



Article

Characterization of Lactic Acid Bacteria for Potential Use as a Direct-Fed Microbial in Food Animals

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Abstract

Zoonotic pathogens could persist in their environment and be introduced into the food-chain. With careful screening and selection, lactic acid bacteria (LAB) could be used as direct-fed microbials (DFMs) to control these pathogens in food animals. Previously isolated LAB (n = 250) were evaluated for inhibition against Shiga-toxigenic *Escherichia coli* (STEC) and *Salmonella enterica*, using agar spot test. Tests revealed that LAB were more effective against *Salmonella* than STEC, with 67% showing excellent (>15 mm) inhibition. LAB (n = 65) exhibiting significant pathogen inhibition (zones > 10 mm) were tested for acid (pH: 2, 4, 5, 7) and bile (0, 0.1, 0.3, 0.5%) tolerance, and biofilm-forming capabilities. About half of the tested LAB exhibited excellent to very good tolerance. All LAB formed biofilms, with 33% forming strong biofilms. LAB (n = 59) were also examined for susceptibility to commonly used antibiotics due to their intrinsic or acquired antibiotic resistance (AR), transferrable to pathogens. Only *S. thermophilus* S-2 showed susceptibility to all the antibiotics. The majority were susceptible to erythromycin (88%), followed by ampicillin (85%), clindamycin (64%), tetracycline (58%), vancomycin (44%), streptomycin (15%), and gentamicin (9%). Overall, LAB showed strong inhibition against pathogens, along with survival capabilities for environmental stress conditions, and could be considered for potential DFM applications.

Keywords: food animals; *Escherichia coli*; *Salmonella enterica*; direct-fed microbial; food safety



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1. Introduction

According to the Centers for Disease Control and Prevention (CDC), approximately 1 in 6 Americans fall ill from foodborne illnesses, leading to approximately 3000 fatalities yearly [1]. Shiga-toxin-producing *Escherichia coli* (STEC) and *Salmonella enterica* have often been implicated in these foodborne illness outbreaks, with the latter being the most reported foodborne pathogen in foodborne illness outbreaks annually. In the United States (US) alone it causes an estimated 1.35 million illnesses, 26,500 hospitalizations, and 420 deaths annually [2]. At the same time, STEC cause an estimated 265,000 foodborne illnesses, 3600 hospitalizations, and 30 deaths annually, imposing an economic burden of over \$271 million [3,4]. Food animals such as cattle, swine, and small ruminants are well-known reservoirs of these pathogens that form part of the commensal microflora and are intermittently shed in the animal feces [5,6]. Research has revealed that despite antimicrobial treatments, these foodborne pathogens can persist long-term within the GI tract of animals due to the formation of biofilms [7]. These biofilms consist of strong, sticky

carbohydrates, known as exopolysaccharides (EPS), that are difficult to remove. Some high-risk food commodities associated with pathogen persistence include beef and meat products, fresh produce, unpasteurized apple juice, and dairy products [2]. Persisting pathogens can be introduced to the food-chain in several ways, such as direct contact with manure, contaminated soil, poor-quality irrigation/processing water, or direct contact with animals, birds, insects, or infected personnel. Reduction of these pathogens in food animals is critical for preventing their introduction into the food-chain. Antibiotics have traditionally been used to treat enteric foodborne pathogens; however, with increased threat of antibiotic resistance (AR), alternatives are being sought. A potential biocontrol method is the utilization of lactic acid bacteria (LAB) as direct-fed microbials (DFMs) in food animals. Lactic acid bacteria are widely used as beneficial bacteria, with early reports showing alleviation and prevention of intestinal disorders, improved growth performance, increased nutrient utilization, and decreased use of antibiotics [8]. Lactic acid bacteria are generally recognized as safe to consume and are known to inhibit STEC and Salmonella. However, some LAB have shown intrinsic or acquired AR, which could be transferred to pathogenic microorganisms [9,10]. Therefore, it is crucial to evaluate the AR of LAB that hold potential for use as probiotics [11]. Additionally, to be effective in the GI tract, they need to be able to survive the unfavorable GI tract environment, such as acid and bile conditions [12]. The objective of this study was to characterize and select LAB for potential application as DFMs in food animals.

2. Materials and Methods

2.1. Viability of LAB Isolates Following Long-Term Storage

Viability of 250 previously isolated LAB of the genus *Lactobacillus*, *Lactococcus*, *Leuconostoc*, or *Streptococcus* was determined after extended frozen storage (-80°C). The isolates had been freeze-dried and stored in sealed glass vials for approximately 15 years prior to use. These isolates were obtained from the culture collection of late Dr. Stanely Gilliland, at the Oklahoma State University, Stillwater, OK, USA. Individual vials of each isolate were broken and one bead was aseptically transferred into 9 mL of sterile de-Man Rogosa Sharpe (MRS) broth (Difco Becton Dickinson Microbiology Systems, Sparks, MD, USA) and anaerobically incubated between 30 and 42°C for 24–48 h, depending on the genus and species of the LAB isolate. If an isolate showed significant growth after 24 h, 1 mL of the culture was transferred to 9 mL of fresh MRS broth and incubated further at the respective temperature for 24 h. If minimal or no growth was observed, the tubes were re-incubated for another 24 h and the above process repeated. Frozen concentrated cultures (FCC) of the revived isolates were prepared for future use. Approximately 1.5 mL of each revived bacterial culture was transferred into a sterile microcentrifuge tube (Eppendorf, Enfield, CT, USA) and centrifuged at $10,000\times g$ for 10 min at 0°C . The liquid supernatant was removed using a micropipette without disturbing the pellet. The pellet was resuspended in 500 μL of sterile 10% non-fat milk solids and vortexed thoroughly to mix the culture. The FCC (5 tubes per isolate) were then stored at -80°C . The isolates with good growth were also identified using the Gram-stain technique to determine if they were Gram-positive rods or cocci.

2.2. Preliminary Screening of LAB for Inhibition of STEC and *S. Typhimurium* Bacterial Cultures

Lactic acid bacteria ($n = 95$) that showed excellent to very good growth after 24 h were screened for their inhibition capability against STEC (O157, O26, O45, O103, O111, O121, and O145) and *S. Typhimurium* DT104 (H2662, 11942A, 13068A, 152N17-1) using an agar spot test. Overnight cultures of individual LAB strains (1×10^8 CFU/mL) were spot

inoculated (3 spots per isolate) onto MRS agar surface, and incubated anaerobically for 18 to 20 h at 37 °C. Individual cocktails (1:1 ratio) of four *S. Typhimurium* DT104 (H2662, 152N17-1, 13068A, 11942A) and *E. coli* O157:H7 strains and twelve Non O157 strains (2 strains per serotype) were prepared using the overnight cultures of each pathogen strain by inoculating 9 mL of tryptic soy broth (TSB; Difco Becton Dickinson Microbiology Systems, Sparks, MD, USA) with working stocks (4 °C) of each strain and incubating at 37 °C for 16–18 h. All pathogen cocktails were diluted into TSB to obtain a concentration of approximately 1×10^5 CFU/mL before transferring to tempered tryptic soy agar (TSA; Difco Becton Dickinson Microbiology Systems, Sparks, MD, USA) to obtain a final concentration of approximately 1×10^4 CFU/mL. These pathogen mixtures were then poured onto the surface of spot-inoculated MRS agar plates containing LAB. After 24 h of incubation (37 °C), the inhibition zones around the LAB were measured at three locations, using vernier calipers, and the average reported as the inhibition value (Figure 1). Clear inhibition zones of >1 mm around the spot were scored as positive [13]. The LAB isolates that exhibited inhibition zones > 5 mm were considered good, >10 mm very good, and >15 mm were considered as excellent for inhibition towards the pathogens. Isolates exhibiting very good and excellent inhibition were further tested for tolerance to acid and bile conditions.



Figure 1. Agar spot test showing pathogen inhibition by lactic acid bacteria.

2.3. Acid and Bile Tolerance

Of the LAB isolates ($n = 95$) screened for initial inhibition, 65 showing zones of >10 mm were tested for their tolerance to acid and bile according to the methods described by Brashears et al. [14]. Overnight cultures of each LAB isolate were gently vortexed and 25 μ L was transferred into 96-well plates containing 225 μ L of MRS broth that was either acidified (pH 2, 4, or 5) or contained bile (0.1, 0.3, 0.5%). Prior to starting the experiment, MRS broth was adjusted to the appropriate pH (2, 4, and 5) using 3M or 5M HCl. The bile concentrations (0.1, 0.3, and 0.5%) were adjusted in the appropriate amount of MRS broth using oxgall (Sigma Bovine Oxgall, Sigma-Aldrich, St. Louis, MO, USA). Controls for each test included MRS broth that was not acidified or adjusted for bile. The plates were incubated for 0, 1, 3, and 6 h at 30, 37 or 42 °C to provide optimal temperatures for specific strains. Tolerance of each LAB to acid or bile conditions was determined by measuring the optical density (OD) at 620 nm or 660 nm, respectively. The acid and bile tolerance of the LAB strains was categorized as follows: (a) excellent—increased growth at all pH levels or bile concentrations over a 6 h period, (b) very good—increased growth at two or more pH levels or bile concentrations over a 6 h period, (c) good—increased growth for all incubation periods at one pH level or bile concentration, and over a period of 3 h at two or more pH levels or bile concentrations, (d) poor—no increase in growth at any pH level or bile concentration over 3 h period. Each isolate was tested in duplicates and all experiments were repeated 3 times.

2.4. Antibiotic Resistance

The LAB strains ($n = 65$) were also screened for their susceptibility to antibiotics of importance in human health [15]. The antibiotic discs used were: 10 µg ampicillin, 2 µg clindamycin, 15 µg erythromycin, 10 µg gentamicin, 10 µg streptomycin, 30 µg tetracycline, and 30 µg vancomycin (Thermo Scientific, Waltham, MA, USA). A standard disk diffusion assay was used to determine the antibiotic susceptibility profile through a modified Kirby Baur method [8,16]. Overnight cultures of each LAB isolate were gently spread across MRS agar plates to create a lawn and were allowed to dry for 2 min. The antibiotic discs were aseptically placed equidistant from each other with 4 on one plate and 3 on another, and the plates pre-incubated for 1 h at 25 °C to ensure proper diffusion. Following pre-incubation, the plates were incubated anaerobically for 24–48 h at 37 °C. The antibiotic susceptibility of the LAB strains was determined by measuring the zone-diameters twice in a cross formation, using vernier calipers. The average of each zone was recorded as the antibiotic susceptibility zone. In accordance with the Clinical and Laboratory Standards Institute [15], strains showing zones > 20 mm were classified as sensitive (S), between 15 and 19 mm as intermediately sensitive (I), and zones ≤ 14 mm as resistant (R).

2.5. Biofilm Formation

The biofilm-forming capabilities of the LAB were evaluated as described previously [17], with some modifications. Overnight culture (20 µL) of each LAB isolate was aseptically transferred to 180 µL MRS broth in 96-well polystyrene plates (Thermo Scientific, Waltham, MA, USA). The plates were anaerobically incubated at 37 °C for 24 h, following which the liquid culture was carefully removed, using a micropipette, without disturbing the biofilm at the bottom of the well. The biofilms were then washed three times with phosphate-buffered saline (PBS; pH 7.0; Fisher Scientific, Fair Lawn, NJ, USA) to remove any planktonic cells. The plates were then dried at 37 °C for 15 min, and 180 µL of crystal violet (CV) added to the wells (2 min) to stain the biofilms. The CV was removed and the wells washed thrice with PBS and dried for 15 min at 37 °C. The stain was released by adding 180 µL of ethanol-acetone (80:20; vol/vol; Fisher, NJ, USA) mixture. The biofilms were quantified by measuring the optical density (A595) using a microplate reader (Spectro-Max M3; Molecular Devices LLC, San Jose, CA, USA). A blank was also prepared by adding 180 µL of ethanol–acetone in empty wells. The optical density (OD) of each well was measured at 595 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). Triplicate assays were performed. Uninoculated TSB was used as negative control. The cut-off (ODC) was defined as the mean OD value of the negative control [18]. Based on the OD, strains were classified as: non-biofilm producers ($OD \leq ODC$), weak ($ODC < OD \leq 2 \times ODC$), moderate ($2 \times ODC < OD \leq 4 \times ODC$) or strong biofilm producers ($4 \times ODC < OD$).

3. Results and Discussion

3.1. Viability of LAB Isolates Following Long-Term Storage

To ensure that LAB selected for use as a DFM could survive shipment and storage and therefore be viable when delivered to the animals, all the LAB isolates were evaluated for viability after frozen storage (−80 °C). Of the 250 LAB isolates, 95 had excellent or very good growth after 24–48 h. The remaining isolates had fair or good growth after 48–72 h and needed extra time to show growth after extended frozen storage. A population of approximately 1×10^9 CFU/mL was obtained for all the isolates after they were sub-cultured in MRS broth. All the isolates were Gram-positive rods or cocci.

3.2. Preliminary Screening of LAB for Inhibition of STEC and *S. Typhimurium*

To be successful as DFM in food animals, selected LAB must be screened for their inhibitory activity against foodborne pathogens. Lactic acid bacteria (n = 95) that showed excellent to very good growth were screened for inhibition towards STEC and *S. Typhimurium* DT104 using the agar spot test. Results are shown in Tables 1 and 2.

Table 1. Preliminary screening of *Lactobacillus acidophilus* (LA) strains for inhibition of STEC and *S. enterica* Typhimurium, using the agar spot test.

LA Strains	STEC		<i>Salmonella enterica</i> Typhimurium			
	* Inhibition Zones (mm)		* Inhibition Zones (mm)			
	O157:H7	Non-O157	H2662	152N17-1	11942A	13068A
LA-1	15.7	16.4	16.2	16.0	17.2	18.5
LA-223	13.7	11.2	9.6	9.9	9.0	9.6
LA-2R	15.9	15.3	16.5	15.3	16.3	16.2
LA-3811C28	12.1	11.4	12.9	10.6	12.9	12.5
LA-381-IL-25	12.2	12.8	14.6	12.6	11.9	11.8
LA-381-IL-27	13.4	16.6	19.4	16.1	13.3	14.1
LA-396-IL-28	14.9	19.1	2.8	4.7	5.4	1.9
LA-6-L4	12.5	11.6	11.8	9.4	17.3	19.3
LA-A-4	8.4	11.1	8.8	7.4	10.8	9.4
LA-B7	20.1	11.9	1.5	3.2	2.2	1.1
LA-C28	12.4	15.8	17.2	12.2	13.6	14.1
LA-D15	9.8	10.0	10.6	10.1	7.1	10.5
LA-D19	6.3	7.8	9.1	10.4	11.2	9.9
LA-DKW7	13.0	13.4	13.5	12.0	13.6	12.8
LA-G5	11.1	9.6	11.6	10.9	9.3	7.9
LA-HLA-1	14.8	14.4	15.4	14.3	15.6	15.4
LA-J17	11.1	12.2	3.4	4.1	2.4	2.3
LA-J18	2.6	3.2	5.1	7.3	7.3	13.8
LA-J19	15.0	12.9	4.1	5.3	3.9	2.8
LA-K10	14.5	14.3	14.5	14.6	16.5	17.4
LA-K14	8.1	7.9	5.5	6.2	4.3	2.6
LA-L-1	7.5	7.0	6.7	6.7	5.6	7.2
LA- L-23	6.6	7.1	6.8	7.8	9.4	10.2
LA-O2	4.0	3.9	4.5	5.9	5.5	8.8
LA-R-2	13.7	14.3	9.7	11.5	10.0	8.6
LA-T3	17.8	12.8	17.7	17.1	14.7	14.8

* Values are the average of three separated trials where inhibition was measured as the size of the clear zone (mm) around the colony at three separate locations on the agar plate.

Against *E. coli* O157:H7, 56% (51 isolates) met or exceeded the inhibition criteria, with inhibition zones of ≥ 10 mm, and 17% (17 isolates) produced inhibition zones of ≥ 5 mm. For inhibition against Non-O157:H7 STEC, 76% (52 isolates) met or exceeded the inhibition criteria, with inhibition zones of ≥ 10 mm, and 10% (6 isolates) produced inhibition zones of ≥ 5 mm. Inhibition of *S. Typhimurium* was tested against 4 separate strains (H2662, 152N17-1, 11942A, and 13068A) and 62–66% LAB isolates showed excellent to very good inhibition, while 22–27% produced inhibition zones of ≥ 5 mm. The observed degree of inhibition of the pathogens by the LAB isolates was not unexpected. Several studies have reported the antagonistic effects of LAB on various other pathogenic microorganisms in broth, agar, food, or live animals, where inhibition zones greater than 11 mm have been observed [14,19–21]. The mechanism of inhibition used by the LAB was not identified in this study. There are several mechanisms that LAB could use to cause in-vitro inhibition and may include production of organic acids, primarily lactic acid, and inhibitory compounds such as bacteriocins, diacetyl, acetaldehyde,

and hydrogen peroxide. Studies have reported lactic acid, produced by LAB, as the major organic compound in pathogen inhibition [22–24]. In the current study, the ability of LAB to inhibit *E. coli* and *S. enterica* Typhimurium could be primarily attributed to the production of lactic acid. It is known that organic acids are most effective at high levels and require large numbers of LAB for sufficient pathogen inhibition [25,26]. In the current study, LAB were grown to a population of approximately 1×10^8 CFU/mL for agar spot tests, which would have produced sufficient inhibitory compound for inhibition of the two pathogens. Previous studies have shown that pathogens do not survive well in matrices with large numbers (6–7 logs) of LAB and $\text{pH} \leq 4$ [25,26]. Infections by foodborne enteric and zoonotic pathogens cause high morbidity and mortality in the food industry. Antimicrobial activity against these pathogens is a major requirement for the potential LAB DFM [13]. The LAB isolates ($n = 30$) that showed the most inhibition (≥ 10 mm) against all STEC and *S. Typhimurium* isolates were selected for further screening of acid and bile tolerance.

Table 2. Preliminary screening of non-*Lactobacillus acidophilus*, *Leuconostoc* and *Streptococcus* strains for inhibition of STEC and *S. enterica* Typhimurium, using the agar spot test.

LAB Strains	STEC		<i>Salmonella enterica</i> Typhimurium			
	* Inhibition Zones (mm)		* Inhibition Zones (mm)			
	O157:H7	Non-O157	H2662	152N17-1	11942A	13068A
<i>L. animalis</i> 35046	15.0	13.7	18.6	19.4	15.2	16.5
<i>L. bulgaricus</i> 05-53	12.8	8.5	15.8	16.1	9.2	9.2
<i>L. bulgaricus</i> LB-18	5.6	6.4	8.3	7.8	7.9	6.4
<i>L. casei</i> A-12	15.5	16.5	14.8	17.0	19.6	19.2
<i>L. casei</i> C-15	17.7	15.3	19.0	15.4	18.9	16.6
<i>L. casei</i> E-14	17.1	15.0	17.3	14.9	16.2	15.8
<i>L. casei</i> E-17	14.4	13.8	16.2	17.4	16.4	16.5
<i>L. casei</i> E-18	15.7	15.6	16.9	16.6	16.8	15.4
<i>L. casei</i> E-20	13.4	15.0	15.4	15.9	16.0	16.3
<i>L. casei</i> GG	16.6	16.3	18.1	18.6	18.7	17.1
<i>L. casei</i> M-5	18.8	17.4	21.0	18.4	16.8	17.3
<i>L. casei</i> N-7	17.5	15.4	18.9	16.7	17.0	17.2
<i>L. casei</i> O1	14.4	15.1	18.5	19.0	16.4	15.0
<i>L. casei</i> P-13	15.5	14.7	19.7	15.6	16.8	20.2
<i>L. casei</i> P-15	14.7	16.1	16.1	20.2	15.6	16.7
<i>L. cellobiosus</i> L-4	13.7	11.6	17.2	13.2	13.0	13.7
<i>L. fermentum</i> L-9/LEC	11.3	10.5	12.9	11.1	10.8	13.5
<i>L. lactis</i> RM2-5	1.3	3.6	4.8	4.1	1.1	3.3
<i>L. lactis</i> T4-1	6.7	4.0	11.1	7.7	10.8	6.8
<i>L. leichmannii</i> 4797	2.2	3.5	3.8	2.6	2.5	2.6
<i>L. plantarum/casei</i> E-16	14.3	15.1	19.6	12.7	13.4	14.6
<i>L. reuteri</i> 23272	18.1	10.9	14.6	13.3	9.7	14.4
<i>L. reuteri</i> X-18	11.5	14.6	17.6	15.4	13.4	18.7
<i>L. salivarius</i> 459	18.4	17.2	18.5	16	19.1	20.7
<i>L. salivarius</i> PIG 3-1	9.0	12.1	9.5	9.4	17.0	10.0
<i>L. salivarius</i> PIG3-3	12.7	12.9	15.2	14.8	14.7	14.3
<i>Leuc. citreum</i> CAE	4.40	4.5	8.0	7.9	5.6	5.8
<i>S. thermophilus</i> 1	12.80	13.9	12.3	12.9	13.2	10.9
<i>S. thermophilus</i> OSU-1	13.80	14.2	6.0	4.4	3.7	2.2
<i>S. thermophilus</i> S-1	11.40	11.6	7.5	8.6	4.8	2.2
<i>S. thermophilus</i> S-2	19.20	20.5	8.4	8.0	7.1	4.9
<i>S. thermophilus</i> SAN3-2	2.80	2.6	7.4	7.0	6.8	4.6
<i>S. cremoris</i> 290-P	1.10	0.8	8.3	6.7	5.1	4.7

* Values are the average of three separated trials where inhibition was measured as the size of the clear zone (mm) around the colony at three separate locations on the agar plate.

3.3. Acid and Bile Tolerance

Based on their strong growth after extended frozen storage and inhibition against STEC, and *S. Typhimurium* DT104, 65 isolates were further screened for acid and bile tolerance. All the isolates were tolerant to acid and bile conditions with 74% and 79% showing excellent to very good and 19% and 25% showing good acid and bile tolerance, respectively. There was no incidence of isolates not growing during the 6 h incubation period (Table 3). Tolerance to acid and bile conditions was examined to understand the possible survival of the microorganisms in the GI tract of food animals. The microorganisms used as DFM are generally delivered to the animals orally through feed or water and therefore should have the ability to tolerate the intestinal tract environment, including acid and bile secretion. Depending on the type of feed, animal intestinal pH, including the rumen pH of ruminant animals, can range from 4.0 to 6.5. Diets including large amounts of starch and soluble carbohydrates could result in lower pH values than those high in cellulose and other complex carbohydrates [12,27]. Bacteria can be subjected to cellular stress arising from low pH values in the rumen and bile secretion in the intestinal tract. The LAB isolates in the current study showed excellent to very good tolerance to the various bile concentrations. Similar results have been obtained in other studies conducted on the survival of LAB in acid and bile conditions [14,28]. Based on the results of acid and bile tolerance tests, these isolates show promise in their use as a DFM in live animals because of their ability to survive potential intestinal conditions. The isolates of LAB that showed excellent and very good tolerance to both acid and bile ($n = 35$) were selected for further evaluation of their biofilm-forming capabilities. The isolates that did not show good tolerance to both acid and bile conditions were not considered good candidates for DFM. Results are presented in Tables 3 and 4.

Table 3. Acid (pH 2, 4, 5) and bile (0.1, 0.3, 0.5%) tolerance of *L. acidophilus* (LA) strains, determined by growth at 0, 1, 3 and 6 h.

LAB Strains	Acid ^a OD (620 nm)	Bile ^b OD (660 nm)	LAB Strains	Acid ^a OD (620 nm)	Bile ^b OD (660 nm)
LA-1	VG	EXC	LA-G5	VG	EXC
LA-223	G	EXC	LA-GP2A	EXC	G
LA-2R	VG	EXC	LA-HLA-1	VG	EXC
LA-3811C28	VG	EXC	LA-J17	G	G
LA-381-IL-25	VG	G	LA-J18	VG	VG
LA-381-IL-27	VG	VG	LA-J19	G	G
LA-396-IL-28	EXC	G	LA-K10	EXC	EXC
LA-6-L4	G	G	LA-K14	VG	EXC
LA-A-4	VG	VG	LA-L-1	EXC	EXC
LA-B7	P	VG	LA-L-23	EXC	VG
LA-C-2	VG	VG	LA-O17	G	G
LA-C28	EXC	EXC	LA-O2	VG	EXC
LA-CL3	VG	G	LA-R-2	P	EXC
LA-D15	EXC	EXC	LA-RAT-1	EXC	G
LA-D19	VG	VG	LA-T-3	VG	G
LA-DKW7	VG	EXC			

Growth of each isolate was measured as the Optical Density (OD): ^a OD = 620 nm for acid tolerance and ^b OD = 660 nm for bile tolerance. The acid and bile tolerance of the LAB strains was categorized as follows: EXC: Excellent—increased growth at all pH levels or bile concentrations over a 6 h period; VG: Very good—increased growth at two or more pH levels or bile concentrations over a 6 h period; G: Good—increased growth for all incubation periods at one pH level or bile concentration, and over a period of 3 h at two or more pH levels or bile concentrations; and P: Poor—no increase in growth at any pH level or bile concentration over 3 h period. Each isolate was tested in duplicates and all experiments were repeated 3 times.

Table 4. Acid (pH 2, 4, 5) and bile (0.1, 0.3, 0.5%) tolerance of non-acidophilus lactic acid bacteria strains of the genus *Lactobacillus sensu lato* (L.), *Leuconostoc* (Leuc.), and *Streptococcus* (S.), determined by growth at 0, 1, 3 and 6 h.

LAB Strains	Acid ^a OD (620 nm)	Bile ^b OD (660 nm)	LAB Strains	Acid ^a OD (620 nm)	Bile ^b OD (660 nm)
<i>L. animalis</i> 35046	VG	EXC	<i>L. lactis</i> RM2-5	EXC	VG
<i>L. bulgaricus</i> 05-53	VG	VG	<i>L. lactis</i> T4-1	VG	G
<i>L. bulgaricus</i> LB-18	VG	VG	<i>L. leichmannii</i> 4797	G	G
<i>L. casei</i> A-12	VG	EXC	<i>L. plantarum/casei</i> E-16	G	VG
<i>L. casei</i> C-15	VG	EXC	<i>L. reuteri</i> 23272	G	EXC
<i>L. casei</i> E-14	VG	EXC	<i>L. reuteri</i> X-18	VG	EXC
<i>L. casei</i> E-17	VG	EXC	<i>L. salivarius</i> 459	VG	EXC
<i>L. casei</i> E-18	VG	EXC	<i>L. salivarius</i> PIG3-1	G	EXC
<i>L. casei</i> E-20	EXC	EXC	<i>L. salivarius</i> PIG3-3	EXC	EXC
<i>L. casei</i> GG	EXC	EXC	<i>Leuc. citreum</i> CAE	EXC	VG
<i>L. casei</i> M-5	VG	EXC	<i>S. cremoris</i> 290-P	EXC	VG
<i>L. casei</i> N-7	VG	EXC	<i>S. thermophilus</i> 1	G	G
<i>L. casei</i> O1	VG	EXC	<i>S. thermophilus</i> OSU-1	F	G
<i>L. casei</i> P-13	VG	EXC	<i>S. thermophilus</i> S-1	G	G
<i>L. casei</i> P-15	VG	EXC	<i>S. thermophilus</i> S-2	G	G
<i>L. cellobiosus</i> L-4	VG	EXC	<i>S. thermophilus</i> SAN3-2	F	F
<i>L. fermentum</i> L-9/LEC	EXC	EXC			

Growth of each isolate was measured as the Optical Density (OD): ^a OD = 620 nm for acid tolerance and ^b OD = 660 nm for bile tolerance. The acid and bile tolerance of the LAB strains was categorized as follows: EXC: Excellent—increased growth at all pH levels or bile concentrations over a 6 h period; VG: Very good—increased growth at two or more pH levels or bile concentrations over a 6 h period; G: Good—increased growth for all incubation periods at one pH level or bile concentration, and over a period of 3 h at two or more pH levels or bile concentrations; and F: Fair—increase in growth at only one pH level over a 6 h period or at two pH levels over a 3 h period. Each isolate was tested in duplicates and all experiments were repeated 3 times.

3.4. Antibiotic Resistance

Selected isolates were tested for resistance or susceptibility to seven antibiotics (ampicillin, clindamycin, erythromycin, gentamicin, streptomycin, tetracycline, and vancomycin). In addition to being used in humans, these antibiotics are also used in food-producing animals. Results are shown in Tables 5 and 6. Of the 59 isolates tested, one isolate (*S. thermophilus* S-2) showed susceptibility to all the antibiotics. Majority of the isolates were susceptible to erythromycin (88%), ampicillin (85%), clindamycin (64%), and tetracycline (58%). Fifty-five (93%) isolates showed resistance to more than one antibiotic, particularly gentamicin, streptomycin, and vancomycin. Only three isolates were intermediately sensitive to gentamicin. Isolates that showed intermittent sensitivity were included in the calculation of sensitivity. Resistance to vancomycin is of concern since it is primarily used to treat serious bacterial infections, particularly those caused by Gram-positive bacteria, including MRSA (methicillin-resistant *Staphylococcus aureus*). On the other hand, gentamycin and streptomycin are aminoglycoside antibiotics used to treat infections of Gram-negative bacteria. Antibiotic resistance of microorganisms used as probiotics or DFM is an area of growing concern. It is suggested that antimicrobial agents used for food-producing animals can promote the emergence of resistance in these bacteria that can lead to the transfer of resistance to other pathogenic bacteria through the exchange of genetic material [29]. However, in the current study, isolates from the genus *Lactobacillus* were primarily evaluated for antibiotic resistance, which are known to demonstrate a large range of antibiotic resistance naturally but are not transmissible and do not form a safety concern [30]. The resistance to vancomycin by lactobacilli has also been described as intrinsic in several studies [31–33]. With some exceptions, when mutations or intrinsic resistance mechanisms are responsible for the resistance phenotype, antibiotic resistance in beneficial microorganisms does not

necessarily constitute a safety concern. Several probiotic strains with intrinsic resistance could be useful for restoring the GI tract microbiota after antibiotic treatment [33]. Therefore, isolates that had a high resistance profile were given lower priority for consideration as a DFM, but were not eliminated based on resistance. Since isolate *S. thermophilus* S-2 was not resistant to any of the antibiotics, it would be considered safer for use in live animals, and its lack of resistance also indicates that it may not contribute to the transfer of resistance to other microorganisms.

Table 5. Antibiotic susceptibility profile of *L. acidophilus* strains using disk-diffusion assay.

LAB Strains	Amp	Clin	Eryth	Gen	Strep	Tet	Van
LA-223	S	R	S	R	I	S	S
LA-1	S	S	S	R	R	R	R
LA-2R	S	S	S	R	R	R	R
LA-3811C28	S	S	S	R	R	S	R
LA-381-IL-25	S	S	S	R	R	R	R
LA-381-IL-27	I	S	S	I	R	S	R
LA-396-IL-28	S	S	S	R	R	I	S
LA-6-L4	S	S	S	R	R	S	S
LA-A-4	R	I	S	I	I	R	S
LA-B7	S	R	R	R	R	S	R
LA-C28	S	S	S	R	R	S	R
LA-D15	S	S	S	R	R	S	R
LA-D19	S	R	S	R	I	R	S
LA-DKW7	S	S	S	R	R	I	R
LA-G5	S	R	S	R	R	S	R
LA-HLA-1	S	R	S	R	R	R	R
LA-J17	S	R	S	R	R	S	I
LA-J18	S	R	S	R	R	S	S
LA-J19	S	R	S	R	R	S	I
LA-K10	S	I	S	R	R	I	S
LA-K14	S	R	S	R	R	S	I
LA-L-1	S	R	R	S	R	R	S
LA-L-23	S	S	R	R	R	R	S
LA-O2	S	S	R	R	I	S	I
LA-R-2	S	R	S	R	R	S	I
LA-T3	I	S	S	R	R	R	R

Amp: Ampicillin; Clin: Clindamycin; Eryth: Erythromycin; Gen: Gentamycin; Strep: Streptomycin; Tet: Tetracycline; Van: Vancomycin. Antibiotic susceptibility was determined by measuring the zone-diameters (mm) twice in a cross formation. Average of each zone was recorded as the antibiotic susceptibility zone. Strains showing zones > 20 mm were classified as sensitive (S), between 15 and 19 mm as intermediately sensitive (I), and zones ≤ 14 mm as resistant (R).

Table 6. Antibiotic susceptibility profile of non-acidophilus LAB strains of the genus *Lactobacillus sensu lato* (*L.*), *Leuconostoc* (*Leuc.*), and *Streptococcus* (*S.*).

LAB Strains	Amp	Clin	Eryth	Gen	Strep	Tet	Van
<i>L. animalis</i> 35046	S	S	S	R	R	S	R
<i>L. bulgaricus</i> 05-53	I	I	S	R	R	R	I
<i>L. bulgaricus</i> LB-18	S	S	S	R	R	S	S
<i>L. casei</i> A-12	I	S	S	R	R	S	R
<i>L. casei</i> C-15	S	S	S	R	R	I	I
<i>L. casei</i> E-14	S	S	S	R	R	S	R

Table 6. Cont.

LAB Strains	Amp	Clin	Eryth	Gen	Strep	Tet	Van
<i>L. casei</i> E-17	S	S	S	R	R	I	R
<i>L. casei</i> E-18	S	S	S	R	R	S	R
<i>L. casei</i> E-20	S	S	S	R	R	I	R
<i>L. casei</i> GG	S	I	S	R	R	S	R
<i>L. casei</i> M-5	I	I	S	R	R	S	R
<i>L. casei</i> N-7	S	I	S	R	R	S	R
<i>L. casei</i> O1	S	R	S	R	R	S	R
<i>L. casei</i> P-13	S	S	S	R	R	I	R
<i>L. casei</i> P-15	S	S	S	R	I	I	R
<i>L. cellobiosus</i> L-4	S	I	S	R	R	R	S
<i>L. fermentum</i> L-9/LEC	I	R	R	R	R	S	R
<i>L. lactis</i> RM2-5	I	S	I	R	R	S	I
<i>L. lactis</i> T4-1	S	S	S	R	R	S	S
<i>L. leichmannii</i> 4797	R	R	S	R	R	S	S
<i>L. plantarum/casei</i> E-16	S	S	S	R	R	I	R
<i>L. reuteri</i> 23272	S	S	S	R	R	I	R
<i>L. reuteri</i> X-18	S	S	S	R	R	I	R
<i>L. salivarius</i> 459	S	S	S	R	R	I	R
<i>L. salivarius</i> PIG 3-1	S	S	R	R	R	R	R
<i>L. salivarius</i> PIG3-3	S	S	S	R	R	R	R
<i>Leuc. citreum</i> CAE	S	S	S	I	S	S	R
<i>S. cremoris</i> 290-P	S	S	S	R	R	S	R
<i>S. thermophilus</i> 1	S	S	S	R	R	S	I
<i>S. thermophilus</i> OSU-1	S	S	S	R	I	S	S
<i>S. thermophilus</i> S-1	S	S	S	R	R	S	S
<i>S. thermophilus</i> S-2	S	S	S	I	I	S	S
<i>S. thermophilus</i> SAN3-2	S	S	S	R	I	S	S

Amp: Ampicillin; Clin: Clindamycin; Eryth: Erythromycin; Gen: Gentamycin; Strep: Streptomycin; Tet: Tetracycline; Van: Vancomycin. Antibiotic susceptibility was determined by measuring the zone-diameters (mm) twice in a cross formation. Average of each zone was recorded as the antibiotic susceptibility zone. Strains showing zones > 20 mm were classified as sensitive (S), between 15–19 mm as intermediately sensitive (I), and zones ≤ 14 mm as resistant (R).

3.5. Biofilm-Forming Capabilities

LAB isolates (n = 36) with excellent or very good acid and bile tolerance were evaluated for their biofilm-forming capabilities. Biofilms formed by LAB have been reported as an effective barrier against the development of spoilage and/or pathogenic microorganisms and their biofilms [34]. The CV staining of the LAB cells within the biofilm matrix can be used as an indirect method to determine the amount of bacterial attachment and further determined with the use of a spectrophotometer [35]. The intensity of the crystal violet stain was correlated to the level of attachment or biofilm formation on the polystyrene wells. Tested LAB isolates were able to form biofilms after 24 h. Of the 36 isolates, 30 (83%) were strong biofilm producers (OD range: 2.476–3.899), 4 were moderate (OD range: 1.652–2.423) and 2 were weak biofilm producers (OD range: 0.815–1.214). In the current study, all the LAB isolates were biofilm producers, which has also been observed in previous studies [36,37]. The formation of the exopolysaccharides (EPS) in biofilms has been shown to enhance the colonization of LAB in the GI tract, thereby benefiting the host [38]. The biofilm formation capabilities are shown in Figure 2.

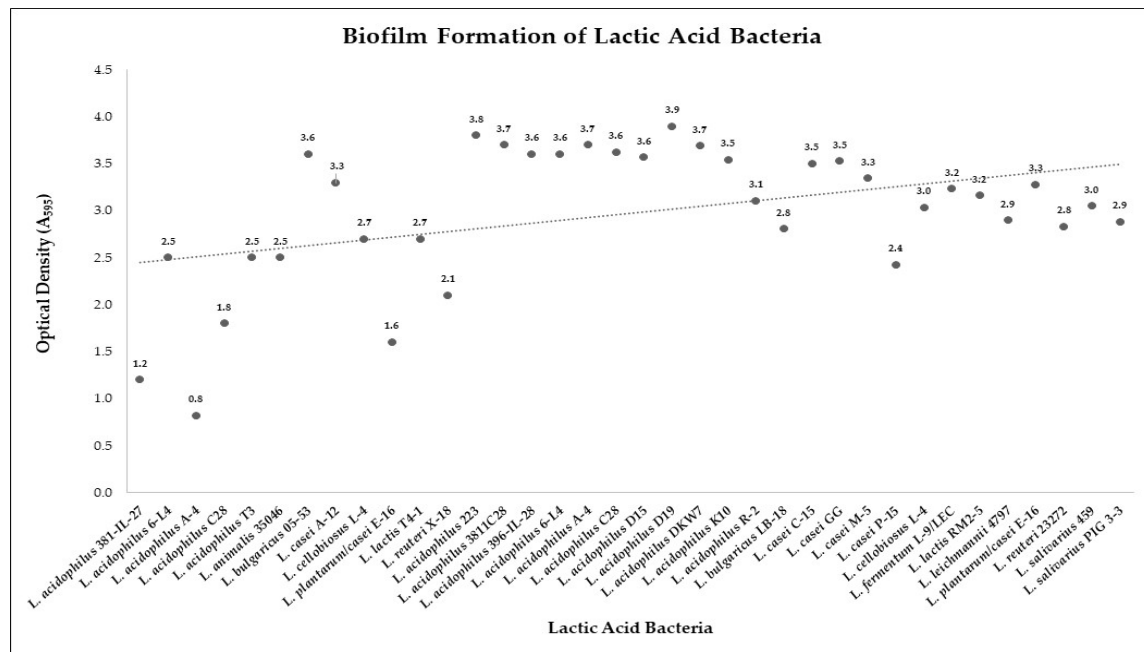


Figure 2. Biofilm-forming capability of LAB over 24 h period. Biofilms were quantified by measuring optical density (OD = A₅₉₅) in triplicate assays. Values are shown as the mean of three assays. The cut-off (ODC) was defined as the mean OD value of the negative control. Strains were classified as: non-biofilm producers (OD ≤ ODC), weak (ODC < OD ≤ 2 × ODC), moderate (2 × ODC < OD ≤ 4 × ODC) or strong biofilm producers (4 × ODC < OD).

4. Conclusions

Since food animals have been identified as a reservoir for common foodborne pathogens such as STEC and *Salmonella enterica*, it is important to focus on control strategies within the animals. The approach of DFM seems to have potential in this regard and LAB could prove to be good candidates. Although the mechanism(s) of action involved in the inhibition of the STEC and *Salmonella* were not identified in the present study, it is speculated that factors such as the production of organic acid and other antimicrobial compounds by the LAB could be responsible. The LAB isolates in this study show promise for selection as a DFM due to their survival in frozen storage conditions, tolerance to acid and bile, susceptibility to common antibiotics, strong biofilm-forming and pathogen inhibition capabilities. Their use as DFM in food animals holds potential for the reduction in STEC and *Salmonella*.

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