Partial characterization of probiotic lactic acid bacteria isolated from Chinese dairy products

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\section*{ABSTRACT}
A total of 246 bacterial isolates were collected from various food and animal sources within Nanning city, China. Sequencing of the 16S rRNA gene revealed that 24.4\% of the isolates were lactic acid bacteria (LAB). Initial screens identified three isolates from semi-hard cheese: \textit{Lactobacillus plantarum} subsp. \textit{plantarum} strains K3 and K4 and \textit{Lb. paracasei} subsp. \textit{tolerans} K8; and one from raw buffalo milk: \textit{Lb. plantarum} subsp. \textit{plantarum} E41 as having the highest levels of cell surface hydrophobicity and auto-aggregation. Further characterization of these strains showed that all four isolates were completely stable after exposure to the pepsin and trypsin for 6 hours, and strains K8 and E41 remained viable after 6 hours of exposure to acidic conditions (pH 2.5) and bile salts (0.3\%). In addition, strains K3 and K4 were shown to inhibit the growth of potential human pathogens, including methicillin resistant \textit{Staphylococcus aureus}, \textit{Escherichia coli} and \textit{Salmonella typhi}, with inhibition zones being >14 mm for each bacterial target. Results from this study suggested that the four characterized LAB strains could survive passage through the gastrointestinal tract, thus supporting the need for additional studies to assess their potential as probiotics.

\section*{INTRODUCTION}
The protective role of a host’s indigenous microbiome against colonization of the gastrointestinal tract by foreign, potentially pathogenic, microorganisms has been considered the scientific basis for the development of probiotics.\textsuperscript{[1]} In 2001, the joint Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) Working Group defined probiotics as “live microorganisms which when administered in adequate amounts confer a health benefit on the host.”\textsuperscript{[2]} In 2014, the International Scientific Association for Probiotics and Prebiotics retained the FAO/WHO definition with a minor grammatical correction as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.”\textsuperscript{[3]} Several bacterial and fungal species have been investigated as potential probiotics, including species of lactic acid bacteria (LAB) and bifidobacteria. A variety of potential health-promoting activities have been attributed to the use of probiotics including: alleviation of inflammatory bowel disease, food allergies, lactose intolerance and hypercholesterolemia; anticancer, immunoregulatory and antioxidant effects; and the prevention of bacterial pathogen colonization and proliferation.\textsuperscript{[4]} Since these bioactivities are often strain dependent,
research continues to identify novel LAB strains capable of serving as probiotics for improving human and animal health.

The joint FAO/WHO Working Group has provided guidelines for assessing the safety of microorganisms for probiotic applications. While most LAB and bifidobacteria species are considered Generally Recognized as Safe (GRAS) microorganisms, they must also be tested for the following criteria: antibiotic resistance; specific metabolic activities (i.e. bile salt deconjugation); epidemiological surveillance for adverse effects in consumers; toxin production; and hemolytic activity.\(^5\) In addition, probiotics need to survive passage through the gastrointestinal tract (GIT) and potentially adhere to gut epithelial tissue and/or co-aggregate with bacteria to ensure their persistence. The adhesion properties have been anticipated as a critical factor for the selection of new probiotics and can easily be investigated using in vitro models of the intestine.\(^6,7\)

Previously, the GIT was considered the main source for isolation of novel probiotic bacteria, but recently attention has shifted to microorganisms present in traditional fermented foods (i.e. dairy products and fermented vegetables). Fermented vegetables have been the focus of several research studies because their indigenous microflora have a broad range of Lactobacillus species, including Lactobacillus (Lb.) plantarum, Lb. paracasei, Lb. casei, Lb. delbrueckii and Lb. brevis, all of which have been reported to have probiotic features.\(^8\)

Therefore, the aim of this study was to screen a variety of food products and fermented forage materials from Nanning city, China for the presence of lactic acid bacteria with promising probiotic traits, specifically: antimicrobial activity, cell surface hydrophobicity, auto-aggregation, acid tolerance, bile salt tolerance, trypsin and pepsin tolerance, and stability under cold storage. Additionally, potential probiotic strains were assessed for production of lactic acid during growth.

**MATERIALS AND METHODS**

**Isolation of lactic acid bacteria**

LAB were isolated from a variety of foods and dairy buffalo swabs in Nanning, China, including: raw buffalo milk (7 samples), semi-hard cheese produced from raw buffalo milk (3 samples), smoked cheese prepared from raw buffalo milk (1 sample), traditional fermented buffalo milk (4 samples), pickled vegetables (4 samples), fermented forage (2 samples); and surface swabs (10 cm\(^2\)) from the area around buffalo udder (4 samples). Samples were serially diluted in phosphate buffered saline (PBS) (8 g NaCl, 0.2 g KCl, 1.44 g Na\(_2\)HPO\(_4\), 0.24 g KH\(_2\)PO\(_4\), 1 L distilled water, pH 7). The pour-plate method was applied to isolate LAB using DeMan, Rogosa and Sharpe (MRS) and M17 agars (Qingdao Hope Bio-Technology Co., Ltd, China). Plates were incubated aerobically at 37°C for 24 h.

**Identification of LAB**

All bacterial isolates were identified according to 16S rRNA gene sequencing. Chromosomal DNA was extracted using Chelex-100 (Bio-Rad laboratories, CA, USA), and the entire 16S rRNA gene (~1.5 kb) was amplified with universal primers: forward: 5’-AGAGTTTTGATCCTGCTCA-3’ and reverse: 5’-GGTTACCTTGGTACAAGCTT-3’. PCR products were visualized using 2% agarose gel electrophoresis, and sequenced by Suzhou Jin Wei Zhi Biotechnology Co. (Nanning, China). The V1-V4 variable regions were compared with known sequences using BLAST (https://www.ncbi.nlm.nih.gov/) to identify the bacterial species. LAB isolates were routinely passaged in skim milk and stored under refrigeration conditions for further studies.

**Blood hemolysis assay**

Blood agar supplemented with sheep blood (5%) was used to characterize the hemolytic activity of LAB isolates. LAB from a 24 h slant culture were used to streak the surface of blood agar and incubated
at 37°C for 48 h. After incubation, the plates were observed for characteristic hemolytic zones (α, β or γ hemolysis).\textsuperscript{[9]}

**Cell surface hydrophobicity assay**

LAB isolates were cultured in MRS broth for 24 h at 37°C. Cells were collected by centrifugation (4000 x g for 10 min), washed twice with sterile saline (pH 7.0), and resuspended in the same solution at an OD\textsubscript{600} of 0.6–0.7 using a UV/Vis spectrophotometer (Metash UV-800, Shanghai, China). Two milliliters of the bacterial suspension were mixed with 2 mL of xylene and vortexed for 2 min. The mixture was then left undisturbed for 30 min to allow the two phases to separate,\textsuperscript{[10]} and the cell surface hydrophobicity (CSH) was calculated as a percentage from three replicates according to the following equation:

\[
\text{CSH\%} = \left[\frac{(A_0-\text{A})}{A_0}\right] \times 100
\]

where A\textsubscript{0} is the OD\textsubscript{600} before mixing and A is the OD\textsubscript{600} after 30 min of mixing.

**Auto-aggregation assay**

LAB broth cultures were centrifuged at 4000 x g for 10 min, and the harvested cells were washed twice and re-suspended in saline solution (OD\textsubscript{600} 0.6–0.7). Suspensions were incubated at 37°C for 1, 2, 3, 4 and 5 hours, after which the auto-aggregation (AA) percentage was calculated from four replicates using the following equation\textsuperscript{[10]}:

\[
\text{Auto-aggregation \%} = [1 - (\text{A_0/\text{A}})] \times 100
\]

where A\textsubscript{0} and A\textsubscript{t} represent OD\textsubscript{600} at zero time and OD\textsubscript{600} after selected time of incubation, respectively.

**Gastrointestinal tolerance**

The tolerance of LAB to conditions and enzymes within the gastrointestinal tract was assessed through exposure to bile salt (0.3%), acidic pH (2.5), pepsin (5 mg/mL) and trypsin (10 mg/mL), separately. For all tests, LAB were collected from an overnight broth culture by centrifugation at 4000 x g for 10 min, washed twice with saline solution and inoculated (10\textsuperscript{7}–10\textsuperscript{8} cfu/mL) into MRS broth supplemented with bile salt, pepsin or trypsin. Additionally, LAB were re-suspended in MRS broth with an adjusted pH to 2.5 to test for acid tolerance.\textsuperscript{[11]} LAB suspensions were incubated at 37°C, with viable cell counts determined using the pour-plate method after 2, 4 and 6 hours.

**Antibacterial activity assay**

The antibacterial activity of LAB isolates was assessed against methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 43300, *Escherichia coli* ATCC 25922 and *Salmonella typhi* ATCC 19430 using a well diffusion assay (cup – plate method).\textsuperscript{[12]} LAB were grown in MRS broth at 37°C for 24 h, after which cells were removed by centrifugation at 8000 x g for 10 min. The cell-free supernatant (CFS) was filtered through a cellulose acetate membrane filter with a pore size of 0.45 µm. The cup – plate method was performed using 3 sterilized Oxford cups placed equidistant on an agar base medium (2% agar). Subsequently, a layer of nutrient agar inoculated with 1% (v/v) of the indicator bacterial strain was overlaid on the agar base. Once solidified, all cups were removed, and the wells were filled with 150 µL of CFS. Plates were incubated at 37°C for 24 h and observed for the appearance of clear zone around the wells. The diameter of each inhibition zone (mm) was measured using vernier caliper and the results were expressed as mean ± SD. Measurement of cell-free supernatant pH was performed in parallel with this experiment using pH meter (PB-10, Sartorius Stedim Biotech GmbH, Gottingen, Germany).
Growth kinetics and acid production

To evaluate the relationship between LAB growth and acid production, MRS broth (150 mL) with an initial pH of 5.3–5.8 was inoculated with 2% (v/v, 3 mL) of an overnight MRS culture for each LAB isolate. The initial optical density was 0.6 at 600 nm to standardize the bacterial count to approximately 10^6 cfu/mL. The growth curve was established by monitoring the optical density (OD_{600}) every 2 hours for 24 hours. At each time point, the pH value was also measured using pH meter (PB-10). The growth rate and generation time were calculated applying the following equations:\(^{[13]}\):
\[
\text{Growth rate}(r) = \frac{\ln(\text{OD}_2/\text{OD}_1)}{T_2-T_1}
\]
As OD\(_1\) and OD\(_2\) are the OD\(_{600}\) values at the time T\(_1\) and T\(_2\), respectively.

\[
\text{Generation time} = \ln 2/r
\]

Statistical analysis

The data were presented as mean ± standard deviations (SD). One-way analysis of variance (ANOVA) was performed by Statistix 8.1 (Analytical Software, Tallahassee, USA) followed by assessment of differences by Tukey’s test at P < .05.

RESULTS

Isolation and identification of LAB

In the present study, a total of 246 bacterial colonies collected from the seven surveyed locations were selected randomly for further analysis by 16S rDNA sequencing. Gene sequencing revealed that 60 of the 246 total isolates were LAB, with the highest number of LAB identified in raw buffalo milk (18 of 106, 16.9%) and semi-hard cheese (18 of 39 isolates, 46.15%) (Table S1). Raw buffalo milk was shown to have the most diverse collection of LAB, which included the following species: Lactobacillus (Lb) plantarum subsp. plantarum (10), Lb. pentosus (2), Lb. paracasei subsp. tolerans (2), Lb. fermentum (2), Lactococcus petauri (1), and Streptococcus parasuis (1). Within semi-hard cheese the LAB isolates represented four Lactobacillus species, including: Lb. plantarum subsp. plantarum (8), Lb. pentosus (5), Lb. paracasei subsp. tolerans (4) and Lb. fermentum (10) (Table S1). In the remaining samples, LAB represented between 20 and 33% of the isolates sequenced. Results from the sequencing of all isolates are summarized in Table S1.

Lb. plantarum subsp. plantarum was the most prevalent LAB species identified from the isolates in raw buffalo milk (55.5%), semi-hard cheese (44.4%) and smoked cheese (100%); followed by Lb. pentosus in buffalo milk (11.1%) and semi-hard cheese (27.8%) (Table 1). Lb. paracasei subsp. tolerans was the dominant LAB isolated from pickled vegetables (75%) and fermented forage (50%) (Table 1). In fermented milk samples, isolates of Lb. paracasei subsp. tolerans, L. petauri and Lb. pantheris were identified, while the surface around the buffalo udder was shown to contain L. petauri (37.5%), Lb. paracasei subsp. tolerans (25%), Lb. fermentum (12.5%), Lb. pantheris (12.5%) and Enterococcus olivae (12.5%) (Table 1). All LAB isolates were screened for hemolytic activity and shown to have no zone or a greenish zone around each colony, suggesting they were non-hemolytic or gamma-hemolytic (data not shown).

Cell surface hydrophobicity assay

The cell surface hydrophobicity was estimated for all LAB isolates, with 10 isolates showing levels ranging between 17.27 ± 5.5 and 97.13 ± 0.8% (Table 2). The highest cell surface hydrophobicities were observed for Lb. plantarum subsp. plantarum K3 (CSH = 93.78 ± 0.6%), Lb. plantarum subsp. plantarum K4 (CSH = 96.5 ± 0.8%) and Lb. paracasei subsp. tolerans K8 (CSH = 97.13 ± 0.8%) from semi-hard cheese, and Lb. plantarum subsp. plantarum E41 (CSH = 93.31 ± 2.1%) from buffalo milk. It was also observed that Lactobacillus plantarum subsp. plantarum F39 from semi-hard cheese
Table 1. Prevalence of LAB in tested samples.

| Sample            | Lb. plantarum subsp. planatarum | Lb. pentosus | Lb. fermentum | Lb. paracasei subsp. tolerans | Lb. pantheris | Lac. petauri | Str. paralis | Ent. olivae | Weissella para-mesenteroides |
|-------------------|---------------------------------|--------------|---------------|-------------------------------|---------------|-------------|-------------|-------------|---------------------------------|
| Raw buffalo milk  | 55.5                            | 11.1         | 11.1          | 11.1                          | –             | 5.6         | 5.6         | –           | –                                |
| Semi-hard cheese  | 44.44                           | 27.8         | 5.6           | 22.2                          | –             | –           | –           | –           | –                                |
| Smoked cheese     | 100                             | –            | –             | –                             | –             | –           | –           | –           | –                                |
| Fermented milk    | –                               | –            | 33.3          | 33.3                          | 33.3          | –           | –           | –           | –                                |
| Pickled vegetables| –                               | 25           | –             | 75                            | –             | –           | –           | –           | –                                |
| Fermented forage  | –                               | 12.5         | –             | 50                            | 25            | –           | –           | –           | –                                |
| Area around the udder | –                               | –            | 12.5          | 25                            | 12.5          | 37.5        | –           | 12.5        | –                                |

Table 2. Percentage of cell surface hydrophobicity and auto-aggregation of isolated LAB.

| Isolate code | Top-hit taxon | Food product | CSH (%) | Auto-aggregation (%) |
|--------------|---------------|--------------|---------|----------------------|
|              |               |              |         | 1 h | 2 h | 3 h | 4 h | 5 h |
| F39          | Lb. plantarum subsp. planatarum | Semi-hard cheese | 28.32 ± 4.88 | 8.4 ± 2.8 | 10.2 ± 3.6 | 15.6 ± 4.6 | 22.2 ± 1.1 | 30 ± 1.1 |
| K3           | Lb. plantarum | 93.78 ± 0.6B | 12.6 ± 3.9 | 23.1 ± 2.3 | 26.7 ± 5.8 | 44.2 ± 14.7 | 52.6 ± 12.1 |
| K4           | Lb. paracasei subsp. tolerans | 96.5 ± 0.8A | 10.9 ± 0.7 | 19.4 ± 1.9 | 21.7 ± 3.5 | 32.6 ± 7.3 | 41.0 ± 1.9 |
| K8           | Lb. paracasei subsp. tolerans | 97.13 ± 0.8A | 10.1 ± 0.9 | 16.5 ± 0.5 | 19.03 ± 2.8 | 16.5 ± 0.5 | 22.9 ± 2.6 |
| K43          | Lb. fermentum subsp. plantarum | 17.27 ± 5.5A | 4.9 ± 1.1 | 7.3 ± 0.5 | 8.2 ± 0.4 | 10.0 ± 1.2 | 9.1 ± 0.5 |
| E41          | Lb. plantarum subsp. planatarum | 93.31 ± 2.1A | 13.0 ± 2.8 | 20.3 ± 6.3 | 27.8 ± 2.7 | 38.04 ± 4.1 | 47.5 ± 2.8 |
| K24          | Lb. pentosus | 72.2 ± 4.7B | 8.8 ± 1.3 | 18.5 ± 2.0 | 24.8 ± 1.6 | 34.1 ± 0.4 | 37.6 ± 3.0 |
| L22          | Lac. petauri | 57.54 ± 3.4A | 12.8 ± 0.9 | 27.1 ± 2.3 | 35.4 ± 4.6 | 38.95 ± 7.8 | 34.3 ± 11.1 |
| K41          | Lb. fermentum subsp. plantarum | 47.54 ± 6CD | 5.95 ± 0.3 | 9.8 ± 1.5 | 13 ± 3.3 | 15.1 ± 4.4 | 12.8 ± 1.5 |
| N9           | Lac. petauri | 73.5 ± 5.8B | 6.6 ± 0.5 | 10.5 ± 1.3 | 14.9 ± 3.5 | 18.1 ± 3.2 | 27.7 ± 1.5 |

Data are means ± SD (n = 3). Different superscript letters in the same column are significantly different (P < 0.05).

had significantly lower cell surface hydrophobicity when compared to isolates K3 and K4. This confirmed the isolation of multiple strains of Lactobacillus plantarum subsp. plantarum from semi-hard cheese, and that the high level of CSH observed for isolates K3 and K4 was strain dependent.

Auto-aggregation assay

The 10 isolates with the highest CSH were further tested for the auto-aggregation (AA) capability (Table 2). Results showed that auto-aggregation increased over time for most strains, with the exceptions being two Lb. fermentum isolates (K41 and K43) and Lactococcus petauri isolate L22, where AA% peaked at 4 h (Table 2). Three Lb. plantarum subsp. plantarum isolates (K3, K4 and E41) and one Lactobacillus paracasei subsp. tolerans isolate (K8) showed optimal CSH and intermediate to optimal AA activity. Lactobacillus plantarum subsp. plantarum K3 and K4 showed the highest levels of CSH and AA, and the variation in their observed levels suggested they were two novel strains of this species. Based on the CSH and AA results, Lb. plantarum subsp. plantarum isolates K3, K4 and E41; and Lactobacillus paracasei subsp. tolerans K8 were selected for further characterization of potential probiotic traits.

Gastrointestinal juice tolerance

Lb. plantarum subsp. plantarum isolates K3, K4 and E41, and Lactobacillus paracasei subsp. tolerans K8 were further evaluated for their ability to survive exposure to stresses present within the GIT,
Table 3. Stability of K3, K4, K8 and E41 isolates to the components of gastrointestinal juice.

| Bacterial count (Log cfu/mL) resistant to acidity (pH 2.5) |
|-----------------|-------|-------|-------|-------|
| Isolate code    | 0 h   | 2 h   | 4 h   | 6 h   |
| Lb. plantarum subsp. plantarum K3 | 7.55 ± 0.11 Da | 7.44 ± 0.12 Da | 7.52 ± 0.13 Da | 7.61 ± 0.07 Da |
| Lb. plantarum subsp. plantarum K4 | 8.21 ± 0.05Ca | 8.16 ± 0.10Ca | 8.03 ± 0.12Ca | 8.07 ± 0.14Ca |
| Lb. paracasei subsp. tolerans K8 | 8.55 ± 0.11Ba | 8.44 ± 0.12Ba | 8.52 ± 0.13Ba | 8.61 ± 0.07Ba |
| Lb. plantarum subsp. plantarum E41 | 8.95 ± 0.10aA | 8.85 ± 0.12aA | 8.81 ± 0.14aA | 8.88 ± 0.09aA |

| Bacterial count (Log cfu/mL) resistant to bile salt (0.3%) |
|-----------------|-------|-------|-------|
| Isolate code    | 0 h   | 2 h   | 4 h   | 6 h   |
| Lb. plantarum subsp. plantarum K3 | 7.72 ± 0.09aA | 6.58 ± 0.08bB | 5.22 ± 0.06cC | 5.21 ± 0.04dc |
| Lb. plantarum subsp. plantarum K4 | 8.68 ± 0.17caA | 8.97 ± 0.11cbB | 7.56 ± 0.13cbB | 7.21 ± 0.08bc |
| Lb. paracasei subsp. tolerans K8 | 8.4 ± 0.11aA | 8.37 ± 0.14Aa | 8.29 ± 0.09aA | 8.12 ± 0.13aA |
| Lb. plantarum subsp. plantarum E41 | 8.25 ± 0.14Ba | 8.24 ± 0.14Ba | 8 ± 0.22aA | 7.98 ± 0.15aA |

| Bacterial count (Log cfu/mL) resistant to pepsin enzyme (5mg/mL) |
|-----------------|-------|------|-------|-------|
| Isolate code    | 0 h   | 2 h   | 4 h   | 6 h   |
| Lb. plantarum subsp. plantarum K3 | 9.02 ± 0.16aA | 8.86 ± 0.06Ba | 8.81 ± 0.07Ba | 8.96 ± 0.18Ba |
| Lb. plantarum subsp. plantarum K4 | 8.95 ± 0.14aA | 8.92 ± 0.12Ba | 8.92 ± 0.19baAb | 8.85 ± 0.20Ba |
| Lb. paracasei subsp. tolerans K8 | 9.12 ± 0.18aA | 9.18 ± 0.1aA | 9.12 ± 0.16aA | 9.22 ± 0.12aA |
| Lb. plantarum subsp. plantarum E41 | 9.03 ± 0.1aA | 8.82 ± 0.12Ba | 8.88 ± 0.13baAb | 9.35 ± 0.56aA |

| Bacterial count (Log cfu/mL) resistant to trypsin enzyme (10 mg/mL) |
|-----------------|-------|------|-------|-------|
| Isolate code    | 0 h   | 2 h   | 4 h   | 6 h   |
| Lb. plantarum subsp. plantarum K3 | 8.74 ± 0.12Ba | 8.45 ± 0.35Ba | 8.51 ± 0.24aA | 8.44 ± 0.2aA |
| Lb. plantarum subsp. plantarum K4 | 8.89 ± 0.25aAb | 8.15 ± 0.13abB | 8.44 ± 0.13aAb | 8.57 ± 0.21abAb |
| Lb. paracasei subsp. tolerans K8 | 9.19 ± 0.07aA | 8.71 ± 0.39aA | 8.68 ± 0.17aA | 9.04 ± 0.1aA |
| Lb. plantarum subsp. plantarum E41 | 8.9 ± 0.05aAb | 8.5 ± 0.18aB | 8.55 ± 0.06aAb | 8.83 ± 0.38aA |

Data are means ±SD (n = 3). For each treatment, different uppercase letters within the same column indicate significant differences (P < 0.05) in the survival of isolates at a single time point. Different lowercase letters within the same row indicate significant differences (P < 0.05) in the survival of each isolate over time.

including: bile salts (0.3%), low pH (2.5), pepsin (5 mg/mL) and trypsin (10 mg/mL) (Table 3). The four strains showed no significant loss in viability after exposure to pH 2.5 for up to 6 h, and appeared resistant to pepsin and trypsin exposure for up to 6 h. However, exposure to bile salts was shown to reduce the viability of Lb. plantarum subsp. plantarum isolates K3 and K4 by approximately 1.14 and 2.81 log CFU/ml respectively, after 2 h. Cell viability continued to drop over time, with both cultures having approximately 5.2 log CFU/ml viable cells after 6 h.

**Antibacterial activity**

Cell-free supernatants collected from overnight cultures of Lb. plantarum subsp. plantarum isolates K3, K4 and E41, and Lactobacillus paracasei subsp. tolerans K8 were screened for antimicrobial activity against methicillin-resistant Staphylococcus aureus (MRSA), Escherichia coli and Salmonella typhi (Table 4). Inhibition zones were observed (≥13 mm) against all three target bacteria with CFS from Lb. plantarum subsp. plantarum isolates K3 and K4, and Lb. paracasei subsp. tolerans K8. CFS from Lb. plantarum subsp. plantarum isolate E41 was not shown to inhibit the growth of any targeted pathogen. CFS from Lb. plantarum subsp. plantarum isolates K3 and K4 had the lowest pH values and the largest inhibition zones, but the difference in pH was negligible for CFS from Lb. plantarum subsp. plantarum and Lb. paracasei subsp. tolerans K8, yet only the later displayed antimicrobial activity. This suggested that low pH contributed to the observed antimicrobial activities; but was solely responsible for inhibition of target cell growth.

**Lactic acid bacterial growth and acid production**

Growth and acid production were monitored for the four LAB isolates in MRS broth for 24 hours (Figure 1). The growth rates of Lb. plantarum subsp. plantarum K3, K4 and E41 were 0.32, 0.3 and 0.51/h; and the generation times were 2.17, 2.3, and 1.4 h, respectively. Lactobacillus paracasei subsp.
toleransK8 had a growth rate of 0.7 h and a generation time of 1.0 h. The culture pH values were also recorded after 13 h and dropped from the initial 5.8–5.9 to between 4.1 and 4.5 by the end of exponential growth (Figure 1), which agreed with results reported in Table 4.

**DISCUSSION**

Extensive research has focused on the isolation and characterization of probiotics from human sources, but recent investigations have emphasized the characterization and identification of probiotic bacteria isolated from other sources such as fermented foods and dairy products. In this study, 60 LAB isolates were obtained from either raw buffalo milk, semi-hard cheese, smoked cheese, yogurt, pickled vegetables, fermented forage and swabs obtained from the area surrounding the buffalo udder.

![Figure 1](image_url) OD600 values and pH changes during the growth of *Lb. plantarum* subsp. *plantarum* K3, *Lb. plantarum* subsp. *plantarum* K4, *Lb. paracasei* subsp. *tolerans* K8 and *Lb. plantarum* subsp. *plantarum* E41 lactic acid bacterial isolates for 24 hours.
Table 4. Antibacterial activity of K3, K4, K8 and E41 isolates.

| Isolate code | Diameter of the inhibition zone (mm) | pH value |
|--------------|-------------------------------------|----------|
|              | MRSA                               | E. coli  | Salmonella typhi |
| Lb. plantarum subsp. plantarum K3 | 17.89 ± 0.35<sup>A</sup> | 16.97 ± 0.44<sup>A</sup> | 17.94 ± 0.75<sup>B</sup> | 3.74 |
| Lb. plantarum subsp. plantarum K4 | 14.39 ± 0.63<sup>B</sup> | 15.88 ± 0.23<sup>B</sup> | 18.12 ± 0.59<sup>B</sup> | 3.79 |
| Lb. paracasei subsp. tolerans K8 | 13.16 ± 0.64<sup>C</sup> | 14.41 ± 0.67<sup>C</sup> | 15.86 ± 0.43<sup>C</sup> | 3.96 |
| Lb. plantarum subsp. plantarum E41 | Resistant | Resistant | Resistant | 4.0 |

Data are means ±SD (n = 3). Different superscript letters in the same column are significantly different (P <0.05).

All isolates were identified using 16S rRNA gene sequencing and shown to be gamma- or non-hemolytic, suggesting they were safe to pursue as potential probiotic strains. All LAB isolates were evaluated for cell surface hydrophobicity (CSH), with the 10 strains showing the highest CSH activity further screened for their auto-aggregation (AA) ability.

CSH and auto-aggregation (AA) are potentially important traits for probiotic candidates, as they would affect the bacterium’s ability to adhere to the intestinal mucosa and form biofilms on different surfaces. Previous studies have suggested cells with a CSH > 40% are considered hydrophobic. In the current study, 80% of tested LAB isolates had a CSH between 47.54 ± 6 and 97.13 ± 0.8%, confirming their hydrophobic nature. Furthermore, seven LAB isolates with the highest CSH activities also showed auto-aggregation levels above 25%. These results are comparable to previous studies which showed LAB with comparable CSH and AA activities survived exposure to gastrointestinal stresses and adhered to epithelial cells. These activities are believed to increase the probiotic potential of LAB allowing them to persist longer within the host to convey their health benefits. CSH and AA results from this study suggested that isolates K3, K4 and E41, identified as novel strains of Lb. plantarum subsp. plantarum, and isolate K8, identified as Lb. paracasei subsp. tolerans, should be further investigated for their potential as probiotics.

In order to convey a health benefit to the host, probiotic bacteria must be able to survive passage through the GIT; and results from this study showed that Lb. plantarum subsp. plantarum isolates K3, K4, and E41; and Lb. paracasei subsp. tolerans K8 showed little or no loss in cell viability after in vitro exposure to low pH and digestive enzymes (pepsin and trypsin). Resistance to bile salts has also been reported to be one of the most essential properties required of a potential probiotic active within the small intestine; and in this study, Lb. plantarum subsp. plantarum E41 and Lb. paracasei subsp. tolerans K8 were resistant to bile salt exposure for up to 6 h. Viability of Lb. plantarum subsp. plantarum isolates K3 and K4 was reduced by 2–3 log CFU/ml after 6 hours of exposure to bile salts. This loss in viability was most likely due to leakage of intracellular material as bile salts have been reported to permeabilize bacterial cell membranes. Previous studies have shown that the antimicrobial activity of bile salts depends on their concentration. High concentrations have been reported to dissolve membrane lipids, causing leakage of cell materials and cell death, while low concentrations may affect membrane fluidity and permeability by altering membrane-bound proteins or increasing the flow of divalent cations through the membrane. Our results suggest that the Lb. plantarum subsp. plantarum isolates K3 and K4 may require protection when being delivered if they are intended to reach the colon to convey health benefits to a host.

Another probiotic trait investigated was the inhibition of human pathogens by Lb. plantarum subsp. plantarum isolates K3, K4 and E41; and Lb. paracasei subsp. tolerans K8. LAB have been reported to display antagonistic activities against pathogenic and food spoilage microorganisms through production of lactic acid, or other antimicrobial agents including carbon dioxide, hydrogen peroxide and low molecular peptides, called bacteriocins. The antimicrobial effect of H₂O₂ may result from oxidation of sulphydryl groups causing denaturation of enzymes, and from the peroxidation of membrane lipids causing permeabilization of the cell membrane. H₂O₂ may also be a precursor for the production of bactericidal free radicals which can damage DNA. Carbon dioxide is mainly produced by heterofermentative LAB and contributes to the creation of an anaerobic environment which inhibits enzymatic
decarboxylation. In addition, accumulation of CO₂ in the lipid bilayer may cause dysfunction of the cell membrane. [30] Bacteriocins are peptides or peptide complexes (usually 30–60 amino acids) naturally produced by some LAB, which exhibit bacteriostatic or bactericidal activity mainly against closely related species. [31] However, some bacteriocins also display broad-spectrum activity, which has resulted in their investigation as alternatives to chemical food preservatives or antibiotics. [29] While several mechanisms of action have been shown for these peptides, many act by forming pores within the bacterial cell membrane, resulting in the leakage of intracellular material and possibly cell death. [32] In this study, cell-free supernatants from *Lb. plantarum* subsp. *plantarum* K3 and K4; and *Lb. paracasei* subsp. *tolerans* K8 were shown to have antimicrobial activity against MRSA, *E. coli* and *Salmonella typhi*. Recent studies reported the antimicrobial activity of *Lb. plantarum* against *Staphylococcus aureus, Salmonella typhimurium, Escherichia coli* O157:H7 and *Listeria monocytogenes*. [33,34] While there are some reports describing LAB bacteriocin activity against Gram-negative foodborne pathogens, [35] these peptides are more often active against Gram-positive pathogens, such as *Listeria monocytogenes*. [36] More studies are required to determine the antimicrobial compounds produced by these three LAB isolates, but the lack of antimicrobial activity for CFS from isolate E41, which had a similar pH to the others, suggests that the production of organic acid alone is not responsible for the broad-spectrum activity.

In addition to surviving passage through the gastrointestinal tract, LAB that are intended to serve as probiotics within food must be able to survive under typical storage conditions for the intended product. In this study, *Lb. plantarum* subsp. *plantarum* isolates K3, K4, E41, and *Lb. paracasei* subsp. *tolerans* K8 were all shown to survive at refrigeration temperature (4°C) without a reduction in viable cell count after 15 days of storage. This suggested that they may be ideal cultures for use in fermented dairy products which are stored under refrigeration. Future studies are needed to investigate if the cultures are capable of surviving at this temperature within a dairy environment.

Finally, the relationship between growth and acid production was assessed for *Lb. plantarum* subsp. *plantarum* isolates K3, K4, and E41, and *Lb. paracasei* subsp. *tolerans* K8. As expected, the greatest drop in pH occurred during the log phase for all isolates as the pH was reduced by 28.4, 27.6, 23.7 and 28.6% for each isolate, respectively. The final pH for all cultures was ≤4.2, which was similar to previous reports for fermented meat products where LAB cell counts reached 6–8 log cfu/g of meat. [37–39]

**CONCLUSION**

In conclusion, the *Lactobacillus plantarum* subsp. *plantarum* isolates K3, K4 and E41, and *Lactobacillus paracasei* subsp. *tolerans* K8 were partially characterized in vitro to demonstrate their potential probiotic traits. Results from these studies suggest that *Lb. paracasei* subsp. *tolerans*, which was isolated from semi-hard cheese, exhibited promising probiotic properties, including antimicrobial activity against MRSA, *E. coli* and *Salmonella typhi*. This isolate also had high cell surface hydrophobicity and auto-aggregation capability; and cell viability was not affected by the gastrointestinal stresses studied. *Lb. plantarum* subsp. *plantarum* E41, isolated from buffalo milk, was also resistant to the gastrointestinal stresses; however, it did not show antimicrobial activity against any of the targeted pathogens. *Lb. plantarum* subsp. *plantarum* isolates K3 and K4 were negatively affected by exposure to bile salts but were resistant to the other GIT stresses tested and displayed broad-spectrum antimicrobial activity against the targeted bacterial pathogens. The results presented in this study are promising for the probiotic potential of these four strains; more studies are required to assess their true potential, including antibiotic susceptibility, co-aggregation capability with pathogenic bacteria, simulated gastrointestinal juice tolerance, and phenol tolerance.

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