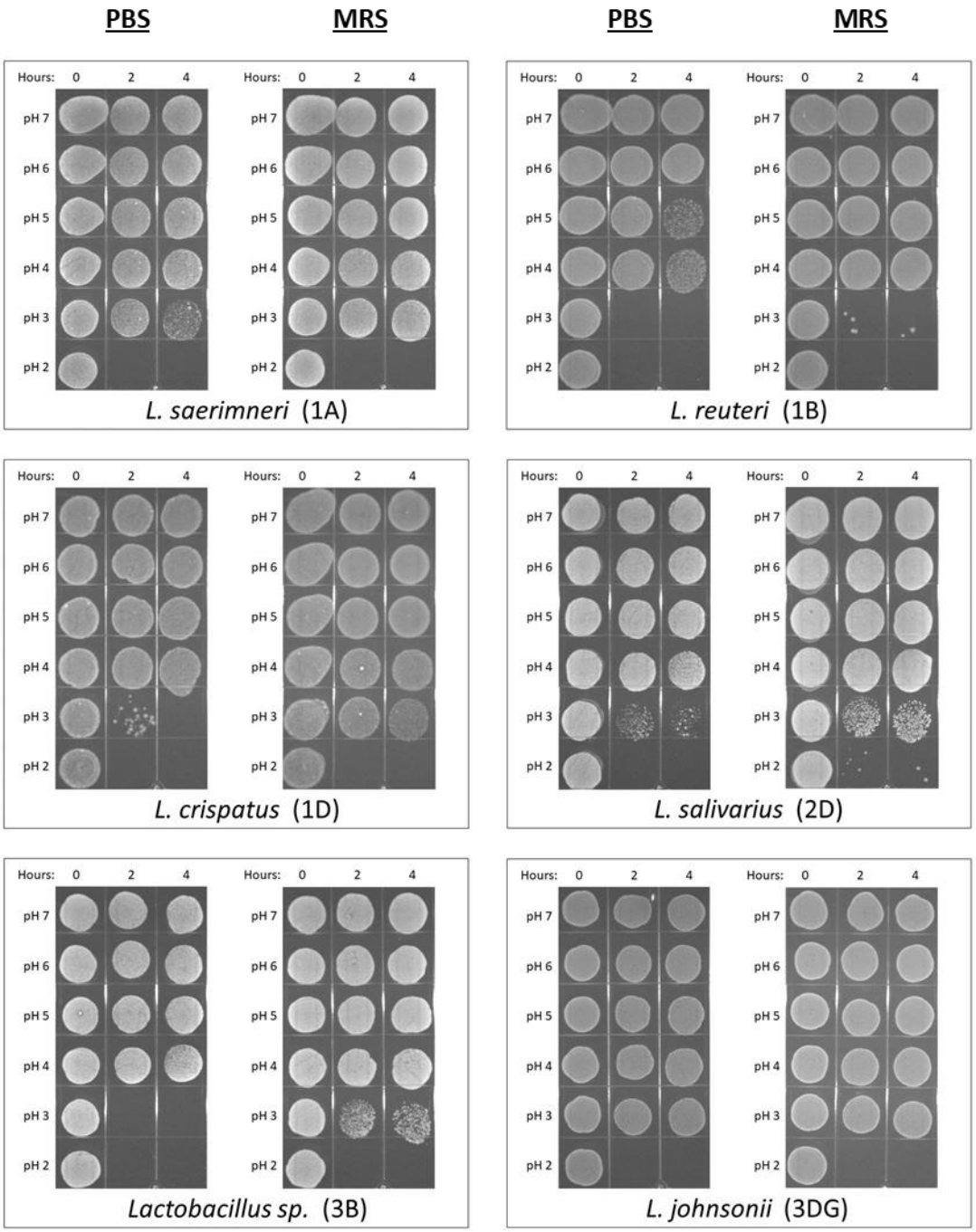
	Carbohydrate	*L. saerimneri* (1A)	*L. reuteri* (1B)	*L. crispatus* (1D)	*L. salivarius* (2D)	*Lactobacillus sp.* (3B)	*L. johnsonii* (3DG)		Carbohydrate	*L. saerimneri* (1A)	*L. reuteri* (1B)	*L. crispatus* (1D)	*L. salivarius* (2D)	*Lactobacillus sp.* (3B)	*L. johnsonii* (3DG)
0	Control	-	-	-	-	-	-	25	Esculin ferric citrate	-	-	+	-	+	-
1	Glycerol	-	-	-	-	-	-	26	Salicin	-	-	+	+	-	-
2	Erythritol	-	-	-	-	-	-	27	D-cellobiose	-	-	+	-	+	+
3	D-Arabinose	-	-	-	-	-	-	28	D-Maltose	+	+	+	+	+	+
4	L-Arabinose	-	-	-	-	+	-	29	D-Lactose	+	-	+	+	+	-
5	D-Ribose	+	-	-	-	-	-	30	D-Melibiose	+	-	+	+	+	-
6	D-Xylose	-	-	-	-	-	-	31	D-Saccharose	+	+	+	+	+	+
7	L-Xylose	-	-	-	-	-	-	32	D-Trehalose	-	-	-	+	-	+
8	D-Adonitol	-	-	-	-	-	-	33	Inulin	-	-	-	-	-	-
9	Methyl-βD-Xylopyranoside	-	-	-	-	-	-	34	D-Melezitose	-	-	-	-	-	-
10	D-Galactose	+	-	+	+	+	-	35	D-Raffinose	+	+	+	+	+	+
11	D-Glucose	+	+	+	+	+	+	36	Amidon	-	-	+	-	-	-
12	D-Fructose	-	+	+	+	+	+	37	Glycogen	-	-	-	-	-	-
13	D-Mannose	-	+	+	+	+	-	38	Xylitol	-	-	-	-	-	-
14	L-Sorbose	-	-	-	-	-	-	39	Gentibiose	-	-	-	-	-	-
15	L-Rhamnose	-	-	-	+	-	-	40	D-turanose	-	+	-	-	-	-
16	Dulcitol	-	-	-	-	-	-	41	D-Lyxose	-	-	-	-	-	-
17	Inositol	-	-	-	-	-	-	42	D-Tagatose	-	-	-	-	-	-
18	D-Mannitol	-	-	-	+	-	-	43	D-Fucose	-	-	-	-	-	-
19	D-Sorbitol	-	-	-	+	-	-	44	L-Fucose	-	-	-	-	-	-
20	Methyl-αD-Mannopyranoside	-	-	-	-	-	-	45	D-Arabitol	-	-	-	+	-	-
21	Methyl-αD-Glucopyranoside	-	-	-	-	-	-	46	L-Arabitol	-	-	-	-	-	-
22	N-Acetylglucosamine	-	-	-	+	-	+	47	Potassium Gluconate	-	-	-	-	-	-
23	Amygdalin	-	-	-	-	-	-	48	Potassium 2-Ketogluconate	-	-	-	-	-	-
24	Arbutin	-	-	+	-	-	-	49	Potassium 5-Ketogluconate	-	-	-	-	-	-

Acid Tolerance

We tested the ability of the isolates to resist acidic pH in PBS (no energy source) and MRS (in the presence of energy source), **Figure 2.** In PBS or MRS, none of the isolates were able to survive an exposure at a pH 2 for 2 hours.

In PBS at pH 3, only *L. saerimneri* (1A) and *L. johnsonii* (3DG) survived exposures for 2 and 4 hours; while *L. crispatus* (1D) and *L. salivarius* (2D) were able to partially survive 2 hours exposures. On the other hand, *L. reuteri* (1B) and *Lactobacillus* sp. (3B) were not able to survive pH 3 exposures for 2 hours. However, exposures to acid in MRS resulted in improving the acid resistance at pH 3 for all the isolates except *L. johnsonii* (3DG). Also, in MRS a few colonies of *L. salivarius* (2D) and *L. reuteri* (1B) survived 2 and 4 hours of exposure at pH 2 and 3, respectively.

Figure 2: Acid Tolerance of the isolates. Effects of exposure to different pH (2,3,4, 5, 6 and 7) and for different times (0, 2, and 4hours) on the survivability of the isolates in PBS (left) and MRS (right).

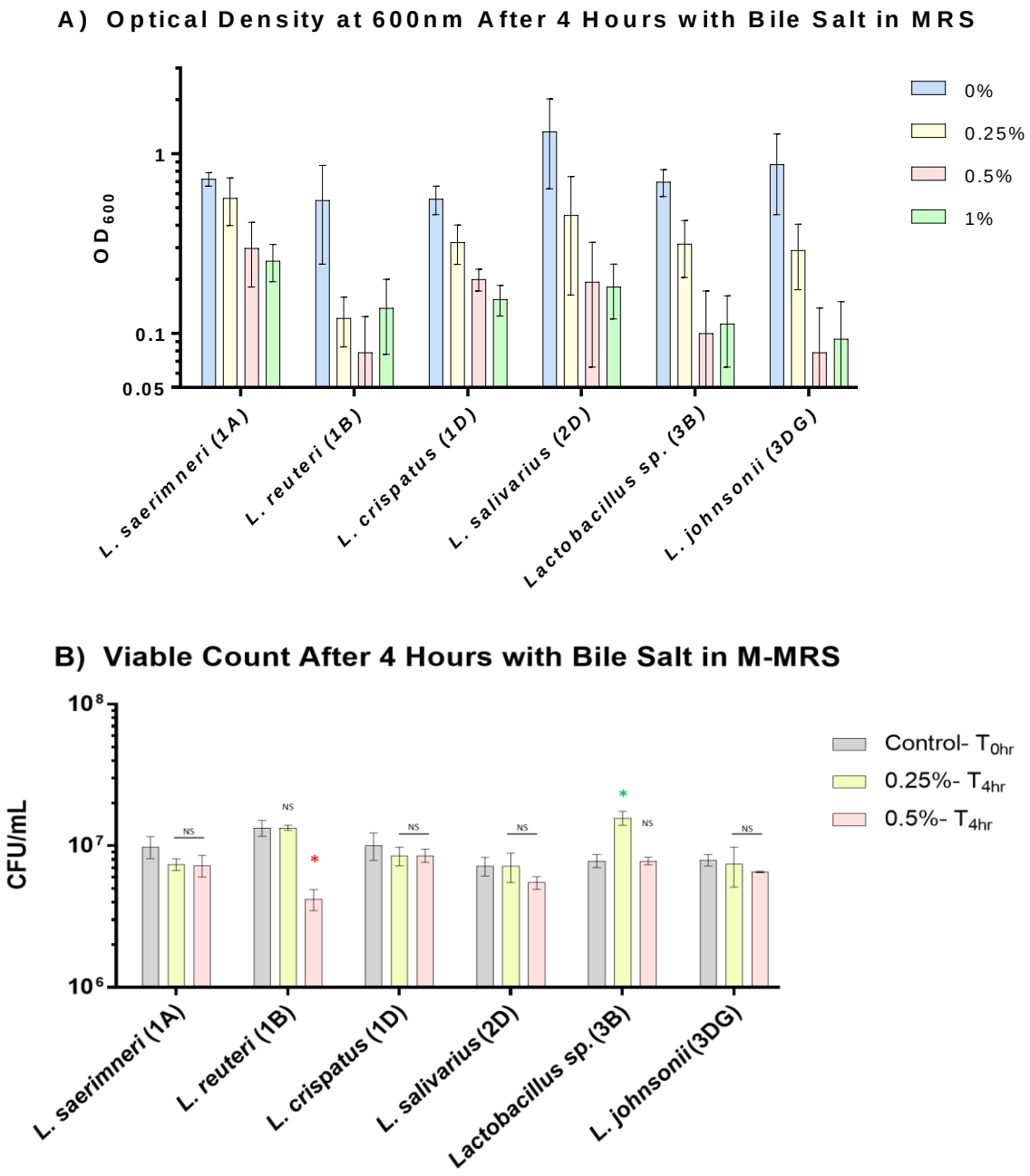


Bile Salt Tolerance

The isolates were able to withstand and continue to grow in varying concentrations of bile salts (**Fig. 3A**). This is important because as probiotic bacteria travel through the upper part of the intestine, they encounter the bile salts. All isolates showed a decrease in growth as the concentration of bile salts increased. *L. saerimneri* (1A) was the most bile tolerant. All isolates were able to grow in the presence of 0.25% (~6.1 µM).

The effect of bile salts on the isolates was bacteriostatic. Data indicate that there was no significant change in the number of CFU/mL after 4 hours of exposure to 0.25% bile salt (**Fig. 3B**). However, isolate 3B was able to grow in presence of 0.25% bile salt. Also, there was no significant inhibition after 4 hours exposures to 0.5% bile salt, except for *L. reuteri* (1B).

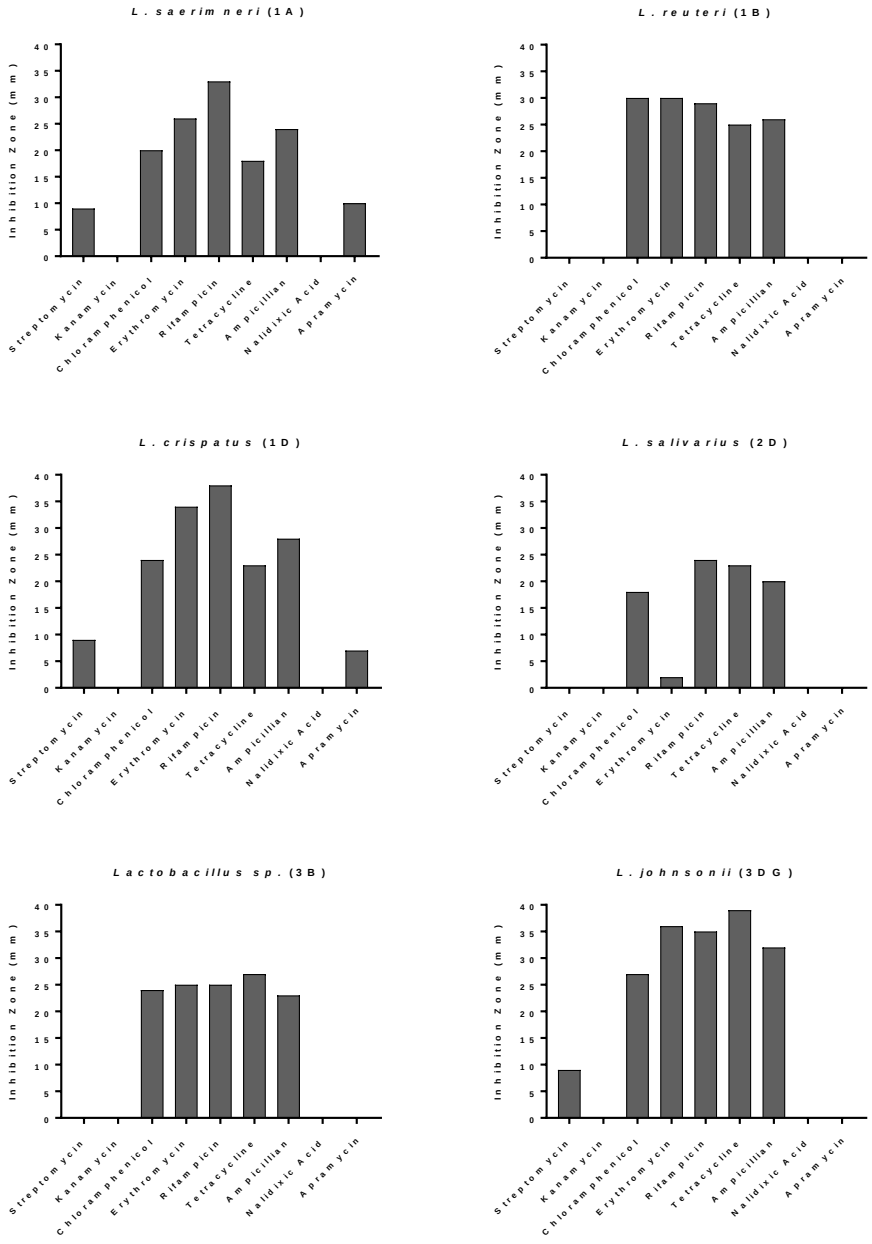
Figure 3. Resistance to Bile Salts. (A) The isolates were exposed to 0, 0.25, 0.5, and 1% Bovine bile salts in MRS and OD_{600} reported after 4 hours; and **(B)** the number of CFU/mL reported after 4 hours of exposure to 0, 0.25, and 0.5% bile salts in M-MRS – ns, >0.05; *(inhibition), <0.05; *(growth),<0.05 .



Antibiotic Resistance.

The antibiotic resistance was analyzed as indicated in the Materials & Methods. Nine antibiotics were tested (**Figure 4**). All isolates were sensitive to Chloramphenicol (Cm), Erythromycin (Erm), Rifampicin (Rif), Tetracycline (Tet), and Ampicillin (Amp); however, *L. salivarius* (2D) was less sensitive to Erm. Also, *L. saerimneri* (1A), *L. crispatus* (1D) and *L. johnsonii* (3DG) were sensitive to Streptomycin (Str), while *L. saerimneri* (1A) and *L. crispatus* (1D) were also sensitive to Apramycin (Apr). In other words, the more sensitive isolates were: 1A =1D > 3DG > 2D, and 1B = 3B. Furthermore, all isolates were resistant to Kanamycin (Kan) and Nalidixic Acid (Nal); while (1A, 1D, and 3DG) were resistant to Str, (1A and 1D) also resisted Apr. In other words, (1B, 1D, and 3B) resisted (Kan, Nal, Str, and Apr), 3DG resisted (Kan, Nal, and Apr), while (1A and 1D) resisted (Kan and Nal).

Figure 4: Susceptibility to antibiotics. The size of the zone of inhibition (mm) was measured in millimeters from the edge of the disc. Discs containing the specified antibiotics (10 µg/disk) were added on the surface of a lawn of each isolate.



Antimicrobial activity evaluation.

The ability of the six *Lactobacillus* isolates to inhibit the growth of *S.* Typhimurium (ATCC 14028) was tested under anaerobic conditions. The results in **Table 7** indicate that all six isolates have a potential to inhibit the growth of *S.* Typhimurium. The inhibitory effects were observed when the cells (the colony) or the supernatants were tested. *L. salivarius* (2D) was most effective in inhibiting *S.* Typhimurium; where 2D>1A>3B>3DG>1D>1B.

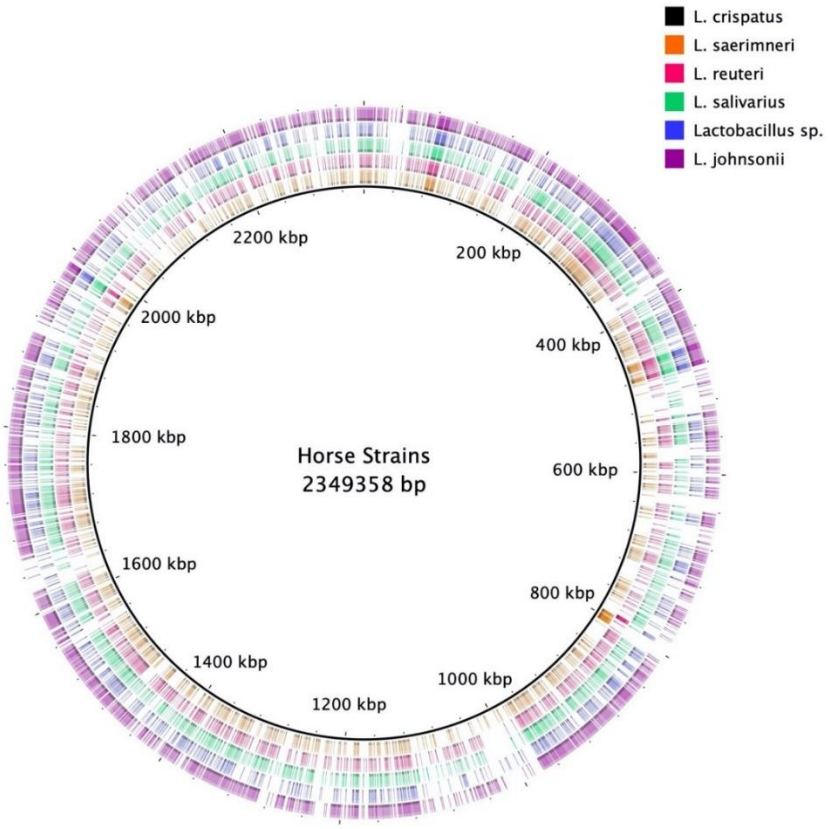
Table 7: Anti-*Salmonella* Activities of the Isolates and Supernatants. The whole cell suspension, cells only, and supernatants of each isolate was overlaid with soft agar containing *S.* Typhimurium. The zone of inhibition was measured in millimeters out from the edge of each colony or the plug. The data represent an average of triplicates +/- Std.

	Zone of Inhibition (mm)		
Isolate	**Cells+Supernatant**	**Cells**	**Supernatant**
***L. saerimneri* (1A)**	15.3 (+/- 0.6)	10.7 (+/- 0.6)	15.0 (+/- 1.0)
***L. reuteri* (1B)**	6.0 (+/- 3.0)	4.7 (+/- 0.6)	4.0 (+/- 1.0)
***L. crispatus* (1D)**	12.0 (+/- 2.0)	9.0 (+/- 0.0)	10.0 (+/- 1.0)
***L. salivarius* (2D)**	19.0 (+/- 0.0)	12.0 (+/- 2.8)	17.5 (+/- 2.1)
***Lactobacillus sp.* (3B)**	15.0 (+/- 0.0)	11.0 (+/- 1.0)	14.0 (+/- 1.7)
***L. johnsonii* (3DG)**	12.5 (+/- 0.7)	9.3 (+/- 1.1)	13.5 (+/- 0.7)

Genomic characterization of the different isolates.

Data in **Figure 5** shows that despite there being variation between the isolate chromosomes on the nucleotide level, the overall alignment is quite high. The reference genome in the figure was selected to be *L. crispatus* since it has the largest chromosome size. The figure indicates that there are areas in the reference strain 1D that diverged greatly from the other five isolates. The nucleotide sequence percent identity between the *L. crispatus* (1D) chromosome and the other five isolate chromosomes was calculated using BLAST[20] and are 92.9% (*L. saerimneri*), 94.7% (*L. reuteri*), 84.8% (*L. salivarius*), 83.5% (*Lactobacillus* sp.) and 83.0% (*L. johnsonii*).

Figure 5. Comparison of the circular chromosomes at the nucleotide level – The reference chromosome is *L. crispatus* (1D). This figure was generated using the Blast Ring Image Generator (BRIG). [24]



Annotation pathways for the isolates.

The annotation pathways were examined using the Rapid Annotation Subsystem Technology (RAST), where the RASTtk annotation scheme was used. [25-27] The Subcategories 'Monosaccharides' (Table_1S), 'Di- and oligosaccharides' (Table_2S), and 'Central carbohydrate metabolism' (Table_3S) from the 'Carbohydrates' Category, as well as the Subcategory 'Resistance to antibiotics and toxic compounds' (Table_4S) from the 'Virulence, Disease, and Defense' Category were examined as they included subsystems of interest. The genomic features, such as protein-coding genes, and their roles could be identified for each of these subsystems.

Discussion

Firmicutes are the main bacterial Phylum found in the horse GI tract, and Lactobacilli are one of the predominant species found in all compartments. [28] This study was designed to isolate Lactobacilli from horses and examine their biochemical and genetic characteristics. Thus, we did not intend to study the horse microbiome, *per se*. Six different *Lactobacillus* spp. were isolated from horses at different stages of development that were fed age-appropriate diets. One isolate *L. salivarius* (2D) was from a foal that has just been weaned for one day, isolates [*L. saerimneri*, 1A; *L. reuteri*, 1B; and *L. crispatus*,1D] were from a horse weaned for 1.5 months, while isolates [*Lactobacillus sp.*, 3B and *L. johnsonii*, 3DG] were from a mature 10-year-old gelding. All isolates were from fresh manure samples, i.e., they already survived passage through the digestive tract. The complete and circularized genome sequences of the six isolates and any plasmid(s) they harbor has been published. [21]

The current study focused on the biochemical properties of these isolates, as well as the identification of potential genes that code for these functions using genomic mining. However, the presence of specific gene(s) does not imply they are functional, i.e., only transcriptomic data can verify functionality (expression) of the genes. We also recognize that we used a limited number of horses (i.e., 3 horses) of different ages. However, this preliminary data provides valuable information for further hypothesis driven studies.

Carbohydrate Metabolism.

The litmus milk test is a simple method that provided preliminary assessment of the isolates. [17,29] All isolates except for *L. reuteri* (1B) and *L. johnsonii* (3DG) were able to reduce litmus. Normally, a combination of the light pink color and white indicates that the isolate can produce acid from lactose. The change in color occurs when the pH reaches a value of around 4. The presence of gas bubbles indicates a hetero-fermentation and the production of CO_2, [30] which was the case for *L. saerimneri* (1A) and *L. reuteri* (1B). The results from this test are consistent with those from the growth on sugars in M-MRS test (**Fig. 1** and **Table 6**), where the growth of *L. reuteri* (1B) and *L. johnsonii* (3DG) on lactose, galactose, and GOS was significantly less than the other 4 isolates. Also, the results agree with the API50 test as *L. reuteri* (1B) and *L. johnsonii* (3DG) did not ferment lactose or galactose (**Table 6**).

The API 50 tests for the utilization of 49 substrates plus a no substrate, as a control. A total of 26 substrates were not utilized by any of the isolates, i.e., only 23 substrates are potentially used by any isolate. Interestingly, isolate 2D (*L. salivarius*) which was isolated from the youngest horse that had been recently weaned from mother's milk and has the smallest genome size (1,700,858 bp, **Table 1**), was the most versatile in the number of sugars utilized (16/23), and it did not utilize D-cellobiose nor L-arabinose - consistent with the diet consumed. Also, isolate 2D was the only isolate to utilize L-rhamnose, D-mannitol, D-sorbitol, and D-arabitol. On the other hand, *Lactobacillus* sp. (3B) and *L. johnsonii* (3DG) that came from an older horse fed grass and hay were able to utilize D-cellobiose; and 3B was the only isolate capable of utilizing D-arabinose. Both D-Cellobiose and D-arabinose are found in cellulose and hemicellulose. Isolate 1D (*L. crispatus*) was the second most versatile in carbohydrate utilization, it used 14/23 substrates including D-cellobiose.

All isolates were able to utilize D-glucose, D-maltose, D-saccharose, and D-raffinose.

At the genome level, it could be seen from the isolate assembly annotations that all isolates except for 2D possessed genes for Lactose transport (i.e., lactose permease, PTS lactose transporter, PTS lactose/cellobiose transporter) and all isolates possessed galactose mutarotase[31] which is a member of the galactose operon and catalyzes the interconversion of alpha and beta anomers of aldoses and participates in the Leloir pathway. [32] This enzyme is required for the metabolism of galactose via its conversion to glucose. Interestingly, *L. johnsonii* (3DG) possess the β-galactosidase gene but was not able to utilize lactose in the Litmus-milk, M-MRS test, or API 50, while *L. salivarius* (2D) was able to use lactose but does not possess the β-galactosidase gene. Further studies are needed to clarify this interesting observation. It is possible that the enzyme for metabolizing lactose 'β-galactosidase' is not required in the old/mature horse whose diet is based on grass and hay. Indeed,

niche-driven metabolic changes has been reported. [33,34] Gene mutation, deletion, and acquisition are part of the isolate adaptation to the host's environment. Ecological adaption has been noted in isolates isolated from cow milk versus those from human. [35]

In the API 50 test, 1B and 3DG (*L. reuteri* and *L. johnsonii*) were negative in D-lactose and D-galactose utilization, while in the M-MRS media they were able to grow on galactose and lower the pH of the medium (**Fig. 1** and **Table 6**) and they possess the genes for doing so. This may be explained by the fact that the API 50 test relies on enough acids (lactic, acetic, butyric acids. etc.) are produced to change the color of the acid indicator used. The lack of enough acid produced could be due to the conversion of the acid(s) to other neutral products. Indeed, *L. reuteri* has been shown to be a heterofermentative organism that uses the phosphoketolase pathway (PKP) to produce lactate, ethanol, and CO_2 from glucose. [36-38] Indeed, *L. reuteri* (1B) isolate possesses the phosphoketolase enzyme. Also, *L. reuteri* are known to produce an antimicrobial compound "reuterin" from glycerol at the expense of pyruvate and lactate. [39,40] Indeed, the genome of *L. reuteri* (1B) contains the gene cluster (pdu B, M, P, and V) for the transformation of glycerol to 3-hydroxypropionaldehyde (Reuterin). Also, isolate *Lactobacillus* sp. (3B) is the only other isolate that contains this gene cluster.

Furthermore, the gene coding for α-acetolactate decarboxylase which is required to produce the buttery flavor "acetoin" from Lactate via α-acetolactate is found in the genomes of *L. reuteri* (1B), *L. salivarius* (2D), and *L. johnsonii* (3DG). The production of acetoin also results in less lactic acid and reduced acidity. Clearly, tests that relying solely on changes in pH (i.e., Litmus milk and API 50) should be interpreted with caution.

Acid Tolerance.

To pass through the GI tract the organism must survive the acidity of the stomach. The stomach pH of the horse fluctuates between 2 and 6, depending on the frequency, amount, and type of feed/ forage. [41] In horses, the passage time through the stomach is short (~15 minutes) due to its relatively small size. [42] Acid tolerance G+ Lactobacilli is mediated by the "Acid Tolerance Response" (ATR), which includes proton pumps, glutamic acid decarboxylase, and cellular repair mechanisms. [43,44] The annotations of the isolates assemblies show that all isolates have Na+: antiporter genes (NhaC) and amino acid related decarboxylases (i.e., amino acid ornithine, phosphopantothenoylcysteine, threonine-phosphate decarboxylases). None of the isolated possessed the glutamate decarboxylase gene. Indeed, the survival in the acidic environments was improved by the presence of energy and nutrient; compare acid resistance in MRS *vs.* PBS (**Fig. 2**).

It was also reported that the ability to co-aggregate is dependent on the production of exopolysaccharides (Eps), which enhances the organism's ability to survive harsh environments (e.g., high acidity, bile salts, antibiotics) as well as to improve their ability to interact with the host's immune system. [45,46] Data in **Table 4** indicate that the isolates were able to co-aggregate. Indeed, all isolates except *L. salivarius* (2D) possessed genes for EPS biosynthesis. Interestingly, data in **Table 4** (% auto aggregation) indicated that 2D had the second highest score, suggesting that other factors might contribute to aggregation.

The data presented indicate the ability of the isolates to resist acidic environment of the stomach and proceed to grow in the small and large intestine where the pH is more favorable, i.e., 6.5-7.0 in the hind gut. [41]

Bile Tolerance.

In the small intestine, isolates encounter equine GI tract bile salts, which are produced by the liver and conjugate with taurine or glycine residues to aid in the emulsification and digestion of fats, and proteins. Since equine do not have a gall bladder, they are constantly producing bile. [47] The concentration of total plasma bile acids is between 1.00 and 5.80 µmol/L in normal adult horses. [48] The concentration of bile acids increases in horses with hepatic and GI disorders. All the isolates had a steady decrease in growth after 4 hours; however, there was no significant effects on the viability of the isolates in presence of 0.25% (6.12 µmol/mL) bile salts, or on 0.5% (12.25 µmol/mL) except for isolate 1B. The data indicate that bile salts affect the growth of the isolates, and it was not bactericidal.

In general, the growth of the isolates was better in the presence of 0.25% than in higher concentrations of bile salt, which is slightly higher than the average bile concentration in healthy horses. Except for 1A, all isolates possessed a linear amide C-N hydrolase gene that may play a role in bile hydrolysis (i.e., (cholylglycine hydrolases belong to this family). All isolate genome assembly annotations included a DHA2 family efflux MFS transporter permease subunit. In addition, except for 3DG, all isolates possessed a Multidrug and toxic compounds extrusion (MATE) family efflux transporter. Indeed, efflux pumps have been shown to play a major role in bile resistance in several strains of *Lactobacillus* and *Bifidobacterium*. [49] In a recent study, [50] where the effect of 0.15% on the transcriptome and proteome of a probiotic *L. salivarius* L101 was

examined, it was demonstrated that resistance to bile salts stemmed from the differential expression of cell envelope proteins and efflux pumps but was not dependent on bile salts degradation. Indeed, to decipher the mechanism(s) for bile salts resistance, future transcriptomic studies are needed.

Antibiotics Resistance.

The topic of antibiotic resistance is controversial in that antibiotic resistance genes could be transferred to other pathogenic organisms which would be detrimental to human and animal health. This resistance could also prove to be beneficial for probiotic bacteria in maintaining the health of the gut during and after a course of antibiotic treatment.

Bacterial antibiotic resistance is accomplished by one of two mechanisms: a) intrinsic, caused by mutation(s) in the chromosomal genes, or b) extrinsic, via acquisition of external elements for resistance. Genomic DNA sequencing is the best way to identify the mechanism of resistance to antibiotics.[51] The data (**Fig. 4**) suggested that *L. reuteri* (1B), *L. salivarius* (1D) and *Lactobacillus sp.* (3B) were most resistant to 4 out of the 9 antibiotics tested, while *L. saerimneri* (1A) and *L. crispatus* (1D) were the least resistant (resisted 2 out of 9 antibiotics tested). From the RAST annotation pathways shown in **Table 4S** for the 'Resistance to antibiotics and toxic compounds' Subcategory, it could be seen that all isolates had a Subsystem for resistance or regulation to cobalt and copper, and all isolates except for *Lactobacillus* sp. (3B) included Subsystem for mercury reductase, in addition, *L. reuteri* (1B) and *L. salivarius* (2D) had a Cadmium resistance Subsystem.

Fig. 5 showed that all isolates were resistant to Nalidixic acid, and from the pathway annotation it could be seen that all isolates possessed the genes Gyrase A and B for the Subsystem 'Resistance to fluoroquinolones' (Table 4S). It important to note that the above-mentioned resistance genes were not found on plasmids or transposable elements, except for isolate 2D which has 'Bile salt hydrolase, copper homeostasis, and Tet resistance – ribosome type protection' present on the larger plasmid #1. However, interestingly, all isolates were sensitive to Tetracycline despite the annotation pathways indicating the presence of genes for Tet resistance (ribosome type protection and elongation factor G). Clearly, the position of Multidrug efflux pumps, and the ability of the isolates to auto-aggregate may also enhance their resistance to antibiotics, survival, and colonization within the gut.

Antimicrobial Activities.

It is also important that isolates be able to compete with pathogenic organisms that maybe encountered in the gut. Probiotic bacteria can produce bacteriocins (ribosomally synthesized antimicrobial peptides). The physiological and ecological function of bacteriocins is not exactly known. However, they may serve to help the producers in colonizing the gut, killing competitor organisms and pathogens, or in communicating with other organisms and the host.[52-54] In the antimicrobial test we used S. Typhimurium Strain ATCC 14028 as a model Gram negative pathogen important in farm and domestic animals. The results of this test (**Table 7**) showed that all isolates were able to inhibit *Salmonella*. This inhibition could be due to the organic acids (lactate, acetate, etc.), bacteriocins production, or antimicrobial compounds like Reuterin or a combination thereof.

All isolates can produce organic acids from monosaccharides by glycolysis via the Embedin-Mayerhoff pathway, isolates 2D and 3DG possessed genes for bacteriocin immunity proteins, and 1D possessed the most loci (6 genes for bacteriocin biosynthesis and 3 genes for bacteriocin immunity proteins), while 1A,1B, and 3B did not possess any bacteriocin genes. Also, isolates 1B and 3B possess the gene cluster (pdu B, M, P, and V) for the transformation of glycerol to 3-hydroxypropionaldehyde (reuterin). Only isolate 1A did not possess any genes for bacteriocin or reuterin.

In summary, current feed additives utilize less than 1% of organisms that came from the same host. Problematically, the bacteria being used are not found in high numbers in the equine's large intestine, where complex carbohydrates are metabolized, and most intestinal diseases occur in this portion of the gut. Recent studies have shown the possibility that *Lactobacillus* isolated and reinoculated into the same host species are more effective than those from other species.[4] This could be in part due to the host-microbiota adaption. The *Lactobacillus* species are known to be transferred during birth and during the early days of life through mother's milk.[55] This study and unpublished data, suggest that isolates *L. salivarius* (2D), *L. reuteri* (1B), *L saerimneri* (1A) and *L. crispatus* (1D) are most likely suited for early life, while *L. crispatus* (1D), *Lactobacillus* sp. (3B), *L. johnsonii* (3DG) are appropriate for later stages of equine's life. Under normal circumstances the gut functions as an anaerobic environment; however, when this is disrupted by using antibiotics or infection with pathogen aerobic conditions can occur. The resulting imbalance is referred to as dysbiosis.[56] The purpose of probiotic bacteria is to prevent and heal the result of this dysbiosis by helping to restore normal function of the gut. To achieve that role, it is important to select and apply host-specific probiotic organisms.[14, 34, 57]

Conclusion

The results of the study indicate the isolates possess different traits that matched the host's environment (i.e., age and diet). The importance of selecting and applying Species-adapted (i.e., Species-specific) Lactobacilli as probiotics was emphasized. The isolates have the characteristics required to enable survival and colonization within the equine GI tract during the different stages of development. Resistance to acids during passage through the stomach is improved by the presence nutrients (e.g., feed). Future studies on the transcriptomes of these isolates, their interactions with each and other members of the microbiome in the GI tract, and their influence on the host's immune system will be of great value. Also, the use of large cohorts and comparisons between different horse breeds will be essential for reaching solid conclusions.

Data availability

The isolate chromosome and plasmid assemblies are available in GenBank. The accession numbers for these sequences are isolate 1A:CP047418, isolate 1B: CP047416, CP047417, isolate 1D: CP047415, isolate 2D: CP047412, CP047413, CP047414, isolate 3B: CP047410, CP047411, and isolate 3DG: CP047409. The SRA accession numbers for the Pacific Biosciences sequencing reads are isolate 1A: SRR10752814, isolate 1B: SRR10752813, isolate 1D: SRR10752812, isolate 2D: SRR10752811, isolate 3B: SRR10752810, and isolate 3DG: SRR10752809.

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Conflict of Interest

All authors declare no conflict of interest

Authors Contributions

Conceptualization, HMH; Methodology, HMH, RBL, MM, AD; Data Curation, RLB, MM, AD; Bioinformatics, AD, HMH; Writing-original draft, RLB, HMH; Reviewing and Editing, HMH, AD, MM; Project Administration, HMH, MM. All authors have read and agreed to publish the final version of the manuscript.

Supplementary Data (Available here)

Table 1S: 'Monosaccharides' Subcategory.
Table 2S: 'Di- and oligosaccharides' Subcategory.
Table 3S: 'Central carbohydrate metabolism' from the 'Carbohydrates' Category.
Table 4S: 'Resistance to antibiotics and toxic compounds' from the 'Virulence, Disease, and Defense' Category.

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