fermentation

MDPI

Article

In Vitro Characterisation of Potential Probiotic Bacteria Isolated from a Naturally Fermented Carrot and Ginger Brine

Nontobeko Cele, Babalwa Nyide and Thandeka Khoza *

Discipline of Biochemistry, School of Life Sciences, University of Kwa-Zulu Natal (Pietermaritzburg Campus), Scottsville, Pietermaritzburg 3209, KwaZulu-Natal, South Africa
* Correspondence: khozat1@ukzn.ac.za

Abstract: Unhealthy dietary patterns have been associated with an increase in mortality rate as well as the high occurrence of nontransmissible chronic degenerative diseases. Subsequently, the development of new functional foods has been proposed to reduce the incidence of non-transmissible chronic degenerative diseases. Probiotics represent a group of functional foods, defined as live microbial feeds, which provide the host with intestinal health benefits. The present study focused on the identification and characterisation of the probiotic potential of lactic acid bacteria isolated from a fermented carrot and ginger brine. Sixteen isolates were identified as *Leuconostoc mesenteroides subsp. mesenteroides* species, following preliminary screening based on 16S rDNA gene sequencing, and were further characterised for probiotic candidature. The probiotic properties tested included resistance towards gastrointestinal conditions (bile, acid, lysozyme tolerance), cell surface hydrophobicity, antioxidant activity, and antagonistic activity against intestinal pathogens. In general, all the isolated *Leuconostoc mesenteroides subsp. mesenteroides* strains exhibited high acid, bile, and lysozyme tolerance. They also showed strong antibacterial activity against common intestinal pathogens, i.e., *Staphylococcus aureus* and *Escherichia coli*, as well as antioxidant activity such as hydroxyl radical-scavenging ability and hydrogen peroxide resistance. Overall, *Leuconostoc mesenteroides subsp. mesenteroides* possesses a great potential as a beneficial strain for functional food.

Keywords: functional foods; lactic acid bacteria; *Leuconostoc mesenteroides subsp. mesenteroides*; probiotics

Citation: Cele, N.; Nyide, B.; Khoza, T. *In Vitro* Characterisation of Potential Probiotic Bacteria Isolated from a Naturally Fermented Carrot and Ginger Brine. *Fermentation* **2022**, *8*, 534. https://doi.org/10.3390/fermentation8100534

Academic Editor: Teresa Gervasi

Received: 11 September 2022
Accepted: 9 October 2022
Published: 12 October 2022

1. Introduction

The increasing incidence of chronic lifestyle diseases and disorders has become a global concern, with the rapid progression of society into the age of digitisation and automation. Central to this issue is the rise of highly processed convenience foods and the sedentary lifestyle of the digital age, which cause frequent imbalances between high-energy intake and reduced physical activity [1,2]. However, the introduction of probiotics as components of functional foods can improve the nutritional and prophylactic value of the modern diet, to curb the incidence of both communicable and noncommunicable diseases [3,4]. The Food and Agriculture Organisation (FAO) and the World Health Organisation (WHO) define probiotics as live microorganisms that confer a health benefit on the host when administered in adequate amounts [5]. As such, probiotics can be used as viable foods or dietary supplements for human and animal consumption [6].

There are several criteria that microbial strains should fulfil to be considered as probiotics [7]. Firstly, probiotic strains should be natural nonpathogenic inhabitants of the host and be generally regarded as safe (GRAS) [8–10]. Moreover, they should be acid- and bile-tolerant to survive the digestive enzymes, stomach acid, and bile salts of the digestive tract [11–13]. Furthermore, probiotic strains must exhibit considerable surface hydrophobicity and good aggregation properties to facilitate colonisation of the lining

of the gastrointestinal tract (GIT) [14]. They must adhere to the mucosal surface to colonize the intestinal wall and stimulate the immune system without effecting inflammatory responses [13,15,16]. Importantly, probiotic strains are expected to prevent adhesion and colonisation by common pathogenic bacteria, through the production of bactericidal metabolites (bacteriocins) [14,15]. Lastly, probiotic strains must exhibit good technological properties such as long-term stability for commercialisation, phage resistance, and large-scale production, and they should have no adverse effects on the organoleptic properties when applied to foods [17–19]. Although in vitro data alone are insufficient to conclude on the eligibility of a strain as a probiotic, it can provide valuable insight as a screening tool.

The origins of probiotics are closely linked to the consumption of fermented food, and, to date, lactic acid bacteria (LAB) from the *Lactobacillus* and *Bifidobacterium* genera are the most common and well-studied probiotic bacteria [20–22]. Some probiotic species of *Lactobacillus* and *Bifidobacterium* are natural inhabitants of the human GIT and have been associated with improving the management of irritable bowel syndrome, antibiotic-associated diarrhoea, cancer, and metabolic disorders [22–24]. However, despite the promising health benefits associated with probiotics in humans, exhaustive research is still required to justify their therapeutic properties; hence, probiotics can be considered as prophylactic agents rather than therapeutics. Nevertheless, this has not impeded the widespread application of probiotics in the food industry, where probiotics are exploited for the enzymes, exopolysaccharides, antimicrobials, aromatic compounds, and nutraceuticals they produce, which exhibit nutritional and biopreservative properties [25].

Today, consumers are aware of the relationship between diet, lifestyle, and health, which explains the growing demand for functional foods with enhanced nutritional value [8,26]. Consequently, the food industry is exploring the probiotification of everyday foods, to create a new generation of "probiotic health foods" [8,26]. However, due to economic challenges, the use of sophisticated techniques for the cultivation, characterisation, and screening of new probiotic isolates remains beyond the scope of most laboratories in developing countries; hence, simple and cost-effective methods are required [27]. Fruits and vegetables are readily accessible and naturally rich sources of vitamins, carbohydrates, and essential nutrients, which makes them a suitable and affordable cultivation media for probiotics [28,29]. The present study sought to isolate and identify potential probiotic LAB strains from a naturally fermented carrot and ginger brine using 16S rDNA gene sequencing and to evaluate the probiotic potential of these LAB isolates using simple in vitro tests. Here, we present LAB isolates identified as *Leuconostoc mesenteroides subsp. mesenteroides* that exhibited desirable probiotic properties under in vitro conditions.

2. Materials and Methods

2.1. Fermentation and Physicochemical Analysis of Carrot and Ginger Brine

Carrot and ginger were washed, peeled, grated, and mixed in a 2.5% brine concentration and left to ferment at room temperature for 14 days. During each day of fermentation, a sample was taken to analyse pH, titratable acidity, and total soluble solids.

pH and Titratable Acidity

The pH value of the brine was determined using a digital pH meter (OHAUS, New Jersey, NJ, USA). Titratable acidity expressed as lactic acid was estimated, as previously described by Tyl and Sadler [30]. Briefly, titratable acidity was determined by titrating the known quantity of carrot and ginger brine (15 mL) against standardised 0.1 M NaOH, using a few drops of 1% phenolphthalein solution as an indicator of the pink endpoint.

Results were expressed as percentage of lactic acid:

$$\% \text{ Lactic acid} = \frac{N \times V_1 \times Eq\ Wt}{V_2 \times 1000} \times 100$$

N = normality of titrant (mEq/mL);
V_1 = volume of titrant (mL);

Eq Wt = equivalent weight of predominant acid (mg/mEq);
V_2 = volume of sample (mL);
1000 = factor relating mg to g (mg/g).

2.2. Enumeration of Yeasts, Coliforms, and LAB

To determine the number of yeasts, coliforms, and LAB from each day of fermentation, tenfold serial dilutions of up to 10^{-6} from the carrot and ginger brine were prepared using sterile saline solution (0.85% w/v NaCl). An aliquot of each dilution was plated out on Yeast Extract Peptone Dextrose (YPD) agar (Sigma-Aldrich, St. Louis, MO, USA), MacConkey agar (Sigma-Aldrich), and de Man, Rogosa, and Sharpe (MRS) agar (Sigma-Aldrich) in triplicates for yeast, coliform, and LAB count, respectively. YPD and MacConkey agar plates were incubated under aerobic conditions, while MRS agar plates were incubated under anaerobic conditions in an Anaerobic Gas-Pack system (ThermoFisher Scientific, Waltham, MA, USA) for 48 h at 37 °C. The total colony forming units per millilitre (cfu/mL) of yeast, coliforms, and LAB were recorded.

2.3. Isolation and Selection of LAB

Colonies with different appearances (colour, shape, size) were picked from the MRS agar plates. The selected colonies were repeatedly streaked on fresh MRS agar plate until single colonies with distinct appearances were obtained. The obtained colonies underwent preliminary tests, namely Gram staining, catalase production, growth at different temperatures, and carbon dioxide production from glucose [31–33].

2.4. Identification of LAB Using API 50 CHL Kits and 16S rDNA Sequencing

Biochemical characterisation of the isolates was performed using the API 50 CH kit (BioMerieux, Marcy-l'Étoile, France), which determines their ability to ferment carbohydrate substrates. All tests were performed in accordance with the instructions of the manufacturer. Further to this, the colonies identified as putative LAB were further confirmed to species level using 16S rDNA gene-targeted colony PCR. Each reaction mixture (final volume 25 µL) contained 0.5 µL of 10 µM primers: 15F (5′-GCTCAGGAYGAACGCYGG-3′) and 687R (5′-CACCGCTACACATGRADTTC-3′), 10.5 µL of distilled water, 1 µL of colony suspension, and 12.5 uL of TAKARA-EmeraldAmpGT PCR master mix (Separations, Johannesburg, South Africa). The PCR conditions were as follows: initial denaturation for 30 s at 94 °C and then 30 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 50 °C, extension for 30 s at 68 °C, and final extension for 5 min at 68 °C. The resulting PCR products were resolved on a 1% (w/v) agarose gel using 1 × TAE buffer and visualised under UV light. The PCR products were then sent for sequencing at the Kwazulu-Natal Research and Innovation Sequencing Platform (KRISP). To identify the LAB isolates, a blast search was performed for the resulting sequences using BLAST search engine http://www.ncbi.nlm.nih.gov/blast (accessed on 3 April 2020).

2.5. Assessment of Probiotic Potential of LAB

2.5.1. Tolerance to Low pH

The acid tolerance test was studied using a slightly modified method, as previously described by Hedin et al. [34]. Briefly, overnight grown cultures of the test strains were centrifuged for 10 min at 8000× *g*, and the resulting pellet was resuspended into MRS broth (Sigma-Aldrich), previously adjusted with hydrochloric acid (HCl) to pH 2.0 and pH 3.0. Bacterial cells that were resuspended in MRS broth (Sigma-Aldrich) containing no HCl were used as the control. The cultures were incubated anaerobically for 3 h at 37 °C. The viable cell count as a measure of bacterial growth was determined by subculturing at 3 h intervals and plating the serially diluted portions on MRS agar medium (Sigma-Aldrich). These were anaerobically incubated at 37 °C for 48 h in an anaerobic jar (ThermoFisher Scientific) with an AnaeroGen 2.5 L sachet (ThermoFisher Scientific). *Lactobacillus reuteri Protectis* DSM17938 was used as the reference strain.

2.5.2. Bile Salt Tolerance

The method of Walker and Gilliland [35] was used with minor modifications to assess bile tolerance of the 16 LAB isolates. Briefly, the isolates were inoculated into MRS-Thio-broth (Sigma-Aldrich), supplemented with or without Oxgall (Sigma-Aldrich), at a concentration of 0.3% or 0.5% (w/v). Then, the bacterial cultures were incubated in an anaerobic jar (ThermoFisher Scientific) with an AnaeroGen 2.5 L sachet (ThermoFisher Scientific) for 4 h at 37 °C. Thereafter, the optical density of the cultures was determined at 620 nm (OD_{620}) as a measure of cell viability, and the bile resistance was calculated using the following equation:

$$\text{Bile resistance } (\%) = \frac{\text{OD620 in MRS} - \text{Thio} - \text{broth with oxgall}}{\text{OD620 in MRS} - \text{Thio} - \text{broth without oxgall}} \times 100\%$$

2.5.3. Lysozyme Resistance

Lysozyme resistance was assessed per the method of Dias et al. [36], with some modifications. Briefly, bacterial isolates were incubated in MRS broth (Sigma-Aldrich) overnight at 37 °C, pelleted by centrifugation (4500× *g*, 6 min, 4 °C), washed twice in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4 pH 7.4), and resuspended in PBS. An aliquot of the bacterial suspension was inoculated into MRS broth (Sigma-Aldrich), supplemented with 50 or 100 µg/mL lysozyme (Sigma-Aldrich), and a bacterial suspension inoculated into MRS broth without lysozyme was prepared as the control. All the samples were incubated for 180 min at 37 °C, and microbial counts were conducted on MRS agar (Sigma-Aldrich) plate after 48 h of incubation at 37 °C in an anaerobic jar (ThermoFisher Scientific) with an AnaeroGen 2.5 L sachet (ThermoFisher Scientific).

2.5.4. Cell Surface Hydrophobicity

The method described by Vinderola and Reinheimer [37] was used to investigate the ability of *L. mesenteroides subsp. mesenteroides* isolates to attach to hydrophobic molecules. Cells from the 16 *L. mesenteroides subsp. mesenteroides* isolates and the reference strain, *Lactobacillus reuteri Protectis* DSM17938, were cultured overnight at 37 °C in MRS broth (Sigma-Aldrich). The cells were then harvested from the overnight cultures using centrifugation (6500× *g*, 4 °C, 6 min), washed twice in PBS, and resuspended in 1.5 mL of PBS. The optical density at 600 nm (A_0) was determined. The cell suspension was then mixed in a 1:1 ratio with ethyl acetate (Sigma-Aldrich), vortexed for 2 min, and incubated at room temperature for 1 h to allow the aqueous and organic phases to separate. Lastly, 1 mL of the aqueous phase was collected for spectrophotometric analysis at 600 nm to determine the absorbance (A_1). Using this data, percentage of hydrophobicity was calculated according to the following formula:

$$\%\ \text{hydrophobicity} = \frac{A_0 - A_1}{A_0} \times 100\%$$

2.5.5. Antibacterial Activity

The antibacterial activity of the isolates was determined using the agar well diffusion technique, as previously described by Mohankumar and Murugalatha [38], with minor modifications. The 16 *L. mesenteroides subsp. mesenteroides* isolates and the reference strain, *Lactobacillus reuteri Protectis* DSM17938, were screened for antibacterial activity against the two commonly known intestinal pathogens, *Escherichia coli* and *Staphylococcus aureus.* The selected isolates were grown in MRS broth (Sigma-Aldrich) at 37 °C for 24 h. Subsequently, the cell-free supernatants were prepared by centrifugation (6500× *g*, 10 min, room temperature), and the supernatants were sterilised using syringe filtration (0.45 µm). The pH of the filter-sterilised supernatants was adjusted to 6.5, using 1 M NaOH to neutralise the inhibitory effect of lactic acid. Diameter wells (6 mm) were then carved into solidified

Mueller–Hinton agar (Sigma-Aldrich), seeded with either *E. coli* or *S. aureus*. Thereafter, 25 µL of each supernatant was added to the wells, and the plates were incubated at 37 °C for 24 h. The antibacterial activities of isolates and the reference strain were assessed by measuring the diameter of the inhibition zones around each well.

2.5.6. Antioxidant Activity

Scavenging of Hydroxyl Radical with Intracellular Cell-Free Extract

The 16 *L. mesenteroides subsp. mesenteroides* isolates and *Lactobacillus reuteri Protectis* DSM17938 were cultured for 18 h in MRS broth at 37 °C. Bacterial cell pellets from each of the isolates and reference strain were collected using centrifugation (6000× *g*, 10 min, 4 °C), washed twice with sterile deionised water and resuspended in sterile deionised water to approximately 10^8, 10^9, and 10^{10} cfu/mL. Intracellular cell-free extracts from each of the samples were then prepared, as described by Lin and Yen [39]. The hydroxyl radical-scavenging assay was performed using the Fenton reaction method [40]. Briefly, a reaction mixture containing brilliant green (0.435 mM, Sigma-Aldrich), $FeSO_4$ (0.5 mM, Sigma-Aldrich), H_2O_2 (3.0%, *w*/*v*), and intracellular cell-free extracts of various concentrations was incubated at room temperature. Absorbance was measured at 624 nm for 20 min. The change in the absorbance of the reaction mixture showed the scavenging ability of the strains for hydroxyl radicals, and hydroxyl scavenging activity was calculated as follows:

$$\text{Scavenging activity (\%)} = [(A_s - A_0)/(A - A_0)] \times 100$$

where A_s is the absorbance with sample, A_0 is the absorbance of the control without sample, and A is the absorbance without the sample and Fenton reaction system.

Resistance to Hydrogen Peroxide

The scavenging ability of hydrogen peroxide by intact cells of *L. mesenteroides subsp. mesenteroides* isolates was determined using the method of Buchmeier et al. [41], with some modifications. Briefly, the overnight cultures of the strains were inoculated into MRS broth and MRS broth containing 0.4, 0.7, or 1.0 mM hydrogen peroxide (30% (w/w) in water, Sigma-Aldrich). The cultures were incubated at 37 °C for 8 h. The optical density of the cultures was measured using a spectrophotometer at 600 nm as an indicator of cell growth. The results were reported as optical density (OD).

2.6. Statistical Analysis

The mean values (±standard deviation) for the pH, TA, TSS, and microbial counts for the brine from every day of fermentation were calculated using Microsoft Excel (2016). Acid tolerance, bile tolerance, lysozyme tolerance, cell surface hydrophobicity, and antimicrobial activity as well as antioxidant activity are presented as mean values ± SD from triplicate samples using Microsoft Excel 2016 (Microsoft Corporation, Washington, DC, USA). Further statistical analysis was performed on GraphPad Prism 7.03 (GraphPad Software, San Diego, CA, USA) to determine the normality of the data using the D'Agostino–Pearson test and the Shapiro–Wilks test. Statistical significance between the data at the 95% level of confidence was then determined using a one-way ANOVA and Tukey's post-hoc test.

3. Results and Discussion

3.1. Genotypic Identification of the Isolates by 16S rDNA Sequencing

Preliminary to the study, carrot and ginger were peeled, grated, and spontaneously fermented in a 2.5% brine solution for 14 days under anaerobic conditions. The physico-chemical properties and microbial diversity of the fermentation process were monitored in order to determine a suitable day for lactic acid bacterial growth (Figures S1 and S2). Based on characteristics such as high titratable acidity, low pH, and increased lactic acid bacterial count, day 5 of the fermentation process was considered suitable for lactic acid bacterial growth (Figures S1 and S2). Subsequently, 18 isolates (L1–L18) from the naturally

fermented carrot and ginger brine sample of day 5 were identified as presumptive LAB species, based on phenotypic identification (Tables S1 and S2).

The identity of the isolates was further confirmed using LAB-specific primers, 15F and 687R, for species-level identification based on the 16S rDNA. As expected, a single discrete PCR amplicon band of approximately 0.75 Kb was observed for all the isolates investigated in this study. This corresponded with the size of the amplicon observed for our reference LAB strain (LR), *Lactobacillus reuteri Protectis* (DSM17938), as shown in Figure 1.

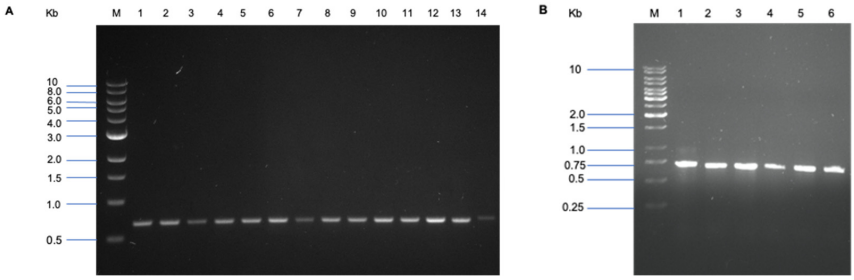

Figure 1. Species-specific PCR amplification with primers 15F and 687R. (**A**) Lane M: (1 Kb ladder), Lane 1: (*Lactobacillus reuteri Protectis* DSM17938), and Lanes 2–14: (isolates L1–L13). (**B**) Lane M: (1 Kb ladder), Lane 1: (*Lactobacillus reuteri Protectis* DSM17938), and Lanes 2–6: (isolates L14–L18).

Then, 16S rDNA sequencing analysis of the isolates showed that, after 5 days of fermentation, the predominant population of bacteria in the carrot and ginger brine were *Leuconostoc* strains, as shown in Table 1. Of the 18 isolates sequenced, an overwhelming majority of the isolates (L1–L16) were identified as *Leuconostoc mesenteroides subsp. mesenteroides*, whereas isolates L17 and L18 were identified as *Bacillus safensis* and *Bacillus licheniformis*, respectively (Table 1). In this regard, the results of the genotypic identification contradicted those of the phenotypic identification, which had suggested that L17 was *Bacillus pumilus* (Table S2). This observation is in agreement with Branquinho et al. [42], who reported on the difficulty of differentiating *Bacillus pumilus* from closely related species, based on phenotypic and biochemical characteristics. Overall, these findings provided strong evidence to warrant further investigation of *Leuconostoc mesenteroides subsp. mesenteroides*, the dominant strain, as a potential probiotic LAB strain. Hence, isolates L1–L16 were selected for further analysis.

Table 1. Identification of LAB isolates from spontaneously fermented carrot and ginger brine by sequencing the 16S rDNA.

Isolate Code	Phylogenetic Affiliation	Accession No.	Similarity (%)
L1	*Leuconostoc mesenteroides subsp. mesenteroides*	NC008531.1	81.25
L2	*Leuconostoc mesenteroides subsp. mesenteroides*	NC008531.1	89.34
L3	*Leuconostoc mesenteroides subsp. mesenteroides*	NC008531.1	94.71
L4	*Leuconostoc mesenteroides subsp. mesenteroides*	NC008531.1	94.79
L5	*Leuconostoc mesenteroides subsp. mesenteroides*	NC008531.1	98.75
L6	*Leuconostoc mesenteroides subsp. mesenteroides*	NC008531.1	98.91
L7	*Leuconostoc mesenteroides subsp. mesenteroides*	NC008531.1	99.06
L8	*Leuconostoc mesenteroides subsp. mesenteroides*	NC008531.1	98.76
L9	*Leuconostoc mesenteroides subsp. mesenteroides*	NC008531.1	99.21
L10	*Leuconostoc mesenteroides subsp. mesenteroides*	NC008531.1	99.21
L11	*Leuconostoc mesenteroides subsp. mesenteroides*	NC008531.1	99.21
L12	*Leuconostoc mesenteroides subsp. mesenteroides*	NC008531.1	99.04
L13	*Leuconostoc mesenteroides subsp. mesenteroides*	NC008531.1	99.20
L14	*Leuconostoc mesenteroides subsp. mesenteroides*	NC008531.1	97.45
L15	*Leuconostoc mesenteroides subsp. mesenteroides*	NC008531.1	97.81
L16	*Leuconostoc mesenteroides subsp. mesenteroides*	NC008531.1	97.81
L17	*Bacillus safensis*	NZCP018191.1	97.50
L18	*Bacillus licheniformis*	NC006270.3	96.89

3.2. Acid Tolerance

The survival of probiotics during gastric transit is important for colonisation of the hosts GIT, in order to exert beneficial effects [43]. Therefore, the acid tolerance of the *Leuconostoc mesenteroides subsp. mesenteroides* isolates (L1–L16) was investigated under pH conditions that mimic the human stomach. Figure 2 shows the survival rates of the isolated strains at pH 2.0 and pH 3.0 after 3 h of incubation. The results showed that the overall survival rate for all 16 isolates and the reference strain was higher at pH 3.0 compared to pH 2.0. These results correlated with those previously reported by Benmechernene and colleagues [44], where a decrease of 21.17% in the viability of *L. mesenteroides* strains isolated from raw camel milk was observed after exposure to acidic pH 2, while an increase of 0.49% was observed at pH 3. In addition, isolates L5, L6, and L14 exhibited a survival rate comparable to that of the known acid-tolerant reference strain (LR) at pH 2.0 and pH 3.0 (Figure 2). Importantly, the survival rates of the three isolates showed no statistically significant difference to the survival rate of the reference strain under these acidic conditions, suggesting that the three isolates closely mimic the reference strain's acid-tolerance capabilities. Notably, there was a sharp decline of viable cells for all the isolates at pH 2, with no growth observed for six strains of *Leuconostoc mesenteroides subsp. mesenteroides* (L8, L9, L12, L13, L15, L16). This observation corroborates the findings of previous studies, which have reported a decrease in the cell viability of probiotic strains at a pH of 2 and lower [44,45]. Furthermore, it is postulated that a pH value of 2.5 or below is very selective for the selection of potential probiotic strains, due to the variation in the pH of the stomach following the consumption of food [46].

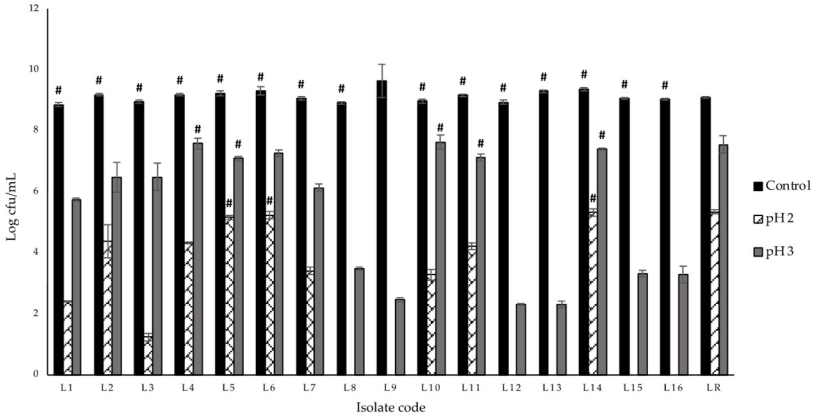

Figure 2. Survival rates of isolates after 3 h of exposure to acidic conditions. (Isolate code: L1–L16: *Leuconostoc mesenteroides subsp. mesenteroides*, LR: *Lactobacillus reuteri Protectis* DSM17938). The results are expressed as a mean ± SD of three independent experiments. Data points denoted with a # bear no statistically significant difference to LR.

3.3. Bile Tolerance

Bile secreted into the small intestine plays an important role in lipid digestion; however, due to its amphiphilic properties, it is cytotoxic to the gastrointestinal microbiota [47]. Therefore, bile salt tolerance is considered a key selection criterion for probiotic strains to preserve the integrity of the lipid bilayer of the bacterial cell membranes, maintain metabolic activity, and facilitate colonisation of the human GIT [47,48]. Although bile concentration is not uniformly distributed in the human GIT, the average intestinal bile concentration is considered to be 0.3%, with a staying time of 4 h [48,49].

The results of the bile salt tolerance assay are shown in Figure 3. All the *Leuconostoc mesenteroides subsp. mesenteroides* strains were able to grow in the presence of 0.3% and 0.5% Oxgall after the 4 h incubation period. As shown in Figure 3, isolates L1 and L5 showed bile tolerance of between 40–50% when exposed to 0.3% and 0.5% Oxgall, whereas all the other isolates had a survival rate of 60% and above. Notably, isolates L4 and L11 exhibited

survival rates ranging closer to 90% for both concentrations of Oxgall, which were comparable to the reference strain (LR). However, statistical analysis of these data found that the difference in bile tolerance between isolates L4 (89.9%) and LR (87.6%) was insignificant in the presence of 0.5% Oxgall, whereas the observed difference between L11 (91.5%) and LR was significant. Similarly, at 0.3% Oxgall, LR (94.3%) was significantly different than isolate L4 (90.6%) and not statistically different to L11 (94.2%) at this concentration. As such, we can infer that isolate L11 showed the most promising results among the test isolates at both 0.3% and 0.5% Oxgall concentrations, when compared to the reference strain. In addition, the bile tolerance of the isolated LAB strains appears to be a strain-specific characteristic, and this is consistent with previously published studies [49,50].

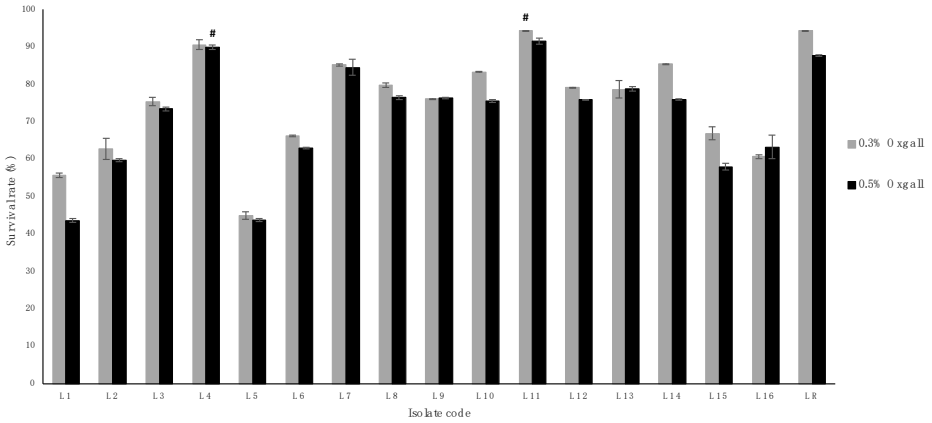

Figure 3. Survival rates of lactic acid bacteria after 4 h of exposure to MRS broth supplemented with Oxgall. (Isolate code: L1–L16: *Leuconostoc mesenteroides subsp. mesenteroides*, LR: *Lactobacillus reuteri Protectis* DSM17938). The results are expressed as a mean ± SD of three independent experiments. Data points denoted with a # bear no statistically significant difference to LR.

Overall, the bile-tolerance data revealed a slight decrease in the survival rate of the isolates after 4 h of exposure to 0.5% Oxgall, when compared to 0.3% Oxgall (Figure 3). The resistance of LAB to high Oxgall concentrations is most likely due to the expression of bile-resistance-related proteins such as Hsp 1, Hsp 3, and ClpP in the bacterial cell [47,51]. The findings of the current study are similar to those reported by Benmechernene et al. [44] and De Paula et al. [52], where it was found that *Leuconostoc mesenteroides* and *Leuconostoc mesenteroides subsp. mesenteroides* strains showed good tolerance to higher bile salt concentrations. Owing to the high tolerance to bile salts of all the isolates, we expect the strains to be effective in bile salt deconjugation and, consequently, effective in lowering serum cholesterol [35].

3.4. Lysozyme Resistance

An important step towards the selection of potentially probiotic bacteria is to investigate the viability of candidate strains in vitro, under conditions that mimic the human GIT. Resistance to lysozyme contained in human saliva is one of the first requirements that potential probiotic bacteria must fulfil, as the lysozyme contained in the oral cavity may lyse Gram-positive bacterial cells [53]. All the isolates exhibited excellent resistance to a 50 µg/mL concentration of lysozyme, after the 3 h incubation period (Figure 4). Notably, at this concentration of lysozyme, only isolates L3, L8, and L12 showed a significant difference in behaviour, when compared to the reference strain, with viable counts lower by 1.1 log cfu/mL. By contrast, all the isolates showed reduced tolerance towards 100 µg/mL of lysozyme. Interestingly, only isolates L3 and L13 showed a statistically significant difference, in terms of survival rate at 100 µg/mL of lysozyme, when compared to the reference strain, with viable counts lower by 1.5 log cfu/mL (Figure 4).

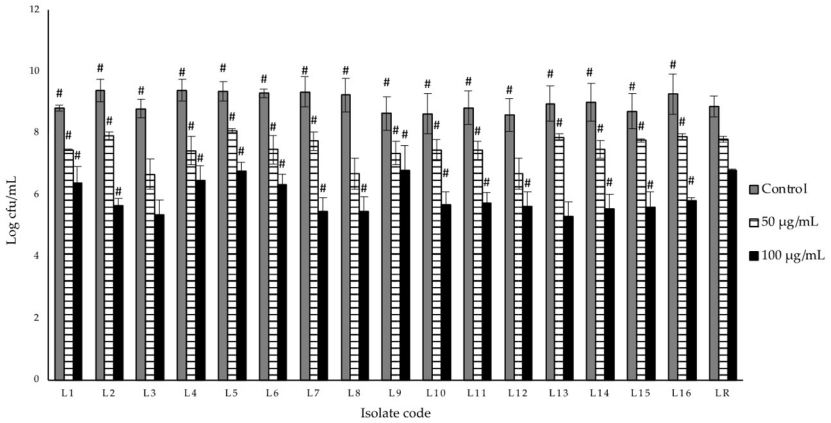

Figure 4. Survival of LAB in the presence of different concentrations of lysozyme. (Isolate code: L1–L16: *Leuconostoc mesenteroides subsp. mesenteroides,* LR: *Lactobacillus reuteri Protectis* DSM17938). The results are expressed as a mean ± SD of three independent experiments. Data points denoted with a # bear no statistically significant difference to LR.

The response of LAB to lysozyme has previously been described to be species- and strain-dependent, and our findings are in agreement with this notion [36]. Overall, our results indicated that all the isolates showed good to moderate resistance to 100 µg/mL lysozyme (ranging between 5.30–7.28 log cfu/mL), being much higher than that of previously reported *Leuconostoc mesenteroides subsp. mesenteroides* strains [54]. This indicates that the lysozyme-resistance capability of the isolates is high enough to survive lysozyme contained in the oral cavity, since 100 µg/mL is the highest level of lysozyme that is normally used to simulate in vivo saliva conditions [14].

3.5. Hydrophobicity

There is evidence to suggest that bacterial cell surface hydrophobicity enhances the adherence of bacteria to epithelial cells of the intestines [55]. Moreover, it has been shown that bacterial colonisation of the human GIT is facilitated by hydrophobic interactions between the epithelial cells of the mucosal surface and the bacterial cell surface [55,56]. Therefore, investigating the cell surface hydrophobicity of potential probiotic strains can yield valuable insights into their ability to adhere to the host epithelial cells and initiate colonisation. In this study, the microbial adherence to solvents test, using ethyl acetate, was used to test the cell surface hydrophobicity of the 16 isolates. As shown in Table 2, isolates L3 and L10 showed greater than 80% hydrophobicity, and the remaining isolates presented a cell surface hydrophobicity ranging between 33% and 56%. More interestingly, the percentage of hydrophobicity of each of the 16 isolates was found to be significantly different to that of the reference strain, which exhibited a cell surface hydrophobicity of 73.3%. In addition, isolates L3 and L10 showed greater cell surface hydrophobicity, when compared to the reference strain (Table 2), suggesting that they could join to epithelium through nonspecific binding [56]. These results are consistent with the findings of De Paula et al. [52], who reported on a strain of *Leuconostoc mesenteroides subsp. mesenteroides* that exhibited a cell surface hydrophobicity of more than 50%. Moreover, it has been suggested that cell surface hydrophobicity is a trait that is characteristic and consistent among isolates of the same bacterial strain. However, this characteristic can be influenced by external factors such as nutrient availability and physical growth conditions, which may affect the expression of genes that influence the adhesion properties of some bacteria [56,57]. Overall, the results presented in the hydrophobicity tests for the *Leuconostoc mesenteroides subsp. mesenteroides* isolates in the present study were higher than those of some other strains of LAB, as previously reported in the literature [56,58].

Table 2. Hydrophobicity (%) of *Leuconostoc mesenteroides subsp. mesenteroides* strains to ethyl acetate, measured as OD_{600nm}.

Isolate Code	Hydrophobicity (%)
L1	45.64 ± 0.58
L2	33.98 ± 2.65
L3	83.68 ± 0.51
L4	56.59 ± 4.59
L5	54.58 ± 0.51
L6	36.28 ± 0.16
L7	36.11 ± 1.58
L8	33.89 ± 5.32
L9	36.74 ± 1.06
L10	84.35 ± 1.85
L11	33.18 ± 1.86
L12	39.07 ± 0.07
L13	38.30 ± 2.04
L14	35.99 ± 1.18
L15	54.93 ± 4.30
L16	51.44 ± 0.87
LR	73.64 ± 1.12

Each value is expressed as the mean ± SD of three independent experiments (n = 3). L1–L16: *Leuconostoc mesenteroides subsp. Mesenteroides*; LR: *Lactobacillus reuteri Protectis* DSM17938.

3.6. Antibacterial Activity Assay

Probiotic bacteria confer immunological benefits on the host, by inhibiting the growth of pathogenic bacteria in their vicinity [59]. Table 3 presents the antibacterial activities against two commonly known intestinal pathogens, *Staphylococcus aureus* and *Escherichia coli*. All 16 *Leuconostoc mesenteroides subsp. mesenteroides* isolates exhibited antibacterial activity against both pathogens, with inhibition zones ranging between 1.0–5.0 mm (low), 5.0–10.0 mm (moderate), and greater than 10.0 mm (high). Notably, 10 of the isolates (L2, L5, L6, L7, L8, L9, L10, L11, L13, L16) exhibited high antibacterial activity against both pathogens, as did the reference strain. Overall, it was observed that *S. aureus* was more sufficiently neutralised by the *Leuconostoc mesenteroides subsp. mesenteroides* isolates, compared to *E. coli*. (Table 3). These results are consistent with the results of previously published studies, which show that LAB isolates display greater influence against *Staphylococcus aureus*, in comparison to other pathogens [60,61].

Table 3. Antibacterial activity of *Leuconostoc mesenteroides subsp. mesenteroides* against two common intestinal pathogens.

Isolate Code	Antimicrobial Activity	
	Escherichia coli	***Staphylococcus aureus***
L1	++	+++
L2	+++	+++
L3	+	+++
L4	++	++
L5	+++	+++
L6	+++	+++
L7	+++	+++
L8	+++	+++
L9	+++	+++
L10	+++	+++
L11	+++	+++

Table 3. *Cont.*

Isolate Code	Antimicrobial Activity	
	Escherichia coli	***Staphylococcus aureus***
L12	++	++
L13	+++	+++
L14	+	+++
L15	++	+++
L16	+++	+++
LR	+++	+++

(+) inhibition zone 1.0–5.0 mm; (++) inhibition zone 5.0–10 mm; (+++) inhibition zone > 10 mm. L1–L16: *Leuconostoc mesenteroides subsp. Mesenteroides*; LR: *Lactobacillus reuteri Protectis* DSM17938.

The growth of pathogenic bacterial species might be inhibited by the secretion of antibacterial compounds such as hydrogen peroxide, organic acids, and bacteriocins produced by the *Leuconostoc mesenteroides subsp. mesenteroides* isolates [62,63]. Interestingly, the antibacterial activity of these compounds can be enhanced by the acidic environment in the stomach, to competitively exclude pathogenic bacterial species in the GIT [62,64]. Similarly, all the *Leuconostoc mesenteroides subsp. mesenteroides* isolates investigated in the present study exhibited strong inhibitory activity against the common intestinal pathogens, *E. coli* and *S. aureus*.

3.7. Antioxidant Activity

Free radicals such as reactive oxygen (ROS) species are important signalling molecules that regulate various redox-dependent cellular pathways. As critical modulators of the redox environment, regulation of free radical concentrations in the intracellular and extracellular environments is imperative for cell survival. Free-radical scavenging is one of the common methods employed by cells to prevent ROS-induced oxidation [65]. The hydroxyl (OH^-) ion is one of the most potent ROS that can induce oxidative injury of biomolecules or even cell death. Published data have shown that when transition metals such as copper and iron are present, hydroxyl ions can be derived from hydrogen peroxide through the Fenton reaction [66]. However, some LAB species including *Lactobacillus casei*, *Bifidobacterium longum*, and *Streptococcus thermophiles* have developed antioxidative mechanisms that eradicate the transition metals that may facilitate these Fenton reactions [39,67]. Based on this premise, we sought to investigate the scavenging activity of our 16 *Leuconostoc mesenteroides subsp. mesenteroides* isolates for two of the most common ROS, hydroxyl radical and hydrogen peroxide.

As shown in Figure 5, all the isolates demonstrated varying levels of hydroxyl radical-scavenging activity within the tested concentration range of 10^8–10^{10} cfu/mL. Furthermore, five of the *Leuconostoc mesenteroides subsp. mesenteroides* isolates (L1, L2, L3, L8, L16), as well as the reference strain *Lactobacillus reuteri Protectis* DSM17938 (LR), showed the highest hydroxyl radical-scavenging activity at 10^{10} cfu/mL, with hydroxyl radical-scavenging rates exceeding 50%. The strong hydroxyl radical-scavenging activity observed from the intracellular cell-free extracts of *Leuconostoc mesenteroides subsp. mesenteroides* in this study is likely due to the ability of these strains to chelate transition metal ions such as Fe^{2+} [67]. We postulate that this may form part of the antioxidant mechanisms of these strains. Lastly, we noted that *Lactobacillus reuteri Protectis* DSM17938 (LR) consistently outperformed all the test isolates at all three concentrations tested, since statistical analysis of the data showed that all the data for *Leuconostoc mesenteroides subsp. mesenteroides* were statistically lower, when compared to the data obtained for the reference strain (Figure 5).

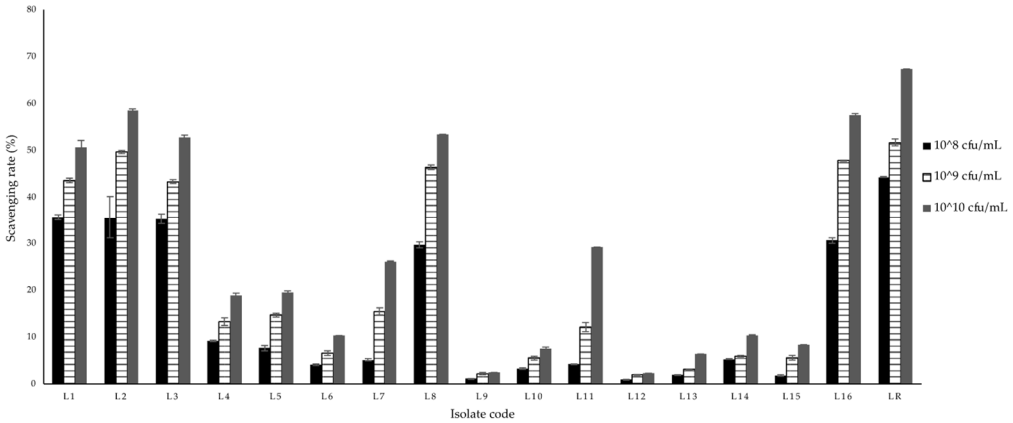

Figure 5. Scavenging of hydroxyl radical by intracellular cell-free extract of *Leuconostoc mesenteroides subsp. mesenteroides* isolates. (Isolate code: L1–L16: *Leuconostoc mesenteroides subsp. mesenteroides,* LR: *Lactobacillus reuteri Protectis* DSM17938). Each value is expressed as mean ± SD (n = 3). Data points denoted with a # bear no statistically significant difference to LR.

Furthermore, compared to the hydroxyl radical, hydrogen peroxide is a weak oxidant that may give rise to a hydroxyl radical that can cause oxidative damage of cells. Moreover, hydrogen peroxide may also cause transition metal ion-dependent and hydroxyl radical-mediated oxidative DNA damage [65,68]. Therefore, the effect of hydrogen peroxide on the viability of *Leuconostoc mesenteroides subsp. mesenteroides* was assessed (Table 4). The data showed that all 16 test isolates could tolerate 8 h of exposure to 0.4 mM hydrogen peroxide, despite varying levels of viability. Importantly, the reference strain outperformed all the test isolates at all the hydrogen peroxide concentrations tested, except isolate L3 at a hydrogen peroxide concentration of 0.4 mM. However, upon analysing the data further, it was found that this observed difference was, in fact, insignificant. At a concentration of 1.0 mM hydrogen peroxide, only two isolates, L2 and L16, were found to be statistically comparable to the reference strain, with optical densities of 0.83 and 0.82, respectively (Table 4).

Table 4. Resistance of *Leuconostoc mesenteroides subsp. mesenteroides* strains to different concentrations of hydrogen peroxide.

Isolate Code	**Hydrogen Peroxide (mM)**			
	0	**0.4**	**0.7**	**1.0**
L1	2.58 ± 0.27 #	2.16 ± 0.02 #	1.41 ± 0.02 #	0.72 ± 0.02
L2	2.67 ± 0.24 #	2.06 ± 0.06 #	1.51 ± 0.05 #	0.83 ± 0.05 #
L3	2.59 ± 0.25 #	2.17 ± 0.11 #	1.46 ± 0.01 #	0.73 ± 0.02
L4	2.54 ± 0.22 #	1.86 ± 0.06	1.08 ± 0.03	0.53 ± 0.17
L5	2.28 ± 0.06 #	1.56 ± 0.08	0.99 ± 0.03	0.41 ± 0.02
L6	2.28 ± 0.13 #	1.56 ± 0.07	0.96 ± 0.01	0.34 ± 0.06
L7	2.07 ± 0.05	1.93 ± 0.02 #	1.34 ± 0.06 #	0.64 ± 0.03
L8	2.60 ± 0.26	2.05 ± 0.05 #	1.42 ± 0.14 #	0.74 ± 0.04
L9	1.96 ± 0.06	1.48 ± 0.10	0.71 ± 0.23	0.37 ± 0.13
L10	1.95 ± 0.07	1.50 ± 0.03	0.75 ± 0.22	0.33 ± 0.12
L11	2.15 ± 0.04	1.92 ± 0.01 #	1.39 ± 0.02 #	0.60 ± 0.10
L12	1.92 ± 0.07	1.52 ± 0.04	0.79 ± 0.16	0.25 ± 0.03
L13	2.28 ± 0.03	1.72 ± 0.04	0.90 ± 0.09	0.32 ± 0.10
L14	2.35 ± 0.04	1.75 ± 0.02	0.94 ± 0.13	0.37 ± 0.02

Table 4. *Cont.*

Isolate Code	Hydrogen Peroxide (mM)			
	0	**0.4**	**0.7**	**1.0**
L15	2.04 ± 0.02	1.76 ± 0.03	1.20 ± 0.11	0.39 ± 0.08
L16	2.01 ± 0.03	1.90 ± 0.05	1.41 ± 0.03 #	0.82 ± 0.14 #
LR	2.68 ± 0.22	2.11 ± 0.13	1.71 ± 0.31	1.01 ± 0.03

Each value is expressed as the mean ± SD of three independent experiments (n = 3). L1–L16: *Leuconostoc mesenteroides* subsp. Mesenteroides; LR: Lactobacillus reuteri Protectis DSM17938; #: not significantly different to LR.

Our findings corroborated the results of previous studies, which reported that *Lactobacillus* strains exhibit varying levels of hydrogen peroxide resistance; Lee et al. [67] found that *L. casei* KCTC 3260 and *L. rhamnosus* GG cells could tolerate exposure to 1.0 mM of hydrogen peroxide for 8 h, whereas *L. casei* 01 showed no resistance. Moreover, it has been shown that *L. fermentum* E-3 can tolerate 1.0 mM of hydrogen peroxide for 180 min. By contrast, *L. fermentum* E-18 cells were only viable for 150 min, and *L. fermentum* E-338-1-1 cells showed no resistance under the same conditions [68]. Similar results were observed in our study, where isolates L1, L2, L3, L8, and L16 exhibited the highest level of resistance against hydrogen peroxide, with optical densities above 0.7 after incubation for 8 h with 1.0 mM hydrogen peroxide. By contrast, isolate L12 was the most sensitive, as it was found to have an optical density below 0.30. Currently, the mechanism that is responsible for the observed variation in hydrogen peroxide tolerance among LAB remains unknown. This can be a subject of investigation for future studies.

4. Conclusions

The 16 LAB strains isolated from a fermented carrot and ginger brine, identified as *Leuconostoc mesenteroides subsp. Mesenteroides*, presented interesting probiotic characteristics. Most of the isolates were found to possess desirable probiotic properties similar or superior to those of the reference probiotic strain, *Lactobacillus reuteri Protectis* DSM17938, under in vitro conditions. All the tested isolates showed good tolerance towards simulated gastrointestinal conditions (high acid, bile, lysozyme tolerance). In addition, they exhibited strong antibacterial activity against resident intestinal pathogens as well as antioxidant activity. Therefore, these strains are good candidates for further investigation with in vivo studies to elucidate their potential health benefits.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8100534/s1. Figure S1: Effect of fermentation duration on pH, titratable acidity, and total soluble solids of fermented carrot and ginger brine; Figure S2: Microbial community dynamics of spontaneously fermented carrot and ginger brine; Table S1: Morphological and physiological characterisation of isolates from a fermented carrot and ginger brine; Table S2: Carbohydrate fermentation profile of lactic acid bacteria isolated from fermented carrot and ginger brine.

Author Contributions: Conceptualisation, N.C. and T.K.; methodology, N.C.; software, N.C. and B.N.; validation, N.C., B.N. and T.K.; formal analysis, N.C. and B.N.; investigation, N.C.; data curation, N.C. and B.N.; writing—original draft preparation, N.C., B.N. and T.K.; writing—review and editing, N.C., B.N. and T.K.; supervision, T.K.; project administration, T.K.; funding acquisition, N.C. and T.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the NRF (grant number 121275) and research grants from the South African Medical Research Council (SAMRC) and the University of KwaZulu-Natal; N.C. was funded by the Department of Science and Technology-National Research Foundation (DST-NRF) and South Africa for Innovation and Scarce Skills Development Master's Scholarship (reference number SFH170717254156).

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the result.

References

1. Caprara, G. Mediterranean-type dietary pattern and physical activity: The winning combination to counteract the rising burden of non-communicable diseases (NCDS). *Nutrients* **2021**, *13*, 429. [CrossRef]
2. Swinburn, B.A.; Caterson, I.; Seidell, J.C.; James, W.P.T. Diet, nutrition and the prevention of excess weight gain and obesity. *Public Health Nutr.* **2004**, *7*, 123–146. [PubMed]
3. Cencic, A.; Chingwaru, W. The role of functional foods, nutraceuticals, and food supplements in intestinal health. *Nutrients* **2010**, *2*, 611–625. [CrossRef] [PubMed]
4. El Sohaimy, S. Functional foods and nutraceuticals-modern approach to food science. *World Appl. Sci. J.* **2012**, *20*, 691–708.
5. Mack, D.R. Probiotics: Mixed messages. *Can. Fam. Physician* **2005**, *51*, 1455. [PubMed]
6. Holzapfel, W.H.; Haberer, P.; Geisen, R.; Björkroth, J.; Schillinger, U. Taxonomy and important features of probiotic microorganisms in food and nutrition. *Am. J. Clin. Nutr.* **2001**, *73*, 365s–373s. [CrossRef] [PubMed]
7. Lee, Y.K.; Salminen, S. *Handbook of Probiotics and Prebiotics*; John Wiley & Sons: Hoboken, NJ, USA, 2009.
8. Anadón, A.; Martínez-Larrañaga, M.R.; Caballero, V.; Castellano, V. *Assessment of Prebiotics and Probiotics: An Overview, in Bioactive Foods in Promoting Health*; Elsevier: Amsterdam, The Netherlands, 2010; pp. 19–41.
9. Fuller, R. A review: Probiotics in man and animals. *J. Appl. Bacteriol.* **1989**, *66*, 365–378. [PubMed]
10. Sarao, L.K.; Arora, M. Probiotics, prebiotics, and microencapsulation: A review. *Crit. Rev. Food Sci. Nutr.* **2017**, *57*, 344–371. [CrossRef] [PubMed]
11. Jin, L.Z.; Ho, Y.W.; Abdullah, N.; Jalaludin, S. Acid and bile tolerance of Lactobacillus isolated from chicken intestine. *Lett. Appl. Microbiol.* **1998**, *27*, 183–185. [CrossRef] [PubMed]
12. Masco, L.; Crockaert, C.; Hoorde, K.V.; Swings, J.; Huys, G. In vitro assessment of the gastrointestinal transit tolerance of taxonomic reference strains from human origin and probiotic product isolates of Bifidobacterium. *J. Dairy Sci.* **2007**, *90*, 3572–3578. [CrossRef]
13. Tuomola, E.; Crittenden, R.; Playne, M.; Isolauri, E.; Salminen, S. Quality assurance criteria for probiotic bacteria. *Am. J. Clin. Nutr.* **2001**, *73*, 393s–398s. [CrossRef] [PubMed]
14. Turchi, B.; Mancini, S.; Fratini, F.; Pedonese, F.; Nuvoloni, R.; Bertelloni, F.; Ebani, V.V.; Cerri, D. Preliminary evaluation of probiotic potential of Lactobacillus plantarum strains isolated from Italian food products. *World J. Microbiol. Biotechnol.* **2013**, *29*, 1913–1922. [CrossRef] [PubMed]
15. Del Carmen, S.; LeBlanc, A.d.M.d.; Levit, R.; Azevedo, V.; Langella, P.; Bermúdez-Humarán, L.G.; LeBlanc, J.G. Anti-cancer effect of lactic acid bacteria expressing antioxidant enzymes or IL-10 in a colorectal cancer mouse model. *Int. Immunopharmacol.* **2017**, *42*, 122–129. [CrossRef] [PubMed]
16. Li, X.; Ringø, E.; Hoseinifar, S.H.; Lauzon, H.L.; Birkbeck, H.; Yang, D. The adherence and colonization of microorganisms in fish gastrointestinal tract. *Rev. Aquac.* **2019**, *11*, 603–618. [CrossRef]
17. Gueimonde, M.; de los Reyes-Gavilán, C.; Sánchez, B. Stability of lactic acid bacteria in foods and supplements. Lactic Acid Bacteria. *Microbiol. Funct. Asp.* **2012**, 361–384.
18. Tripathi, M.K.; Giri, S.K. Probiotic functional foods: Survival of probiotics during processing and storage. *J. Funct. Foods* **2014**, *9*, 225–241. [CrossRef]
19. Vasiljevic, T.; Shah, N.P. Probiotics—From Metchnikoff to bioactives. *Int. Dairy J.* **2008**, *18*, 714–728. [CrossRef]
20. Lilly, D.M.; Stillwell, R.H. Probiotics: Growth-promoting factors produced by microorganisms. *Science* **1965**, *147*, 747–748. [CrossRef]
21. Collins, J.; Thornton, G.; Sullivan, G. Selection of probiotic strains for human applications. *Int. Dairy J.* **1998**, *8*, 487–490. [CrossRef]
22. Piqué, N.; Berlanga, M.; Miñana-Galbis, D. Health benefits of heat-killed (Tyndallized) probiotics: An overview. *Int. J. Mol. Sci.* **2019**, *20*, 2534. [CrossRef]
23. Bogere, P.; Choi, Y.; Heo, J. Probiotics as alternatives to antibiotics in treating post-weaning diarrhoea in pigs. *S. Afr. J. Anim. Sci.* **2019**, *49*, 403–416. [CrossRef]
24. Ouwehand, A.C.; Salminen, S.; Isolauri, E. Probiotics: An overview of beneficial effects. In *Lactic Acid Bacteria: Genetics, Metabolism and Applications*; Springer: Berlin/Heidelberg, Germany, 2002; pp. 279–289.
25. Giraffa, G. Overview of the Ecology and Biodiversity of the LAB. In *Lactic Acid Bacteria*; John Wiley & Sons: Hoboken, NJ, USA, 2014; pp. 45–54.
26. Cassani, L.; Gomez-Zavaglia, A.; Simal-Gandara, J. Technological strategies ensuring the safe arrival of beneficial microorganisms to the gut: From food processing and storage to their passage through the gastrointestinal tract. *Food Res. Int.* **2019**, *129*, 108852. [CrossRef] [PubMed]
27. Yadav, R.; Shukla, P. An overview of advanced technologies for selection of probiotics and their expediency: A review. *Crit. Rev. Food Sci. Nutr.* **2017**, *57*, 3233–3242. [CrossRef] [PubMed]

28. De Corato, U. Improving the shelf-life and quality of fresh and minimally-processed fruits and vegetables for a modern food industry: A comprehensive critical review from the traditional technologies into the most promising advancements. *Crit. Rev. Food Sci. Nutr.* **2020**, *60*, 940–975. [CrossRef] [PubMed]
29. Yildiz, F.; Wiley, R.C. *Minimally Processed Refrigerated Fruits and Vegetables*; Springer: Berlin/Heidelberg, Germany, 2017.
30. Tyl, C.; Sadler, G.D. pH and Titratable Acidity. In *Food Analysis*; Springer: Berlin/Heidelberg, Germany, 2017; pp. 389–406.
31. Kavitha, J.R.; Devasena, T. Isolation, characterization, determination of probiotic properties of lactic acid bacteria from human milk. *OSR J. Pharm. Biol. Sci.* **2013**, *7*, 1–7. [CrossRef]
32. Müller, T. Comparison of methods for differentiation between homofermentative and heterofermentative lactic acid bacteria. *Zent. Mikrobiol.* **1990**, *145*, 363–366. [CrossRef]
33. Nelson, G.M.; George, S.E. Comparison of media for selection and enumeration of mouse fecal flora populations. *J. Microbiol. Methods* **1995**, *22*, 293–300. [CrossRef]
34. Hedin, C.; Whelan, K.; Lindsay, J.O. Evidence for the use of probiotics and prebiotics in inflammatory bowel disease: A review of clinical trials. *Proc. Nutr. Soc.* **2007**, *66*, 307–315. [CrossRef] [PubMed]
35. Walker, D.K.; Gilliland, S.E. Relationships among bile tolerance, bile salt deconjugation, and assimilation of cholesterol by Lactobacillus acidophilus. *J. Dairy Sci.* **1993**, *76*, 956–961. [CrossRef]
36. Dias, R.; Vilas-Boas, E.; Campos, F.M.; Hogg, T.; Couto, J.A. Activity of lysozyme on Lactobacillus hilgardii strains isolated from Port wine. *Food Microbiol.* **2015**, *49*, 6–11. [CrossRef]
37. Vinderola, C.G.; Reinheimer, J.A. Lactic acid starter and probiotic bacteria: A comparative "in vitro" study of probiotic characteristics and biological barrier resistance. *Food Res. Int.* **2003**, *36*, 895–904. [CrossRef]
38. Mohankumar, A.; Murugalatha, N. Characterization and antibacterial activity of bacteriocin producing Lactobacillus isolated from raw cattle milk sample. *Int. J. Biol.* **2011**, *3*, 128. [CrossRef]
39. Lin, M.Y.; Yen, C.L. Antioxidative ability of lactic acid bacteria. *J. Agric. Food Chem.* **1999**, *47*, 1460–1466. [CrossRef]
40. He, Z.S.; Luo, H.; Cao, C.H.; Cui, Z.W. Photometric determination of hydroxyl free radical in Fenton system by brilliant green. *Am. J. Chin. Med.* **2004**, *243*, 236–237.
41. Buchmeier, N.; Bossie, S.; Chen, C.Y.; Fang, F.C.; Guiney, D.G.; Libby, S.J. SlyA, a transcriptional regulator of Salmonella typhimurium, is required for resistance to oxidative stress and is expressed in the intracellular environment of macrophages. *Infect. Immun.* **1997**, *65*, 3725–3730. [CrossRef] [PubMed]
42. Branquinho, R.; Meirinhos-Soares, L.; Carriço, J.A.; Pintado, M.; Peixe, L.V. Phylogenetic and clonality analysis of Bacillus pumilus isolates uncovered a highly heterogeneous population of different closely related species and clones. *FEMS Microbiol. Ecol.* **2014**, *90*, 689–698. [CrossRef]
43. Trabelsi, I.; Bejar, W.; Ayadi, D.; Chouayekh, H.; Kammoun, R.; Bejar, S.; Salah, R.B. Encapsulation in alginate and alginate coated-chitosan improved the survival of newly probiotic in oxgall and gastric juice. *Int. J. Biol. Macromol.* **2013**, *61*, 36–42. [CrossRef] [PubMed]
44. Benmechernene, Z.; Chentouf, H.F.; Yahia, B.; Fatima, G.; Quintela-Baluja, M.; Calo-Mata, P.; Barros-Velázquez, J. Technological aptitude and applications of Leuconostoc mesenteroides bioactive strains isolated from Algerian raw camel milk. *BioMed Res. Int.* **2013**, *2013*, 418132. [CrossRef] [PubMed]
45. Gupta, P.K.; Mital, B.K.; Garg, S.K. Characterization of Lactobacillus acidophilus strains for use as dietary adjunct. *Int. J. Food Microbiol.* **1996**, *29*, 105–109. [CrossRef]
46. Argyri, A.A.; Zoumpopoulou, G.; Karatzas, K.-A.G.; Tsakalidou, E.; Nychas, G.-J.E.; Panagou, E.Z.; Tassou, C.C. Selection of potential probiotic lactic acid bacteria from fermented olives by in vitro tests. *Food Microbiol.* **2013**, *33*, 282–291. [CrossRef]
47. Yu, Z.; Zhang, X.; Li, S.; Li, C.; Li, D.; Yang, Z. Evaluation of probiotic properties of Lactobacillus plantarum strains isolated from Chinese sauerkraut. *World J. Microbiol. Biotechnol.* **2013**, *29*, 489–498. [CrossRef] [PubMed]
48. Dunne, C.; Murphy, L.; Flynn, S.; O'Mahony, L.; O'Halloran, S.; Feeney, M.; Morrissey, D.; Thornton, G.; Fitzgerald, G.; Daly, C.; et al. Probiotics: From myth to reality. Demonstration of functionality in animal models of disease and in human clinical trials. *Lact. Acid Bact. Genet. Metab. Appl.* **1999**, 279–292.
49. Hawaz, E. Isolation and identification of probiotic lactic acid bacteria from curd and *in vitro* evaluation of its growth inhibition activities against pathogenic bacteria. *Afr. J. Microbiol. Res.* **2014**, *8*, 1419–1425.
50. Mishra, V.; Prasad, D.N. Application of *in vitro* methods for selection of Lactobacillus casei strains as potential probiotics. *Int. J. Food Microbiol.* **2005**, *103*, 109–115. [CrossRef] [PubMed]
51. Hamon, E.; Horvatovich, P.; Izquierdo, E.; Bringel, F.; Marchioni, E.; Aoudé-Werner, D.; Ennahar, S. Comparative proteomic analysis of Lactobacillus plantarumfor the identification of key proteins in bile tolerance. *BMC Microbiol.* **2011**, *11*, 63. [CrossRef] [PubMed]
52. De Paula, A.T.; Jeronymo-Ceneviva, A.B.; Silva, L.F.; Todorov, S.D.; Franco, B.D.G.M.; Penna, A.L.B. Leuconostoc mesenteroides SJRP55: A potential probiotic strain isolated from Brazilian water buffalo mozzarella cheese. *Ann. Microbiol.* **2015**, *65*, 899–910. [CrossRef]
53. Joel, T.J.; Suguna, S.; Steffi, S.R. Antimicrobial activity of lysozyme against oral pathogens. *Asian J. Pharm. Res. Health Care* **2016**, *8*, 42–46. [CrossRef] [PubMed]
54. Surono, I.S. *In vitro* probiotic properties of indigenous dadih lactic acid bacteria. *Asian-Australas. J. Anim. Sci.* **2003**, *16*, 726–731. [CrossRef]

55. Pérez, P.F.; Minnaard, Y.; Disalvo, E.A.; Antoni, G.L.D. Surface properties of bifidobacterial strains of human origin. *Appl. Environ. Microbiol.* **1998**, *64*, 21–26. [CrossRef]
56. Raghavendra, P.; Halami, P.M. Screening, selection and characterization of phytic acid degrading lactic acid bacteria from chicken intestine. *Int. J. Food Microbiol.* **2009**, *133*, 129–134. [CrossRef] [PubMed]
57. Ouwehand, A.C.; Vesterlund, S. Antimicrobial components from lactic acid bacteria. *Food Sci. Technol.-N. Y.-Marcel Dekker* **2004**, *139*, 375–396.
58. Aswathy, R.G.; Ismail, B.; John, R.P.; Nampoothiri, K.M. Evaluation of the probiotic characteristics of newly isolated lactic acid bacteria. *Appl. Biochem. Biotechnol.* **2008**, *151*, 244–255. [CrossRef] [PubMed]
59. Woodmansey, E.J. Intestinal bacteria and ageing. *J. Appl. Microbiol.* **2007**, *102*, 1178–1186. [CrossRef] [PubMed]
60. Abushelaibi, A.; Almahadin, S.; El-Tarabily, K.A.; Shah, N.P.; Ayyash, M. Characterization of potential probiotic lactic acid bacteria isolated from camel milk. *LWT-Food Sci. Technol.* **2017**, *79*, 316–325. [CrossRef]
61. Ren, D.; Zhu, J.; Gong, S.; Liu, H.; Yu, H. Antimicrobial characteristics of lactic acid bacteria isolated from homemade fermented foods. *BioMed Res. Int.* **2018**, *2018*, 5416725. [CrossRef] [PubMed]
62. Paula, A.T.d.; Jeronymo-Ceneviva, A.B.; Silva, L.F.; Todorov, S.D.; Franco, B.D.G.d.M.; Choiset, Y.; Haertlé, T.; Chobert, J.M.; Dousset, X.; Penna, A.L.B. Leuconostoc mesenteroides SJRP55: A bacteriocinogenic strain isolated from Brazilian water buffalo mozzarella cheese. *Probiotics Antimicrob. Proteins* **2014**, *6*, 186–197. [CrossRef]
63. Li, L.; Shin, S.Y.; Lee, K.W.; Han, N.S. Production of natural antimicrobial compound d-phenyllactic acid using L euconostoc mesenteroides ATCC 8293 whole cells involving highly active d-lactate dehydrogenase. *Lett. Appl. Microbiol.* **2014**, *59*, 404–411. [CrossRef] [PubMed]
64. Ouwehand, A.C.; Salminen, S.J. The health effects of cultured milk products with viable and non-viable bacteria. *Int. Dairy J.* **1998**, *8*, 749–758. [CrossRef]
65. Hazra, B.; Sarkar, R.; Biswas, S. Nripendranath Mandal Comparative study of the antioxidant and reactive oxygen species scavenging properties in the extracts of the fruits of Terminalia chebula, Terminalia belerica and Emblica officinalis. *BMC Complement. Altern. Med.* **2010**, *10*, 20. [CrossRef] [PubMed]
66. Kao, T.H.; Chen, B.H. Functional components in soybean cake and their effects on antioxidant activity. *J. Agric. Food Chem.* **2006**, *54*, 7544–7555. [CrossRef] [PubMed]
67. Lee, J.; Hwang, T.K.; Chung, M.Y.; Cho, D.H.; Park, C.S. Resistance of Lactobacillus casei KCTC 3260 to reactive oxygen species (ROS): Role for a metal ion chelating effect. *J. Food Sci.* **2005**, *70*, m388–m391. [CrossRef]
68. Kullisaar, T.; Zilmer, M.; Mikelsaar, M.; Vihalemm, T.; Annuk, H.; Kairane, C.; Kilk, A. Two antioxidative lactobacilli strains as promising probiotics. *Int. J. Food Microbiol.* **2002**, *72*, 215–224. [CrossRef]