Isolation and characterisation of *Lactobacillus kimchicus* for use as a potential probiotic.

Abstract

This study aimed to screen for *Lactobacillus* species with probiotic potential for birds. *Lactobacillus*-specific media (LBS) was used to isolate *L. kimchicus* 5.1 from faecal droppings of the great tit bird (*Parus major)*. This isolate was identified using phenotypic testing and 16S rRNA sequencing. A potential probiotic needs to survive the adverse conditions of the GIT, therefore, *in vitro* tests that simulated the GIT were conducted. *L. rhamnosus* GG was used as a reference strain. The characterisation tests included bile salt and acidity tolerance testing, antimicrobial agent production, and pathogen inhibition, amongst others. The study found that this organism is a homofermentative LAB that produces D- lactic acid. Very slight inhibitory activity was observed against *S. simulans* and *E. coli*, using spot and well diffusion assays. After 5 hrs of exposure to bile salts (0.5%), 95.2% of *L. kimchicus* 5.1 cells survived. The lowest pH that this strain could tolerate was 2.5 since cell viability at this pH was maintained at 10^7 CFU/ml for 6 hrs. The susceptibility of *L. kimchicus* 5.1 to various antibiotics was tested according to EFSA guidelines. *L. kimchicus* 5.1 was sensitive to ampicillin, chloramphenicol, erythromycin, clindamycin, tetracycline, gentamycin, kanamycin, and vancomycin but was found to be slightly resistant to streptomycin. The complete genome sequence of *L. kimchicus* 5.1 consists of 2,535,859 bp with no plasmids or transposable elements. The majority of the genes identified were associated with carbohydrate metabolism (19.6%), amino acid (18.7%), and protein metabolism (14.3%). This study contributes to the body of knowledge about *Lactobacillus* and helps improve understanding of novel *Lactobacillus* isolated from bird faeces, which may have potential probiotics characteristics to enhance avian and animal health and nutrition.

Materials and Methods

Isolation of *Lactobacillus*

Bird droppings were obtained from Duke Wood, from a nestling of the great tit (*Parus major*) and were inoculated into BD Difco *Lactobacillus* broth de Man, Rogosa, and Sharpe (MRS; Difco Laboratories, Detroit, MI). This was incubated anaerobically at 37°C overnight and serially diluted using phosphate-buffered saline (PBS), then spread onto *Lactobacillus* Selective (LBS) agar (Difco Laboratories, Detroit, MI) plates and incubated under different conditions, i.e., anaerobically at 30°C and 37°C, and aerobically at 30°C and 37°C for two days. Colonies with different morphologies were streaked and re-streaked on LBS to obtain pure cultures. The pure LAB cultures were subsequently kept in LBS broth supplemented with 35% (v/v) glycerol and frozen at −80°C until further analysis.

Identification of putative *Lactobacillus* isolates

Preliminary identification consisted of phenotypic methods where the colony characteristics in terms of colour, size, shape, and texture were recorded after examining 1-2 day old cultures on LBS agar. Subsequently, catalase testing was performed by placing a drop of 3% (v/v) hydrogen peroxide solution on a pure, single colony and examined on a glass slide. Immediate gas formation (bubbles) was considered a positive result. Microscopic analysis was carried out on 2-day-old pure colonies that were Gram-stained and viewed under a 1000× magnification on an Olympus light microscope. Only the Gram-positive, catalase-negative, rod-shaped bacteria were taken forward for analysis. For comparison, *Lactobacillus rhamnosus* GG (APC 2740) was obtained from the APC/ DPC culture collection (Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland) and treated similarly to the isolate.

Genomic DNA extraction for 16S rRNA sequencing

Genomic DNA was extracted (strains grown anaerobically overnight in 10 ml MRS broth at 37°C) using the GenElute bacterial genomic DNA kit (Sigma). The extracted DNA was quantified using the Qubit 2.0 fluorometer (ThermoFisher Scientific, 168 Third Avenue Waltham, MA, USA 02451). Polymerase Chain Reaction (PCR) was performed using Biomix red (Meridian Bioscience, Dublin, Ireland). For amplification of the 16S rRNA gene, universal primers B27F (5-AGAGTTTGATCCTGGCTCAG-3) and U1492R (5-GGTTACCTTGTTACGACTT-3) (Sigma-Aldrich) were used with the following run conditions: initial denaturation: 94°C × 5 mins, cycling conditions: 30 cycles of 94°C × 40 s,

55°C × 30 s, 72°C × 1 min, final extension: 72°C × 10 mins. PCR products were purified using the GenElute PCR Clean-Up Kit (Sigma) and sent for sequencing to Genewiz (Hope End, Takeley, Essex, CM22 6TA, United Kingdom). The resulting sequences were compared to existing genomic data using the Basic local alignment search tool (BLAST) on the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/). The identity of the isolates was determined based on the highest scores (≥ 98%).

Whole Genome sequencing (WGS)

To prepare for WGS, *L. kimchicus* 5.1 was streaked on LBS plates from frozen stocks and incubated anaerobically at 37°C overnight. A single colony was suspended in 200 µl of sterile PBS buffer. From this, 100 µl was used to inoculate LBS broth, while the remaining culture suspension was streaked out again to ascertain the purity of the culture. The broth with culture was incubated overnight and harvested at the exponential phase as indicated by absorbance of 0.5 at OD_{600}. Cells were harvested by centrifugation at 4000 × g for 10 mins at 4°C. The pellet was washed with 1 ml sterile PBS and centrifuged again at 12000 × g for 3 mins. Finally, the concentrated pellet was suspended in 0.5 ml of 1× DNA/RNA shield buffer and sent for sequencing at MicrobesNG (Microbes NG, 97 Vincent Drive, Birmingham, United Kingdom).

Production of antimicrobial compounds

Screening for bacteriocins

The isolated colonies were overlaid with an indicator organism to check for the production of antimicrobial agents using spot assays. The indicator culture had been grown anaerobically at 37°C overnight. Briefly, the isolate was grown overnight in MRS broth. Thereafter, 10 µl was spotted onto LBS agar, incubated overnight, and overlaid with 0.75% sloppy MRS agar, seeded with the appropriate indicator. The indicator that the isolate was tested against was *Lactobacillus bulgaricus delbrueckii* ssp. *bulgaricus* LMG 6901, which is an acid-resistant strain.

Antipathogen spectra

Seven bacterial strains were used to investigate the antagonistic activity of *L. kimchicus* 5.1 against pathogens, using spot assays as detailed above. The chosen pathogens were based on immediate availability in the lab. These strains were *Staphylococcus epidermis, Escherichia coli* (K12), *Staphylococcus simulans*, *Streptococcus lutetiensis*, *Enterobacter cloacae*,

Enterococcus faecium, and *Streptococcus agalactiae*. The growth conditions of the various pathogens are listed in Table 2.1. Cell-free supernatant (CFS) from *L. kimchicus* 5.1 was obtained through centrifugation (Sorvall Legend RT) at 4000 × g for 20 mins at 4°C, repeated twice. To eliminate inhibition from acid production, the CFS was neutralised to a pH of 6.9 – 7.2 using 1 M Sodium Hydroxide (NaOH). Tempered sloppy media (0.5% agar) was seeded with 0.25% (v/v) of the appropriate indicator pathogen inoculum. Wells (7 mm) were bored into the sloppy agar plates and 50µl of the neutralised and filter sterilised supernatant was added to the wells, and the plates incubated accordingly. A zone of inhibition was suggestive of antimicrobial activity against that particular pathogen.

Table 2.1: Growth conditions for the different target organisms

Target organism	**Media**	**Growth conditions**
S. epidermis	Brain Heart Infusion (BHI)	Aerobic 37°C
E. coli	Luria Broth (LB)	Aerobic 37°C
S. simulans	BHI	Aerobic 37°C
S. lutetiensis	Tryptic Soy Agar (TSA)	Aerobic 37°C
E. cloacae	LB	Anaerobic 37°C
E. faecium	McConkey broth	Anaerobic 37°C
S. agalactiae	TSA	Aerobic 37°C

Fermentation characteristics

A standard growth curve was generated with measurements based on optical density (OD_{600}). Overnight culture (1 ml) was inoculated into 9 ml LBS and incubated anaerobically at 37°C. OD_{600} was measured over time intervals for 48 hrs, and absorbance was recorded in triplicate. This was also done for the reference strain *L. rhamnosus* GG. Growth and acid production were determined using a homofermentative-heterofermentative differential (HHD) agar medium, according to McDonald et al. (1987). The concentration of reagents required for the formulation of HHD agar plates is detailed in Table 2.2. The bromocresol green stock solution was prepared by dissolving 0.1 g bromocresol green powder in a 30 ml solution of 0.01N

NaOH. When all the reagents in Table 2.2 had been combined, the solution was adjusted to pH 7, using NaOH (0.01N), and autoclaved (121°C, 15 mins). The inoculum for the assay on HHD media was prepared by growing bacterial cells in LBS under the appropriate conditions until they reached the early stationary phase. The cells were harvested by centrifugation and washed twice with sterile molecular grade water. The cells were then washed in PBS, streaked onto the HHD agar plates, and incubated at 37°C under anaerobic and aerobic conditions. The cultures were also inoculated into liquid HHD media. For comparison, a known heterofermentative LAB (*L. rhamnosus* GG) and a known homofermentative LAB (*Lactococcus lactis*) were used because they produce colonies with distinct morphologies.

Table 2.2: Composition of HHD media for the differentiation of homofermentative and heterofermentative LAB.

Component	**Amount per litre**	**Supplier**
Trypticase peptone	10 g	Becton, Dublin, Ireland
Casamino acids	3 g	Merck, Darmstadt, Germany
Fructose	2.5 g	Sigma Aldrich, Wicklow, Ireland
Phytone peptone	1.5 g	Thermo Fisher Scientific, Dublin, Ireland
KH_2PO4	2.5 g	Sigma Aldrich, Wicklow, Ireland
Tween 80	1 ml	Sigma Aldrich, Wicklow, Ireland
Yeast extract	1 g	VWR International Limited, Dublin, Ireland
Bromocresol green	20 ml	Sigma Aldrich, Wicklow, Ireland
Agar	20 g	Sigma Aldrich, Wicklow, Ireland

Lactic acid configuration

The configuration and relative amounts of L (+) and D (-) lactic acid isomers produced and released into broth by the isolate were determined using the D-lactic and L-lactic acid assay kit (Megazyme inc., Bray, Ireland), following the protocol as outlined by the supplier. Two-day-old cultures (1 ml) were centrifuged to remove the pellet at 14,000 × g for 5 mins. Thereafter, the assay procedure was performed according to the manufacturer's instructions with a 2.24 ml

reaction volume. Absorbance was read at 340 nm using a spectrophotometer. The standard in the kit was D/L Lactic acid supplied at a concentration of 0.15 mg/ml. LBS media (broth) was used as the control while molecular grade water was the blank.

Characterisation of *L. kimchicus* 5.1 as probiotic

Antibiotic susceptibility

The minimum inhibitory concentration (MIC) was determined using Epsilometer-test (E-test) strips (bioMérieux; Marcy-l'Étoile, France) of ampicillin, streptomycin, chloramphenicol, erythromycin, clindamycin, tetracycline, gentamycin, kanamycin, and vancomycin (0.016 to 256 µg/ml). For MIC assays, the isolate *L. kimchicus* 5.1 and the reference strain *L. rhamnosus* GG (APC 2740) were grown overnight to densities corresponding to a McFarland standard of 0.5 or a spectrophotometric equivalent (3×10^8 CFU/ml). *Lactobacillus* sensitivity media (de Jong et al.), supplemented with L-cysteine-HCl, was prepared according to Klare et al. (2006) by combining Iso-Sensitest broth (90%), MRS broth (10%), agar (15 g/l), and L-cysteine–HCl (0.3 g/l). LSM plates (approximately 4 mm thick) were swabbed with culture, rotating at 60° angles to ensure even distribution of the inoculum, using a sterile swab. Excess moisture on the surface of the plate was allowed to be absorbed for approximately 15 mins. Thereafter, the E-test strips were applied, MIC scale facing upward. The plates were incubated anaerobically at 37°C for 48 hrs. The MIC was defined by the intersection of the growth eclipsing the margin of the E-test strip.

Stress tolerance

Exopolysaccharide (EPS) production

The loop touch test (Ruas-Madiedo and de los Reyes-Gavila´n, 2005) was used to determine if *L. kimchicus* 5.1 could produce exopolysaccharides (EPS) by spotting 5µl of overnight culture on MRS agar plates supplemented with cysteine-HCl ((0.05%) w/v) and glucose ((5%) w/v). EPS production was also tested in MRS broth. Agar plates and broths were incubated anaerobically (anaerobic jars with Anaerocult® A gas packs (Merck, Darmstadt, Germany) for three days at 37°C. In this case, known EPS producer *Lactobacillus mucosae* (DPC 6426) (London et al., 2014) was used as a positive control.

Heat tolerance

Thermotolerance of the isolate was determined by incubating 1 ml aliquots of the overnight culture in different temperatures, i.e., 37, 40, 45, 50, and 60°C for 30 mins. After thermal treatment, surviving microorganisms were serially diluted tenfold using PBS and subsequently plated out and incubated anaerobically at 37°C for two days.

Acid tolerance

Tolerance to low pH was assessed as described by Kobierecka et al. (2017), with minor modifications. Briefly, 1 ml of overnight culture, diluted to OD_{600} of 1.0, was inoculated into 9 ml of *Lactobacillus* broth (Difco MRS) that had been pH adjusted with 1M HCl, which was filter sterilised using a 200 µM membrane filter. The pH conditions tested were pH 2, 2.5, 3, 4, 5, 6 and untreated control broth at 6.67. Enumerations were performed every 2 hrs until 6 hrs. Cultures were serially diluted tenfold at each enumeration point, plated on LBS agar, and incubated under the appropriate conditions. Plating was performed in duplicates.

Bile salt tolerance

The bile tolerance assay was carried out according to Shokryazdan et al. (2014), with modifications. Resistance to bile salts was assessed in triplicates in terms of viable colony counts and enumerated after anaerobic incubation at 37°C at 0 and 5 hrs, in 0.3% and 0.5% Oxgall (Sigma Aldrich, Wicklow, Ireland). The control was MRS broth without bile. Tenfold serial dilutions were prepared using PBS, then spread plated on LBS agar plates without bile. The control samples were incubated in MRS broth without adding bile for 0 and 5 hrs, then plated. Bile tolerance was calculated by comparing viable cell counts of the cells incubated in broth with bile salts and those without bile salt.

Deconjugation of bile salts

Bile salt hydrolase activity testing was based on the method of Ahn et al. (2003). Difco *Lactobacillus* MRS (55 g/l) was supplemented with L-cysteine-HCl (0.5 g/l) and calcium chloride (0.37 g/l). This was mixed well while heating and boiled for a min to allow reagents to get into the solution. Bacteriological agar (12.5 g/l) was then added. Thereafter, it was autoclaved for 15 mins at 121°C. A membrane filter (0.20 µM) was used to sterilise conjugated bile salts (taurodeoxycholic acid (TDCA) and taurocholic acid (TCA). To prepare the BSH assay plates, 0.25 g of each salt was dissolved in 2 ml of deionised water. Thereafter, 2 ml was added per 40 ml of tampered agar to achieve a final concentration of 0.3%. The plates with no

bile served as control plates. When plates were dry, 10 µl of culture was spotted onto the plate and left to dry. Subsequent cultivation of the bacterial strains was carried out at 37°C for 72 hrs under an anaerobic atmosphere. A visible halo around the colony was used to indicate positive BSH activity of the strains. Lack of a halo was considered a negative result for BSH activity.

Viability as a freeze-dried powder

L. kimchicus 5.1 was grown from frozen stock in Difco MRS media under the appropriate cultivation conditions. It was subcultured 2-3 times before the freeze-drying process. The culture was routinely streaked out to check for purity. Using a 1% (v/v) inoculation, *L. kimchicus* 5.1 was grown in 800 ml of modified MRS broth (Difco) supplemented with 0.05% L-cysteine HCl (Sigma) and incubated anaerobically at 37°C for 24 hrs. The culture was centrifuged (Sorvall Lynx 600 centrifuge) at 4000 × g for 20 mins at 4°C, with slow deceleration. The supernatant was removed by pouring it off gently, ensuring that the pellet stayed intact. The remaining pellet was washed by resuspending it in PBS and then centrifuged using the same conditions stated above. The washing step was repeated once again. The bacterial cells were concentrated (×20) by finally suspending the pellet in 10% (w/v) trehalose (Sigma Aldrich, Wicklow, Ireland). The suspension (1 ml) was transferred to FD vials, placing the cap loosely. These were loaded to a decontaminated (with 70% ethanol) freeze-dryer and allowed to freeze-dry for ~24 hrs. Thereafter, the dry weight of the freeze-dried bacterial powder was measured. Subsequently, the powder was suspended in PBS, serially diluted, and plated onto LBS plates to determine the viable cells (CFU/g) of *L. kimchicus* 5.1 post-freeze-drying. Viable cell counts were determined before freezing and after freeze-drying.

To determine if freeze-dried *L. kimchicus* 5.1 could maintain viability long-term, a 6-week stability trial was performed under different conditions, i.e., at room temperature, at 4°C and -20°C. Approximately 50 g of powder from one freeze-drying batch was split into three and stored under the above conditions. The powders were stored in sealed sterile plastic bags. During each sampling episode, one gram of powder was suspended into 9 ml PBS and serially diluted accordingly. The dilutions were spread onto LBS agar plates and incubated anaerobically at 37°C. Duplicate plates were used for each dilution. Colony-forming units (CFUs) were calculated and presented as means when colonies had fully grown. For comparison, *L. rhamnosus* GG (APC 2740) was included in the stability trial and treated the same as *L. kimchicus* 5.1.

Statistical Analysis

Statistical analysis was conducted using GraphPad Prism version 9.1.0 (GraphPad Software, La Jolla, California, USA). The results were expressed as means ± standard deviations of at least two replicates. An unpaired T-test was used to identify any statistically significant differences in the experiments, and differences were considered statistically significant at $P<0.05$.

Results

Strain isolation and characterisation

A faecal sample received from the wild nestling great tits (*Parus major*) in Duke Wood, Cork, Ireland, was inoculated into *Lactobacillus* MRS broth which supports the cultivation of *Lactobacillus* species and incubated overnight. After this enrichment, the inoculated broth was serially diluted and plated on LBS plates in different incubation conditions (anaerobic 37°C, aerobic 37°C, anaerobic 30°C, and aerobic 30°C). The plates were checked intermittently after 24 and after 48 hrs of incubation. Colonies of different morphologies and colours were streaked for purity and incubated again under the conditions previously described. There were five phenotypically different colonies initially isolated. The best growth of colonies was observed when the plates were incubated anaerobically at 37°C for 48 hrs. The colonies of the isolated bacteria were small to medium, cream, and white coloured, and smooth in texture. When the Gram-stained colonies were viewed under the light microscope (×1000), the cells were Gram-positive, short rods that occurred single or in pairs (Figure 2.1). Only one isolate tested catalase positive, which was immediately excluded from the study.

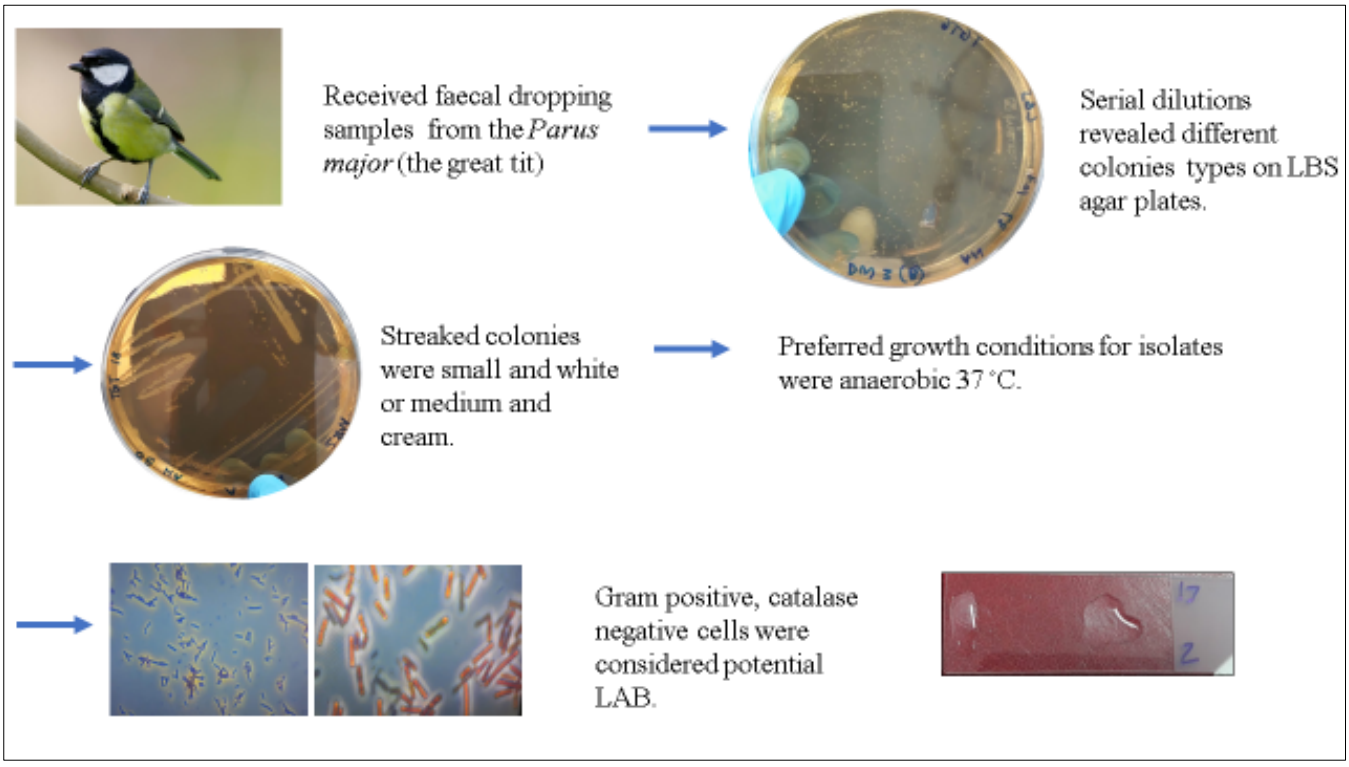

Figure 2.1: Isolation and preliminary identification of *L. kimchicus*.

Genomic identification

16S rRNA sequencing

The isolates were further identified based on 16S rRNA sequencing. The sequences obtained from the four colonies were compared to an existing NCBI genomes database and found to have 98 to 98.86% similarity to *Lactobacillus kimchicus,* confirming the species identity. Because all the isolates had exhibited genotypic similarity, only one of the original five isolates was taken forward for analysis. This isolate was thus designated *as L. kimchicus* 5.1. This isolate had small to medium, round, cream-white, and convex colonies.

Whole Genome sequencing (WGS)

To expand on the knowledge base of this *Lactobacillus* species, WGS was conducted on *L. kimchicus* 5.1. The annotation (Table 2.3) was performed with RAST (version 2.0) (Aziz et al., 2008), and assembly was carried out using tools available on the public server at http://cgview.ca. The complete genome of *L. kimchicus* 5.1 consists of 2,535,859 bp and has no plasmids or transposable elements (Figure 2.2(A)). The G+C content of this strain is 46.4 mol%. Using RAST software, 2730 coding sequences were found, including 71 RNAs and 955 protein-coding open reading frames (ORFs) divided into 27 subsystem groups. The majority of the genes identified were associated with carbohydrate metabolism, amino acid and protein metabolism (Figure 2.2(B)). Other notable regions were protein-encoding genes for ldhD (D-lactate dehydrogenase) and the acid-resistant locus fragment (Arl7), which is 759 bases long. A coding region for the general stress protein family Gls24 was found in contig 5. The 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (MetE) gene was also present. Heat shock-related protein sequences that were found include GroES and GroEL, which are heat shock protein family chaperones and are 10 kDa and 60 kDa, respectively. The S4 paralogue, a ribosome-associated heat shock protein implicated in the recycling of the 50S subunit, was found between positions 9664 and 9936. A coding sequence for leucocin A was found in the genome. The coding sequence between nucleotide 83594 and 85531 was for tetracycline resistance-related proteins.

Table 2.3: General features of *L. kimchicus* 5.1.

Feature	**Size**
Genome length (bp)	2,535,859
Number of contigs with Protein Encoding Genes (PEGs)	39
G+C content (%)	46.4
Number of coding sequences (CDs)	2730
Number of plasmids	0
Number of Subsystems	237
Number of RNAs	71

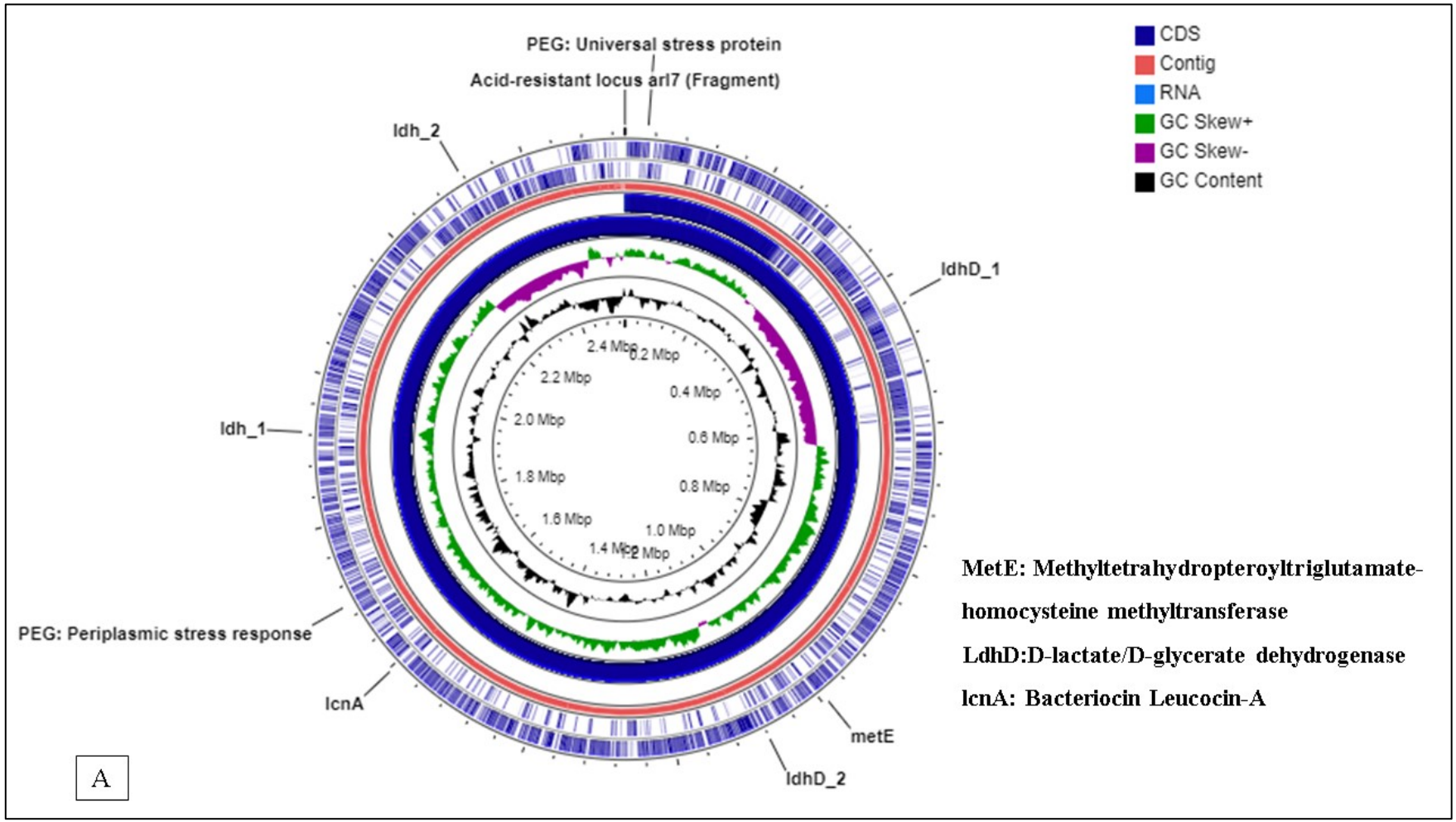

Figure 2.2(A): Circular genome of *L. kimchicus* 5.1 with a subset of representative genes highlighted.

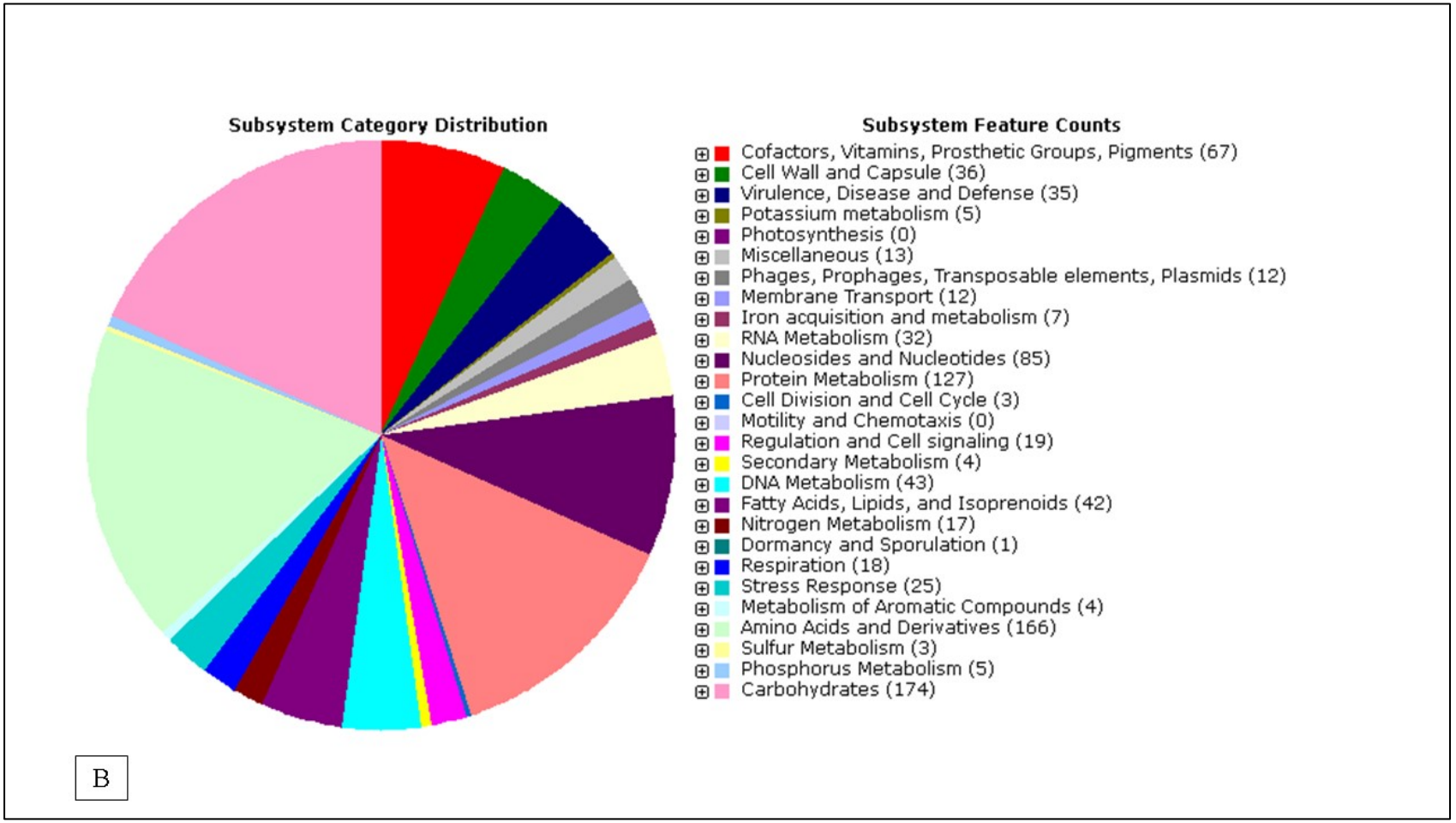

Figure 2.2(B): Pie graph of the gene subsystems for different functions in *L. kimchicus* 5.1.

In silico screening for bacteriocins

Screening the entire genome of *L. kimchicus* 5.1 revealed that the bacteriocin-encoding locus (*LcnA* locus) occurred between positions 1898 and 2291. RAST showed this as encoding for the bacteriocin Leucocin A. Bagel was used to identify bacteriocin encoding gene clusters and showed coding regions for leucocin C and plantaricin 423 (Figure 2.3). The results of the antiSMASH analysis showed a ribosomally synthesised and post-translationally modified peptide (Ripp) like region and no other secondary metabolites in the genome.

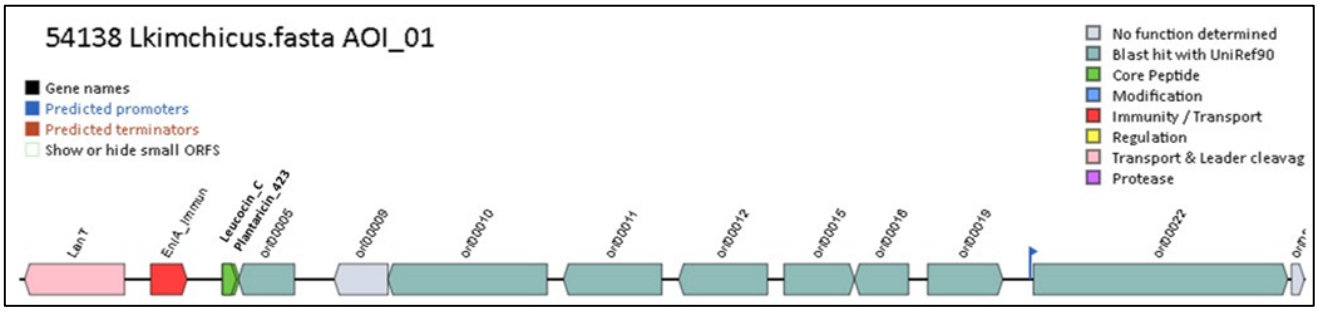

Figure 2.3: Potential bacteriocin operon in *L. kimchicus* 5.1 as shown by Bagel4.

In silico screening for antibiotic resistance genes

The genome sequence of *L. kimchicus* 5.1 was analysed using an online resource for identifying antibiotic resistance genes known as Resfinder (https://cge.cbs.dtu.dk/services/ResFinder/). When antibiotic resistance was initially investigated using Resfinder, no genes known to be involved in antibiotic resistance were detected. Therefore, the Comprehensive Antibiotic resistance database (CARD; http://arpcard.mcmaster.ca) was used to verify these results. No antibiotic resistance genes were detected in the genome using CARD when the resistance gene identifier was set at selection criteria to find only "Perfect and strict complete genes". An antibiotic resistance mechanism with a 31.9% match to a set of genes for the antibiotic efflux pump that confers tetracycline resistance was found when the resistance gene identifier criteria were set to "loose."

In vitro screening for bacteriocins

Antimicrobial activity is important for LAB to colonise the intestinal mucosa effectively, enabling them to defend against potential pathogens (Vijayabaskar and Somasundaram, 2008). This study examined the antimicrobial activity of LAB against common pathogens using the

spot assay and the well diffusion method. When tested against *L. delbrueckii* subsp. *bulgaricus* LMG 6901, *L. kimchicus* 5.1, did not demonstrate any bacteriocin production. Of all the indicators used (Table 2.1), *L. kimchicus* 5.1 exhibited a slight inhibition against *S. simulans* (Figure 2.4(A)) and a slightly more potent inhibition against *E. coli* (Fig 6B). A subsequent well diffusion assay using neutralised CFS demonstrated continued inhibitory activity against *E. coli* (Figure 2.4(C)); however, against *S. simulans,* inhibitory activity was lost.

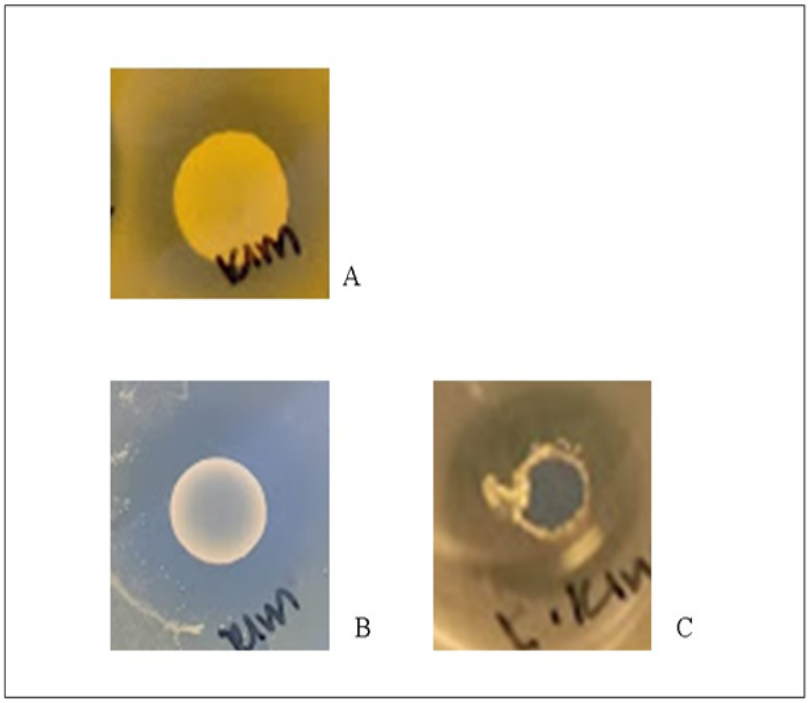

Figure 2.4: Antimicrobial activity using a spot assay against *S. simulans* (A) and *E. coli* (B) and well diffusion against *E. coli* (C).

Growth and Fermentation pattern determination

To establish the growth rate of the newly isolated *L. kimchicus* 5.1 strain, a pure colony was inoculated into 10 ml Difco MRS broth and incubated in anaerobic 37°C, and turbidity (OD_{600}) was regarded as an indicator of growth. This was also performed for *L. rhamnosus* GG (APC 2740). Growth was measured at various time intervals using a spectrophotometer (Ultraspec 10 Cell density meter) (Biochrom, Cambridge, United Kingdom) for 48 hrs. A growth curve was generated for both *L. kimchicus* 5.1 and *L. rhamnosus* GG (Figure 2.5). *L. kimchicus* 5.1

displayed a lag period of 8 hrs and a doubling time of 4.65 hrs, and *L. rhamnosus* showed a doubling time of 3.64 hrs. The formula (1) stated below was used to calculate doubling time.

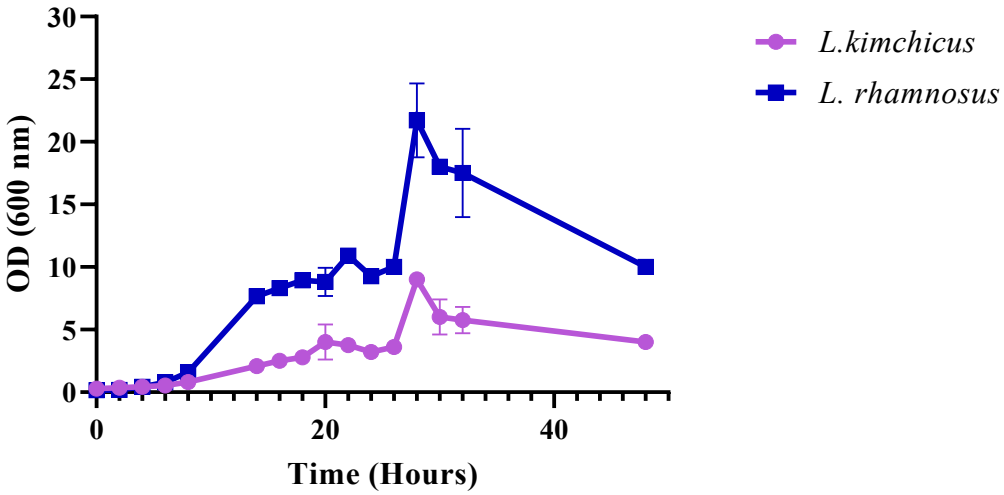

Figure 2.5: Representative growth curves using optical density.

$$(1)\ Y = Y0 * exp(k * x)$$

Y0 = Starting OD

K= Rate constant (*L. kimchicus* 5.1: 0.071; *L. rhamnosus* GG: 0.060)

Y= Population

X= Time (hrs)

The *L. kimchicus* 5.1 strain was tentatively identified based on phenotypic characteristics by determining growth and acid production on HHD agar medium. After two days of incubation, *L. rhamnosus* GG, a known heterofermentative organism, produced white colonies (Figure 2.6(A)), while the known homofermentative *L. lactis* produced blue/green colonies (not shown). *L. kimchicus* also produced blue colonies (Figure 2.6(B)) on HHD media, suggesting that the organism is a homofermentative LAB.

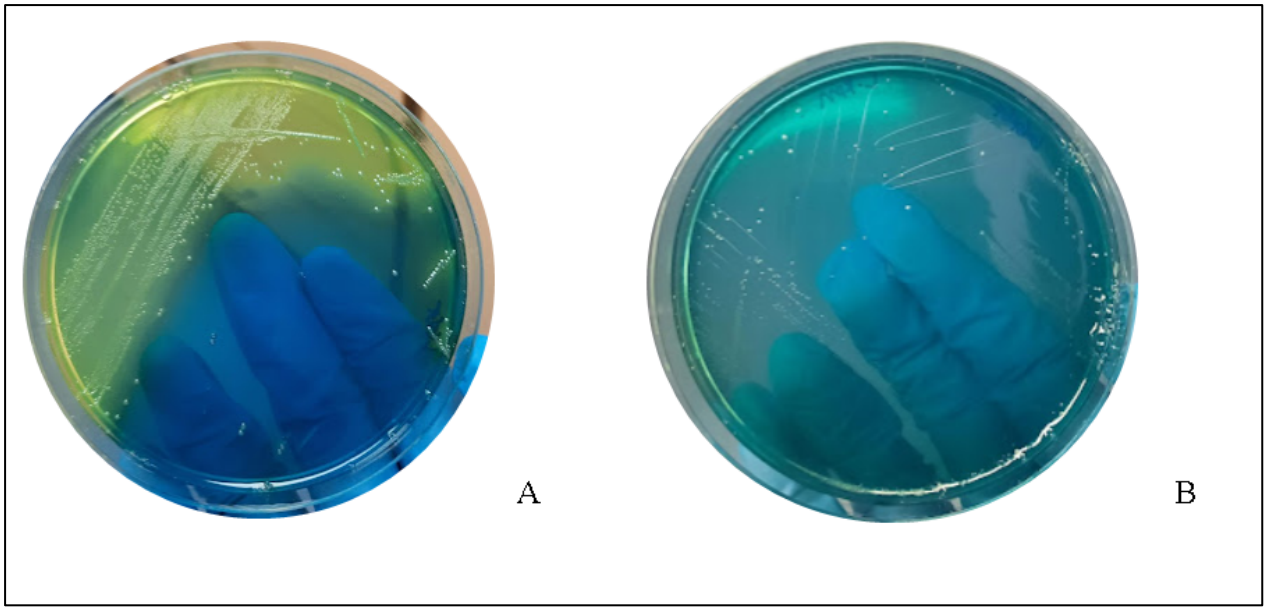

Figure 2.6: Growth and acidification on HHD agar medium of *L. rhamnosus* GG (A) and *L. kimchicus* 5.1 (B).

Lactic acid isomers

A rapid assay kit (Megazyme, Bray, Ireland) was used to calculate the ratio of each of the optically active forms of lactic acid produced by *L. kimchicus* 5.1 (Table 2.4). Each sample was analysed in duplicate, and the assay was performed twice. The accuracy of the assay was confirmed by using *L. rhamnosus* GG as a positive control and comparing the results to the supplied lactic acid standard. Only the D-lactic acid isomer was detected at 0.82 g/l when *L. kimchicus* 5.1 was grown in LBS.

Table 2.4: The optically active isomer of lactic acid produced by LAB.

Organism	**Lactic acid isomer (g/l)**	
	L-lactic acid	**D-lactic acid**
L. kimchicus 5.1	-0.002 ± 0.001	0.82 ± 0.0009
L. rhamnosus GG	0.199 ± 0.001	0.612 ± 0.0003

Characterisation of *L. kimchicus* 5.1 as a probiotic

Antibiotic susceptibility

Table 2.5 shows the antibiotic susceptibility of the two LAB against eight antibiotics using LSM plates. In this study, *L. kimchicus* 5.1 was sensitive to all the necessary antibiotics as specified by the European Food Safety Authority (EFSA) except streptomycin, where the cut-off was 16 µg/ml, and the bacteria was inhibited at 32 µg/ml. *L. rhamnosus* GG was used as the positive control and was susceptible to all the specified antibiotics. Figure 2.7 shows an example of the results obtained using the E-test strips.

Table 2.5: MIC (µg/ml) testing using E-test strips on *Lactobacillus* Sensitivity Media (LSM).

Antibiotic	***L. rhamnosus GG***		***L. kimchicus* 5.1**	
	MIC (µg/ml)	Result	**MIC** (µg/ml)	Result
Chloramphenicol	0.19	**S**	CI	**S**
Erythromycin	0.125	**S**	0.125	**S**
Gentamycin	1.5	**S**	0.38	**S**
Clindamycin	1	**S**	1	**S**
Kanamycin	24	**S**	32	**S**
Streptomycin	6	**S**	32	**R**
Ampicillin	1.9	**S**	0.125	**S**
Tetracycline	0.5	**S**	4	**S**

Key: CI: Complete inhibition; S: Susceptible; R: Resistant.

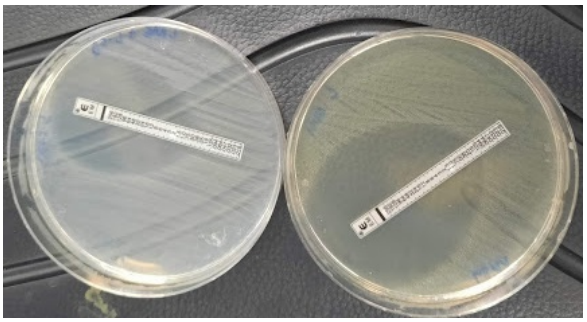

Figure 2.7: An example of MIC testing of *L. kimchicus* 5.1 using E-Test strips.

Stress tolerance

Exopolysaccharide (EPS) production

EPS production is identified by the ropiness or mucoid nature of bacterial colonies when lifted with an inoculation loop. As expected, EPS production was observed in the positive control strain *L. mucosae* (DPC 6426), which was used as a reference strain as this organism is a well-documented EPS producer (Ryan et al., 2019). No EPS production was observed for *L. kimchicus* 5.1 when inoculated with 5% (w/v) glucose.

Heat tolerance

Tolerance to heat was evaluated by incubating the cultures at increasing temperatures from 40°C to 60°C for 30 mins (Figure 2.8). Incubation at 37°C was considered the control, as it had already been established that this strain grows well when incubated at this temperature. When *L. kimchicus* 5.1 culture was incubated at 40 to 45°C, viable cell counts remained at 10^7 CFU/ml, similar to the growth observed at 37°C. At 50°C, there was a log reduction in viable cells compared to 37°C. However, upon incubation at 60°C, there was a dramatic 5 log reduction in viable cells, from 10^7 to 10^2 CFU/ml, compared to the 37°C control temperature. *L. rhamnosus* GG tolerated incubation at 50°C better than *L. kimchicus* 5.1, as viable cell counts were 5.1 $\times 10^6$ and 1.8 $\times 10^6$ CFU/ml for *L. rhamnosus* GG and *L. kimchicus* 5.1 respectively. The tolerance of *L. rhamnosus* GG to 60°C was significantly better than the test isolate *L. kimchicus* 5.1 (P= 0.0198).

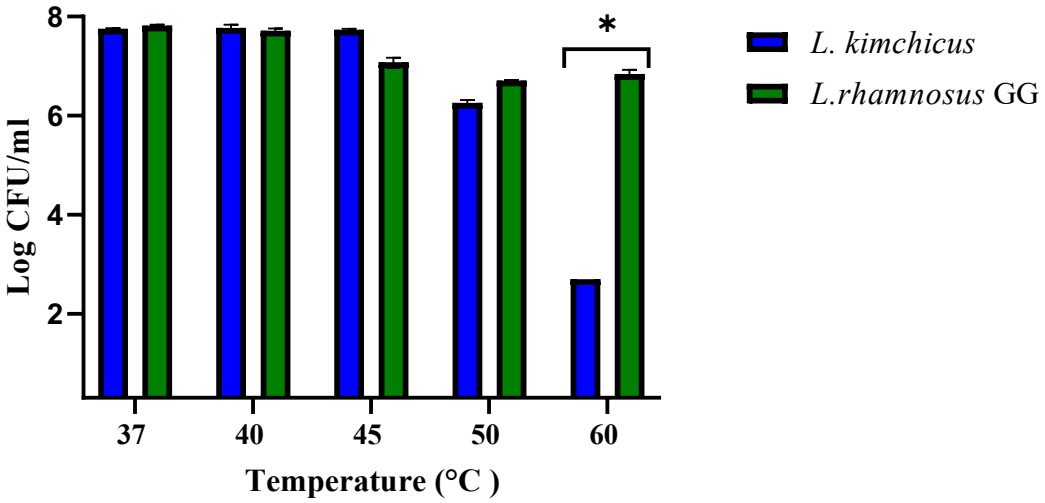

Figure 2.8: Cell counts (log CFU/ml) of *L. kimchicus* 5.1 and *L. rhamnosus* GG after heat treatment challenge at 37°C to 60°C incubation for 30 mins.

Tolerance to acidic conditions

The *L. kimchicus* 5.1 isolate was tested for its ability to survive under different acidic conditions, at pH ranging from 2-6, and control media (pH 6.7) was included. After 4 hrs of exposure to pH 2, *L. kimchicus* 5.1 viable cells were 1×10^3 CFU/ml, while at T_0, the number of bacteria was at 10^7 CFU/ ml for all the pH values (Figure 2.9). Therefore pH 2 appears to be the cut-off for *L. kimchicus* 5.1 since growth at pH 2.5 was maintained at 1×10^7 CFU/ml for 6 hrs. For the reference strain used in this study, *L. rhamnosus* GG counts were maintained at 10^7 CFU/ml for 6 hrs for pH values between 3-6. There was a 3-log reduction of *L. rhamnosus* GG viable cells at pH 2 after 2 hrs of incubation.

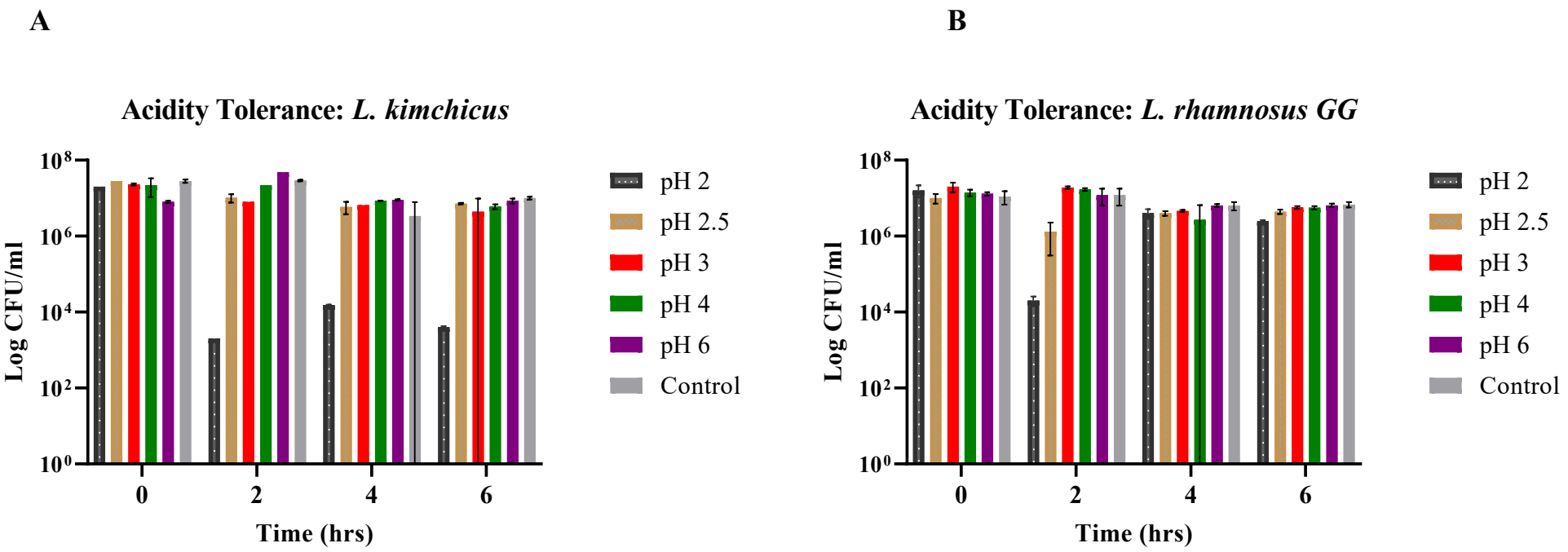

Figure 2.9. Acidity tolerance testing on isolate *L. kimchicus* 5.1 (A) and reference strain *L. rhamnosus* GG (B).

Error bars represent standard deviation.

Bile salt tolerance

After incubation in 0.3% and 0.5% (v/v) bile (oxgall), viability of bacterial cells was assessed by colony counts (CFU/ml) on plates incubated at 37°C (Figure 2.10). Bile tolerance was calculated by comparing viable cell counts in MRS with and without bile. Survival ratios were calculated using Formula 2. *L. kimchicus* 5.1 survived up to 5 hrs in 0.3% and 0.5% bile salt. After 5 hrs of exposure, 98.5% of cells exposed to 0.3% bile salts had survived, while 95.2% of those exposed to 0.5% bile salt remained. The reference strain *L. rhamnosus* GG was unaffected by the presence of 0.3% bile salts, as indicated by 100% recovery after 5 hrs of exposure.

$$\text{Survival ratio} = (\text{Log CFU}_{\text{MRS}} - \text{Log CFU}_{\text{Test bile}}) / \text{Log CFU}_{\text{MRS}}$$

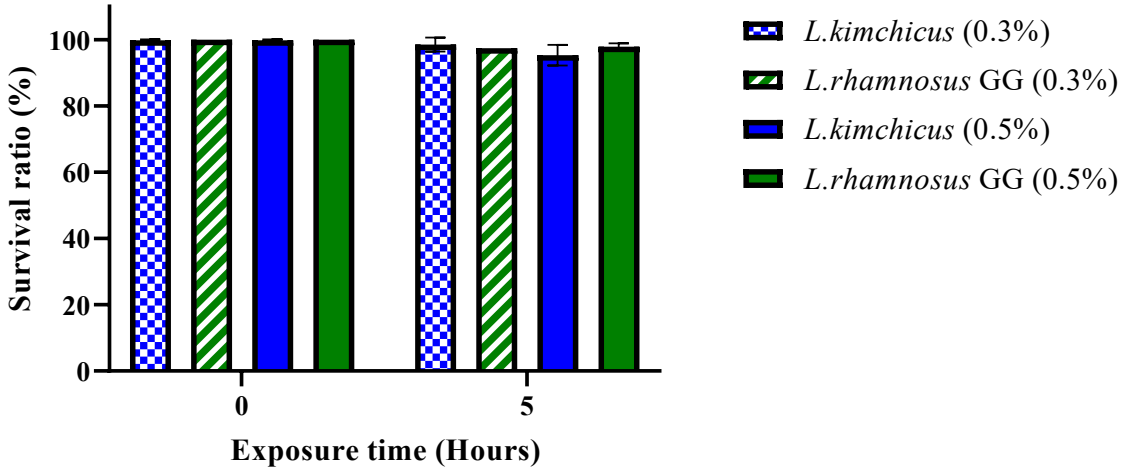

Figure 2.10: Percentage survival ratios of *L. kimchicus* 5.1 and *L. rhamnosus* GG in 0.3 and 0.5% bile salts. The error bars represent the standard deviation.

Bile salt hydrolase activity

The direct plate assay method was used to assess the BSH activity of *L. kimchicus* 5.1 qualitatively. Overnight cultures were spotted (10 µl) in triplicate on LBS agar that had been supplemented with conjugated bile salts: taurodeoxycholic acid (TDCA) and taurocholic acid (TCA). No halos were observed in *L. kimchicus* 5.1, which indicates an absence of bile salt hydrolase activity (Figure 2.11).

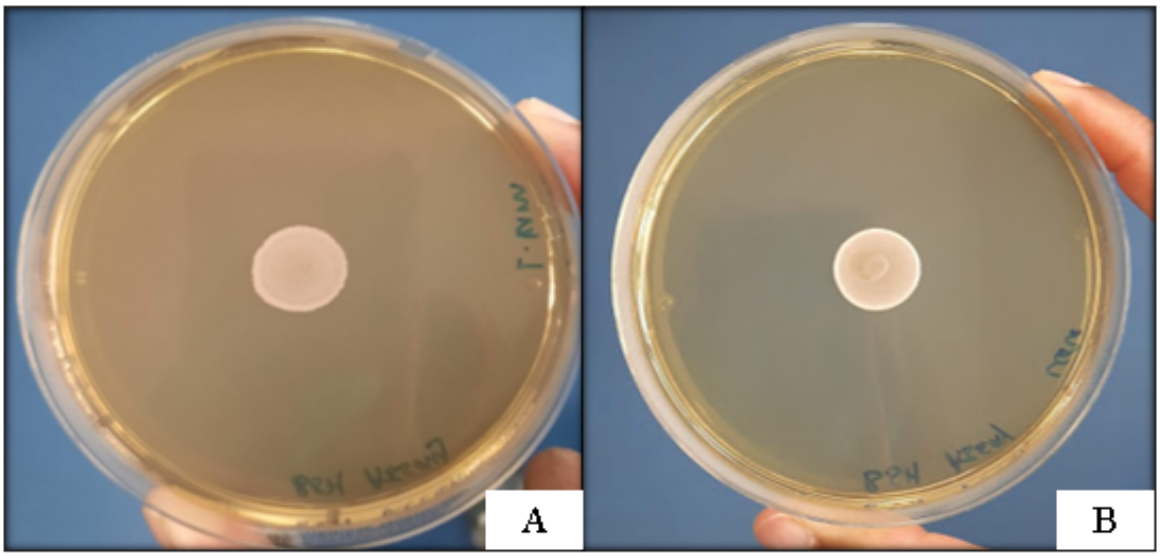

Figure 2.11: *L. kimchicus* 5.1 spotted on BSH assay plates on (A) TDCA and (B) TCA supplemented MRS plates.

Stability of freeze-dried *L. kimchicus* 5.1 at different temperatures

Freeze-dried powders obtained from *L. kimchicus* 5.1 and *L. rhamnosus* GG were stored at different conditions for six weeks, i.e., at room temperature (RT), 4°C, and -20°. Enumerations were performed on the first day of the stability trial, after 1, 4, and 6 weeks. At each time point, the number of viable *Lactobacillus* cells was determined by the plate count method and presented as CFU/ g. The initial viable colonies at T_0 were 1.1×10^{11} and 3.52×10^{11} CFU/ g for *L. kimchicus* and *L. rhamnosus* GG, respectively. After six weeks, the overall decrease in the viability of *L. rhamnosus* GG and *L. kimchicus* 5.1 was less than 1 log CFU/g when the freeze-dried powder was stored at 4°C and -20°C (Figure 2.12 (B and C)). When *L. kimchicus* 5.1 was kept at room temperature (Figure 2.12(A)), a decrease of up to 3 log CFU/g was observed after four weeks of storage. A further sharp decline in cell viability of 2 log CFU/g was observed between week four and week six. A similar trend was observed for *L. rhamnosus* GG.

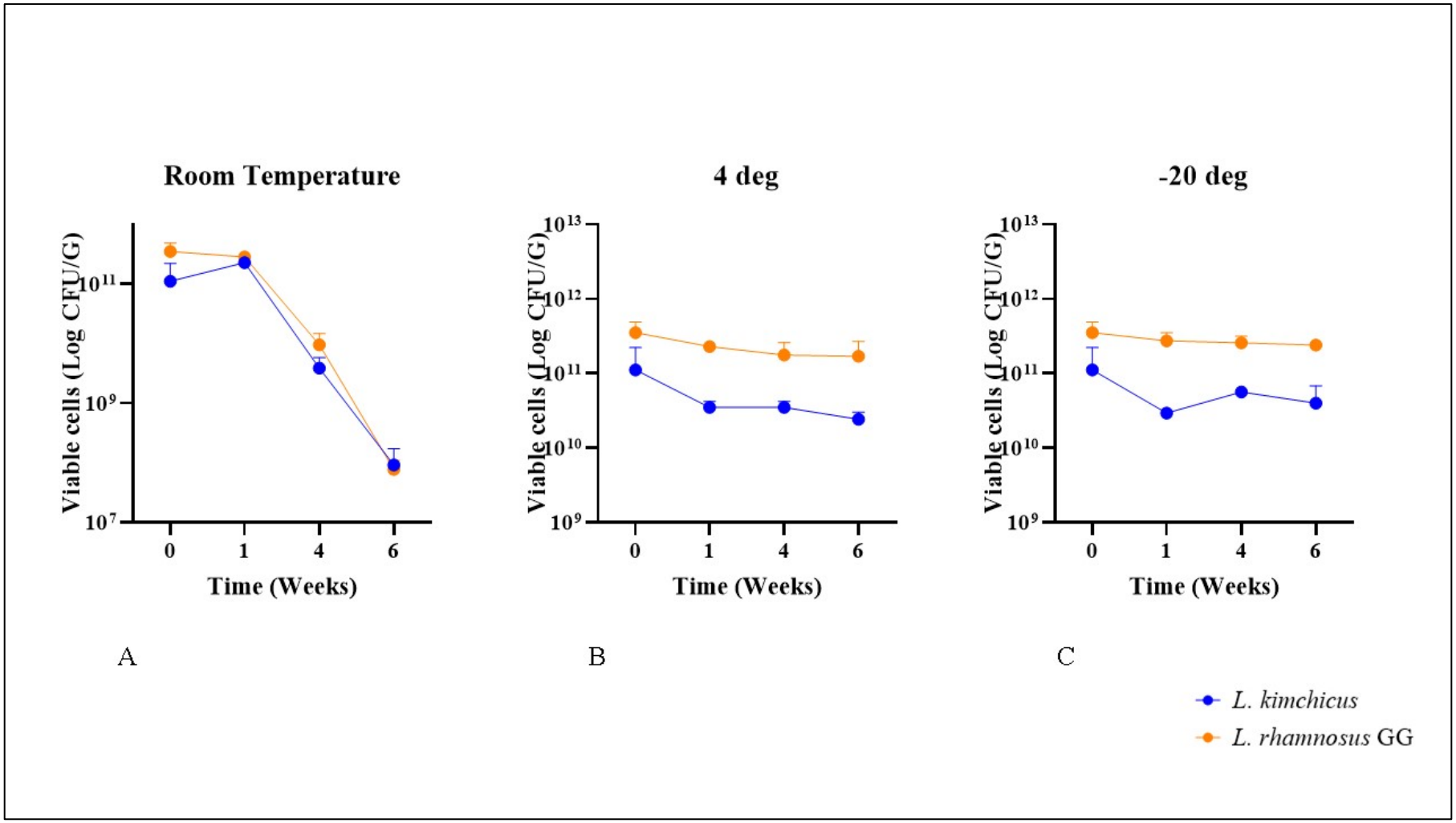

Figure 2.12: Cell viability of *L. kimchicus* 5.1 and control strain (*L. rhamnosus* GG) stored at room temperature (A), 4°C (B), and -20°C (C) for six weeks.

Discussion

Lactobacillus colonies were isolated from bird faeces and characterised for probiotic potential using various *in vitro* tests and *in silico* screening approaches. The first step involved the isolation and identity confirmation of the *Lactobacillus* sp., and the second step was an investigation of the probiotic characteristics of the isolated strain. *Lactobacillus* sp. are the most widely used probiotic group and have an essential role in gut microbiome restoration and maintaining balance after antibiotic treatment of a host (Das et al., 2020).

The isolated colonies grew best under anaerobic conditions, at 37°C, which is characteristic of *Lactobacillu*s, as their optimal growth temperature range is 40°C (Slizewska and Chlebicz-Wojcik, 2020). Initially, five different colonies were identified, but upon testing, one isolate was excluded due to testing catalase positive. Only Gram-positive and catalase-negative isolates were taken forward for analysis. Physiological and biochemical tests confirmed that the isolate was indeed a LAB and 16S rRNA sequencing revealed that the organism is *Lactobacillus kimchicus*. Although there was some slight phenotypic variation in the colour of the colonies, genotypic identification showed that the four colonies sequenced were all *L. kimchicus*. As such, one colony designated *L. kimchicus* 5.1 was carried forward, and all analysis was performed on this strain. *L. kimchicus* is a novel organism that was first isolated from Korean kimchi. The biochemical identification results were similar to those of Liang et al. (2011). The isolate was found to have a long lag phase of 8 hrs at 37°C and a doubling time of 4.65 hrs (Figure 2.5). Evaluating the growth patterns of this organism was important to enable ease of use during processes such as freeze-drying and ensuring that the organism is given sufficient time to grow for other *in vitro* tests.

To gain a thorough insight into the genetic composition of this isolate, WGS was performed on *L. kimchicus* 5.1. There were 2730 coding sequences found, including 71 RNAs and 955 protein-coding open reading frames (ORFs) divided into 27 subsystem groups. The largest subsystem of genes (174 of 955) in the *L. kimchicus* 5.1 genome is dedicated to carbohydrates (Figure 2.2(B)). The nutritional requirements of Lactobacilli could be due to their various habitats, which are typically rich in carbohydrates and proteins (Mayo et al., 2008). A preferred trait when selecting a probiotic strain is the demonstrated ability to produce bacteriocins to positively influence the host's health (Dobson et al., 2012). *Lactobacillus* species primarily exert their essential role in GI tract function by producing bacteriocins that generally have

potent activity against intestinal pathogens (Dempsey and Corr, 2022). Environmental factors such as temperature, pH, culture incubation time, and media composition affect the extent to which bacteriocins are produced (Bibalan et al., 2017). Challenges related to the conventional methods of bacteriocin screening can be overcome by using *in silico* screening (Bibalan et al., 2017). In this study, the draft genome was analysed using BAGEL4 to search for potential antimicrobial-encoding operons. Gene clusters were identified containing putative genes for the production of leucocin C and plantaricin 423 (Figure 2.3). One drawback of *in silico* screening methods is that they cannot be used in isolation to confirm bacteriocin production conclusively (Walsh et al., 2015). Although no bacteriocin activity was detected *in vitro*, there was slight inhibition against *S. simulans* and *E. coli* (Figure 2.4). This inhibitory activity against *E. coli* could not be attributed to lactic acid production as it was maintained despite CFS being neutralised. However, activity was lost against *S. simulans* upon neutralising the CFS (Figure 2.4). This could mean that inhibition was due to the production of another antimicrobial agent such as H_2O_2 (Reis et al., 2012). Antimicrobial agent production could enable the strain to competitively exclude potential pathogens in the GIT when used as a probiotic.

HHD media was developed to enable differential enumeration of heterofermentative and homofermentative LAB based on media and colony colour (Soro-Yao et al., 2014). Fructose was used in this media as the sole carbohydrate source. Heterofermentative organisms reduce fructose to mannitol while producing lactic acid, CO_2, and acetic acid, whereas homofermentative bacteria produce two moles of lactic acid from fructose. This study used known heterofermentative organisms, *L. rhamnosus* GG (APC 2947), and homofermentative *L. lactis* (SL242) (Golomb and Marco, 2015), as the controls for comparison. *L. rhamnosus* produced the expected white colonies, while the media surrounding the colonies turned from blue to light green. The test organism exhibited homofermentative characteristics as blue colonies grew and the media remained blue (Figure 2.6). The D/L lactate assay confirmed the finding of Liang et al. (2011) that this species only produces D-lactate. LAB metabolises sugars via lactate dehydrogenase, which reduces pyruvic acid to lactate. Both the *ldh1* and *LdhD* genes were present in the genome of *L. kimchicus* 5.1. The *ldh1* gene codes for L-Ldh, which synthesises L-lactate in bacteria (Rico et al., 2008). *LdhD,* the D-lactate dehydrogenase gene, is involved in D-lactate catabolism. Similarly, *Lactobacillus helveticus* possesses both L and D-lactate dehydrogenases (Kochhar et al., 1992). *L. helveticus,* a homofermentative LAB, can produce large amounts of lactic acid. However, the expression of ldhD and ldhL genes does

not always correlate to changes in the amounts of D and L- lactate produced (Nikkila et al., 2000).

The emergence and transfer of antibiotic resistance genes is a major concern. The potential to transfer resistance factors to pathogenic bacteria in the gut is a real threat if antibiotic-resistant strains are used as probiotics (Gueimonde et al., 2013). It was therefore, necessary to test the strain *L. kimchicus* 5.1 for antibiotic susceptibility, according to EFSA guidelines. The susceptibility of *L. kimchicus* 5.1 to the various antibiotics as stipulated by EFSA guidelines was evaluated by phenotypic testing. This test revealed that although the organism was susceptible to all the antibiotics tested (ampicillin, chloramphenicol, erythromycin, clindamycin, tetracycline, gentamycin, kanamycin, and vancomycin); it exhibited a slight resistance toward streptomycin. Some *Lactobacillus* strains have a naturally high resistance to streptomycin (Campedelli et al., 2019). This natural resistance of *Lactobacillus* to streptomycin was also documented by Danielsen and Wind (2003). Antibiotic resistance is conferred by the presence of antibiotic resistance genes (Zhang and Zhang, 2019). Antibiotic resistance was investigated using *in silico* methods such as Resfinder and the Comprehensive Antibiotic resistance (CARD) database because it was critical to evaluate whether this putative probiotic has any transferable resistance genes. Although no genes encoding antibiotic resistance were initially found when the selection criteria were set to "perfect and strict hits only" on the resistance gene identifier (RGI) on CARD, RGI found 31.9% similarity to genes encoding for tetracycline resistance when RGI was set to "loose" parameters. This was in agreement with the findings from RAST, which revealed a match to a set of genes encoding for the antibiotic efflux pump that confers tetracycline resistance. No transposable or mobile elements were detected in the *L. kimchicus* 5.1 genome, meaning that any resistance genes present could not be transferred horizontally.

Low pH environments can impair the metabolism and inhibit the growth and viability of lactobacilli (Corcoran et al., 2005). The persistence of a potential LAB in acidic conditions is crucial for it to survive passage through the GIT. A 4 log decrease in *L. kimchicus* 5.1 viable cells was observed after incubation at pH 2 for 4 hrs. At pH 2.5, although there was a decrease in the viable cell numbers, this difference was less than 1 log indicating that this organism is tolerant to this pH value. These results are in agreement with those obtained from a study by Faye et al. (2012), where the viability of *Lactobacillus* strains *(L. rhamnosus* GG and *L. paracasei* INF-448) decreased from log 7 to about log 4 CFU/ml after 3 hrs of exposure at pH

2. The acid-resistant locus (Arl7) located between positions 35676 and 36425 of the *L. kimchicus* 5.1 genome was also found to be part of unique genes belonging to *Streptococcus thermophilus* ACA-DC 2, which is a strain that was isolated from traditional Greek yoghurt by Alexandraki et al. (2017). It can be hypothesised that Arl7 contributes to *L. kimchicus* being able to survive under acidic conditions. LAB utilise fermentable carbohydrates to produce lactic acid. Therefore, acid resistance in LAB is vital for their growth, fermentation, and use as probiotics. LAB employ various mechanisms to regulate acid resistance and survive in low pH environments. These include changes in cell membrane composition, DNA and protein repair (Wang et al., 2018). The protein-encoding gene (PEG) for (Gls24), which is defined as a "general stress protein," was identified in the genome of *L. kimchicus* 5.1. This could have contributed to the varying degrees of tolerance to acidic conditions of this strain when exposed to pH 2- 6 for 6 hrs (Figure 2.9(A)).

Another criterion for assessing potentially probiotic bacteria is the ability to survive the hostile environments in the GIT. In particular, they must be bile tolerant to survive in the small intestine. The stress caused by incubation in 0.3% bile salt resulted in the death of less than 3% of the *L. kimchicus* 5.1 cells (Figure 2.10). This concentration of bile is considered physiologically relevant and is often regarded as critical for evaluating strain resistance to bile (Gharbi et al., 2018). After 5 hrs of incubation at 0.5% bile salts, greater injury to the cells was observed as a 4.8% reduction in viable cells occurred. Bile salt hydrolase, also known as cholylglycine hydrolase, plays a role in deconjugating bile salts (Chand et al., 2017). BSH has various essential functions in colonising the host and is involved in host physiological processes (Grimm et al., 2014). *Lactobacillus* strains with this ability have the added advantage of colonising the small intestine where the enterohepatic cycle occurs (Kumar et al., 2011). Other functions include a nutritional role, where the amino acids released from bile salt deconjugation can be used as nitrogen and carbon for bacterial survival and growth. BSH can also facilitate incorporating cholesterol or bile into bacterial membranes, increasing the membrane tensile strength of bacteria and enabling gastrointestinal persistence (Begley et al., 2006; Chand et al., 2017; Tanaka et al., 1999). BSH activity confers a benefit to the host by reducing serum cholesterol levels in the body (Sedláčková et al., 2014*),* and BSH activity is perceived as a beneficial probiotic trait (Begley et al., 2006). For bacteria to be useful as a potential probiotic strain, the organism should survive in the presence of bile salt in the GIT. Because we could not find the *bsh* gene in the genome of *L. kimchicus* 5.1, this strain was not expected to survive well under conditions with bile. There are some reports of studies that do

not correlate the bile tolerance of strains with BSH activity (Moser and Savage, 2001, Usman and Hosono, 1999). For example, using tauroconjugated bile acids instead of glycoconjugate bile acids may yield differing results (Begley et al., 2006). Although no BSH activity was observed for *L. kimchicus* in the qualitative plate assay, it is important to note that the presence and genetic organisation of *bsh* genes in lactobacilli are quite variable. Furthermore, in some *Lactobacillus* strains, enzyme activity may depend on substrate specificity, temperature and pH range (Fang et al., 2009).

Temperature variation is probably the most common stress bacteria encounter in the natural environment. Some LAB can survive at high temperatures between 45 and 80°C (Mbye et al., 2020). *L. kimchicus* 5.1 cells showed a moderate level of tolerance to high temperatures. For *L. kimchicus* 5.1 cells, exposure to 50°C for 30 mins reduced viable cell numbers by less than 1 log compared to incubation at the control temperature (37°C). At 60°C, there was a log reduction in viable cells, suggesting that the highest temperature that *L. kimchicus* 5.1 can tolerate is 50°C.

Lyophilisation is a commonly used method for preservation of biological material such as viable bacteria to safeguard proteins and liposomes, using sugars such as trehalose and sucrose as the main protectants (Zhang et al., 2017). Freeze-drying can result in cell damage and loss of viability. When *L. kimchicus* 5.1 was freeze-dried using trehalose as a cryoprotectant, the resulting powder consistently harboured *L. kimchicus* 5.1 at levels between log 10 and log 11 CFU per gram. Trehalose was useful in providing the necessary protection for *L. kimchicus* 5.1. Trehalose was chosen because it had been reported by Li et al. (2011) to protect *Lactobacillus reuteri* during freeze-drying sufficiently. Another important characteristic of probiotic bacteria is maintaining viability during the product life cycle (Muller et al., 2013). The therapeutic potential of these bacteria in the GIT depends on their survival during manufacture and storage (Kailasapathy and Chin, 2000). Determining the appropriate conditions for storage of a probiotic powder increases the survival of LAB and, consequently, its effectiveness in the host. There was a 3-log loss of viability of *L. kimchicus* 5.1 during six weeks of powder storage at room temperature. After six weeks of storage at room temperature, the number of viable cells remained above the desired minimum of 10^6 CFU per gram viable cells. However, storage at 4°C resulted in higher viability (log 10) CFU over six weeks (Figure 2.12). This indicates that *L. kimchicus* 5.1 can be stored as a probiotic powder for six weeks at refridgerated temperature.

Conclusion

In this study, small to medium, cream and white coloured, and smooth colonies, which grew best when incubated anaerobically at 37°C, were successfully isolated from the great tit bird faeces using LBS. Genomic sequencing was used to identify the four isolates as *L. kimchicus.* A representative isolate, *L. kimchicus* 5.1, was found to have a doubling time of 4.65 hrs and was characterised as a D-lactate producing homofermentative strain. Although the isolate exhibited slight resistance to streptomycin, *in silico* screening for antibacterial genes using CARD found an antibiotic resistance mechanism that had a 31.9% match to a set of genes for the antibiotic efflux pump that confers tetracycline resistance. WGS also showed that *L. kimchicus* 5.1 harbours no transposable elements or plasmids. The strain was capable of survival under acidic conditions down to pH 2.5 as cell viability was maintained at $1x10^7$ CFU/ml after 6 hrs of exposure. Furthermore, tolerance to *in vitro* digestive conditions was demonstrated in that the strain survived at 98.5% in the presence of 0.3% bile salts for 5 hrs. *L. kimchicus* 5.1 did not exhibit an ability to deconjugate bile salts TCA and TDCA, which was in agreement with WGS results where there were no detectable bile salt hydrolase genes. *L. kimchicus* demonstrated noticeable tolerances to high temperatures. When incubated at 40-45°C for 30 mins, viable cell counts remained at 10^7, similar to the growth observed at 37°C. As a freeze-dried powder, the strain maintained high viability when stored at 4°C for six weeks. Although more research is needed for product development technology, evaluation of extended shelf-life properties, and the actual health benefits conferred to the host, this study lays the foundation for the use of *L. kimchicus* 5.1 as a potential probiotic strain.

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