Vol. 18(3), pp. 72-80, March 2024
DOI: 10.5897/AJMR2024.9742
Article Number: ADEC41772018
ISSN: 1996-0808
Copyright©2024
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJMR

ACADEMIC JOURNALS
expand your knowledge

African Journal of Microbiology Research

Full Length Research Paper

Characterization of novel potential probiotic strains of lactic acid bacteria from rat faeces

Dieudonné Nyamaifofe[1]*, Amos Mbugua[2] and Kevin Mbogo[3]

[1]Department of Molecular Biology and Biotechnology, Pan African University Institute for Basic Sciences, Technology and Innovation, Juja, Kenya.
[2]Department of Medical Laboratory Sciences, Jomo Kenyatta University of Agriculture and Technology, Juja, Kenya.
[3]Department of Biochemistry, Jomo Kenyatta University of Agriculture and Technology, Juja, Kenya.

Received 14 February, 2024; Accepted 14 March, 2024

This study aimed to isolate and characterise novel probiotic strains from the faeces of healthy albino Wistar rats. Lactic acid bacteria were isolated on MRS agar, and their probiotic properties were assessed through *in vitro* tests, including tolerance to simulated gastrointestinal juices, auto-aggregation assays, and antimicrobial activity. The antibiotic susceptibility and haemolysis tests were performed to assess the safety of the isolates. Isolates with probiotic potential were selected and identified by 16S rRNA gene sequencing. Five strains, R11, R21, R52, R71 and R81, were Gram-positive and catalase-negative, and they were able to survive the simulated digestive conditions with digestive enzymes and 1% bile salts and could auto-aggregate. All the strains inhibited the growth of indicator pathogens. Additionally, all five strains did not exhibit haemolytic activity and were sensitive to most test antibiotics.These five strains were identified as *Enterococcus faecalis* (R11), *Enterococcus hirae* (R21), *Lactococcus garvieae* (R71), *Lactococcus garvieae* (R52), and *Enterococcus faecalis* (R81). These strains hold potential as probiotic candidates, and further *in vivo* studies are necessary to evaluate their safety and establish putative health benefits.

Key words: Probiotic, *Lactococcus*, *Enterococcus*, rat faeces, haemolytic activity.

INTRODUCTION

The widespread use of antibiotics has resulted in the emergence of multidrug-resistant microbes, which pose a significant threat to human health. Consequently, there is increasing interest in exploring probiotics and related products as potential antibiotics alternatives (Bazireh et al., 2020). Probiotics are living microorganisms, including bacteria, yeasts, and moulds, that confer benefit to the host's health when consumed in sufficient amounts (Byakika et al., 2019). These microorganisms are crucial in providing significant health benefits to their host and are generally necessary for human health and nutritional needs. Probiotic bacteria, such as *Lactobacillus*, *Pediococcus,* *Bifidobacterium*, *Lactococcus*, *Streptococcus*, *Leuconostoc*, and *Enterococcus* have been identified in fermented foods as well as in the gastrointestinal tracts of animals and humans (Ayivi et al.,

*Corresponding author. E-mail: nyamaifofed@gmail.com. Tel: +254795151066.

Author(s) agree that this article remain permanently open access under the terms of the Creative Commons Attribution License 4.0 International License

2020).

Putative probiotic strains require thorough *in vitro* testing to evaluate their safety and functional properties. These evaluations include survival under gastrointestinal conditions, acid and bile salt tolerance, antibacterial activity, antibiotic susceptibility, and haemolytic activity (Byakika et al., 2019). Lactic acid bacteria (LAB) are a prominent group of probiotic bacteria commonly used in various applications (Shehata et al., 2016). LAB are Gram-positive, catalase-negative, facultative anaerobic bacteria and can be either cocci or rod-shaped. During carbohydrate metabolism, LAB produce lactic acid as the primary fermentation by-product (Quinto et al., 2014). LAB are safe for human consumption due to their long history of use in the production of fermented foods, and are generally recognized as safe (GRAS) microorganisms (Shehata et al., 2016). Probiotic Lactic Acid Bacteria can confer numerous health benefits to the host, including anti-diabetic activity (Rittiphairoj et al., 2021; Wang et al., 2020), prevention of allergies (Lei et al., 2021), cholesterol-lowering effects (Gil-Rodríguez and Beresford, 2021), protection against pathogens (Barcenilla et al., 2022), immunomodulatory attributes (Shahbazi et al., 2021), and disease risk reduction (Paiva et al., 2020). LAB are therefore important in the development of functional foods and dietary supplements that promote health and prevent diseases.

Probiotics derived from the intestinal microbiota of both humans and animals exhibit distinct characteristics when compared with probiotics sourced from dairy products. One of the most prominent attributes of non-dairy probiotics is their adhesion properties. Such intestinal isolates often display a greater degree of adhesion activity than dairy isolates (Sornplang and Piyadeatsoontorn, 2016). Probiotic strains should be isolated from the same species as their intended host to elicit appropriate host-specific responses. These probiotics showed a higher likelihood of colonising and persisting in the gastrointestinal tract, thus promoting the growth of helpful microorganisms. Consequently, their usage is considered acceptable from an ethical point of view and potentially more effective than their exogeneous counterparts (Gopal and Dhanasekaran, 2021). Probiotic strains not isolated from their intended host may exhibit transient efficacy as they might not colonise the host gut for extended period (Kort, 2014).While rats are commonly used as animal models in probiotic research, most of these studies do not utilise probiotic strains sourced from rats. Moreover, there is a lack of research on isolating novel probiotic strains from rats, leading to a dearth of commercially available rat-derived probiotics for use in rat model studies. Jena et al. (2013) isolated and characterised probiotic lactic acid bacteria from the rat faecal microbiota. However, their study was limited only to lactobacilli. This research study aimed to isolate and characterise novel potential probiotic strains of lactic acid bacteria from albino Wistar rat faeces.

MATERIALS AND METHODS

Animals and ethical considerations

Ten eight-week-old male Wistar rats weighing 203 to 294 g, were procured from the Laboratory of Animal Production Unit of the University of Nairobi, Nairobi, Kenya. A veterinarian examined the rats before housing them in the Small Animal Facility for Research and Innovation (SAFARI) at the Jomo Kenyatta University of Agriculture and Technology (JKUAT), Kenya, under a 12 h light/12 h dark cycle at a temperature of 25°C and humidity of 50%. They had access to autoclaved food (chow pellets) and water *ad libitum*. Prior to the commencement of the experiment, the rats were acclimatized for ten days. The study was approved by the JKUAT Institutional Scientific and Ethical Review Committee (approval number JKU/ISERC/02316/1043).

Isolation and identification of lactic acid bacteria

The animals were separately placed in pre-disinfected cages and left to defecate normally. Two pellets of fresh faecal samples were aseptically collected from healthy rats in the morning and put in sterile 1.5-ml Eppendorf tubes. The samples were serially diluted ten-fold (down to a 10^{-7} dilution) in phosphate-buffered saline (PBS). To only select acid-tolerant lactic acid bacteria isolates, each dilution was inoculated in acidified (pH 2.5) de Man Rogosa and Sharpe (MRS) broth (HiMedia Ltd., Mumbai, India, Cat. M369) for 3 h. Subsequently, appropriate dilutions were plated on MRS agar (HiMedia Ltd., Mumbai, India, Cat. M641) supplemented with 0.5% (w/v) of calcium carbonate and incubated anaerobically at 37°C for 48 h (Gupta et al., 2023; Jena et al., 2013). Potential LAB isolates were identified as round, white to cream-coloured colonies with clear halos. The selected colonies were purified on MRS agar by streaking, and subsequently Gram staining and catalase test were performed following the protocol outlined by Ngene et al. (2019). Gram-positive, cocci in shape, and catalase-negative isolates were selected and stored in MRS broth containing 20% glycerol for further analysis.

Survival in simulated gastrointestinal conditions

The ability of the selected isolates to withstand the gastrointestinal conditions was assessed following the methodology outlined by Saboori et al. (2022) and Celiberto et al. (2018), with minor adjustments. Overnight cultures of the selected isolates were centrifuged at 6000 × g for 10 min at 4°C. The pellets were washed with PBS (pH 6.5) and resuspended in 3 mL of the same buffer. A 1 mL aliquot of bacterial culture suspension (10^{-9} CFU/mL) was then added in 9 mL of simulated gastric fluid containing NaCl 125 mM, $NaHCO_3$ 45 mM, KCl 7 mM, and 3 g/L of pepsin from porcine (Solarbio Ltd., Beijing, China) at pH 2.5. The suspensions were incubated at 37°C (150 rpm) for 3 h and then centrifuged at 3000 × g for 10 min. The supernatants were discarded. These pellets were washed again three times with PBS and resuspended in 9 mL of simulated intestinal fluid (pH = 8.0), containing bile salt (Oxgall Powder, Sigma Aldrich, USA) 10 g/L and pancreatin (Solarbio Ltd., Beijing, China) 1 g/L. The suspensions were incubated at 37°C for another 3 h to complete the 6-h gastrointestinal phase. The number of viable bacteria was counted and expressed as log CFU/mL, and the survival rates of bacteria (in percentage) were calculated using the following formula:

$$\text{Survival rate (\%)} = \frac{\text{CFU count after assay (6h)}}{\text{CFU count before assay (0h)}} \times 100$$

Auto-aggregation assay

The ability of the LAB isolates to auto-aggregate was assessed by following the method described by Bazireh et al. (2020), with minor adjustments. Briefly, overnight cultures of the LAB isolates were centrifuged at 6000 × g for 10 min at 4°C. The pellets were washed three times with PBS and resuspended in 9 mL of the same buffer. The initial absorbance was immediately measured at 600 nm (Ao). The mixture was then incubated at 37°C, and the absorbance was measured again at 600 nm (At) at different time intervals (6 and 12 h). The auto-aggregation ability expressed in percentages (%) was calculated using the following formula (Pessoa et al., 2017):

$$\text{Auto-aggregation}(\%) = [(Ao - At)/Ao] \times 100$$

Antimicrobial activity

The antimicrobial activity of the isolates was assessed *in vitro* following to the protocol outlined by Jena et al. (2013) with minor adjustments. Initially, LAB isolates were cultured in MRS broth overnight and subsequently centrifuged at 6,000 × g for 10 min at 4°C. The resulting supernatants were neutralised to pH 7 using 5 N NaOH and filtered through a 0.22 µm filter for sterilisation. Wells with a diameter of 7.8 mm were created in agar plates using a cork borer. Then, 100 µL of overnight culture of each indicator pathogen (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 43300, *Pseudomonas aeruginosa* ATCC 27853, and *Candida albicans* ATCC 64124), diluted to a turbidity corresponding to 0.5 McFarland, was spread on the agar plates, followed by the addition of 100 µL of each supernatant into the wells. The plates were then incubated at 37°C for 24 h, and the diameters (mm) of inhibition zones were subsequently measured. The test pathogens were sourced from the Laboratory of Molecular Biology and Biotechnology of the Pan African University Institute for Basic Sciences, Technology, and Innovation (Nairobi, Kenya).

Antibiotic susceptibility test

The antibiotic susceptibility of the LAB isolates was evaluated using the disc diffusion method following the protocol outilined by Barzegar et al. (2021), with slight modifications. Briefly, overnight cultures of LAB isolates were diluted in PBS to a concentration equivalent to 0.5 McFarland (107-108 CFU/mL) and then spread on MRS agar plates. Ten antibiotic discs were placed on the agar plates, which were then incubated at 37°C for 24 h. The diameters of the inhibition zones were measured in millimetres (mm), and the results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute, 2020). The antibiotic discs utilised in this study included erythromycin (15 µg), gentamicin (10 µg), tetracycline (30 µg), amoxicillin (30 µg), vancomycin (30 µg), amikacin (30 µg), ceftriaxone (30 µg), ampicillin (10 µg), ciprofloxacin (5 µg), and ceftazidime (30 µg).

Haemolytic activity

The haemolytic activity of the LAB isolates was assessed *in vitro* following the methodology outlined by Bazireh et al. (2020). Concisely, fresh bacterial cultures were streaked onto blood agar media supplemented with sheep blood (10% v/v) and the plates were then incubated for 24 h at 37°C. The appearance of colonies was examined, with beta haemolysis characterised by clear zones around the colonies and alpha haemolysis by greenish zones around the colonies. No change in the appearance of the agar indicated no hemolysis. *Listeria monocytogenes*, sourced from the Laboratory of Food Microbiology at JKUAT, served as a positive control.

Molecular identification and phylogeny

Genomic DNA was extracted from selected isolates with probiotic potential using a Bacterial Genomic DNA Extraction Kit (Solarbio Co. Ltd., Beijing, China), following the manufacturer's instructions. PCR was performed to amplify the 16S rRNA gene using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R(5'-ACGGCTACCTTGTTACGACTT-3') (Fan et al., 2022). The PCR reaction with a total volume of 25 µL, comprised 1 µL of both forward and reverse primers (10 µM), 12.5 µL of OneTaq Quick-Load 2X Master Mix (New England BioLab Inc., Ipswich, USA), 1.0 µL DNA template, and 9.5 µL Nuclease-free water. The PCR conditions were set as follows: initial denaturation at 94°C for 30 s, denaturation at 94°C for 30 s, annealing at 55°C for 1 min, extension at 68°C for 1 min, 30 cycles, and a final extension at 68°C for 5 min. The PCR products (1490 bp) were detected by 2% agarose gel electrophoresis. They were purified and sequenced by Macrogen Europe (Amsterdam, Netherlands). BioEdit 7.7.1 (Hall, 1999) was used for sequence analysis, and the sequence similarity comparison was performed using Basic Local Alignment Tool (BLAST) (Altschul et al., 1990) of the National Centre for Biotechnology Information (https://www.ncbi.nlm.nih.gov/). The sequences obtained from this study were aligned together with their closely related sequences in the NCBI database using Multiple Sequence Comparison by Log-Expectation (MUSCLE) software (Edgar, 2004). The phylogenetic tree was constructed using MEGA 11 (Tamura et al., 2021). The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980) at 1000 bootstrap replications.

Statistical analysis

The data were analysed using one-way analysis of variance (ANOVA). Duncan's post hoc test was performed to compare multiple means. Statistical analyses were conducted with SPSS 29.0.1.0 (IBMSPSS Statistics, Chicago, USA), and statistical significance was established at $p < 0.05$. All experiments were performed in triplicate, and the results were expressed as mean ± standard deviation (SD) with superscript (a, b, c, d, and e).

Ethical approval

This study was reviewed and approved by the JKUAT Institutional Scientific and Ethical Review Committee and granted the approval number JKU/ISERC/02316/1043.

RESULTS

Isolation and survival of LAB strains in gastrointestinal conditions

Five isolates (R11, R21, R52, R71 and R81) were cocci-shaped, Gram-positive,and catalase-negative. These strains underwent exposure to simulated gastric fluid (pH 2.5) and intestinal fluid (pH 8.0) to assess their survivability in gastrointestinal conditions. Remarkably, all five isolates were resilient under the simulated conditions

Table 1. Survival rate of LAB strains in simulated gastrointestinal conditions.

Strain	Morphology	Gram	Catalase	log CFU/mL		Survival rate in GIT (%)
				0 h*	6 h**	
R11	cocci	+	-	8.21 ± 0.08^{ab}	7.17 ± 0.05^{a}	87.41
R21	cocci	+	-	8.22 ± 0.13^{ab}	6.99 ± 0.06^{b}	85.04
R52	cocci	+	-	8.38 ± 0.08^{a}	6.89 ± 0.05^{b}	82.25
R71	cocci	+	-	8.30 ± 0.03^{ab}	6.35 ± 0.10^{d}	76.51
R81	cocci	+	-	8.14 ± 0.05^{b}	6.57 ± 0.03^{c}	80.68

*CFU counts before the *in vitro* gastrointestinal survival test. **CFU counts after6 hours of simulated gastrointestinal conditions. Data are expressed as mean ± SD. Mean with different superscripts in a column are significantly different (p<0.05).

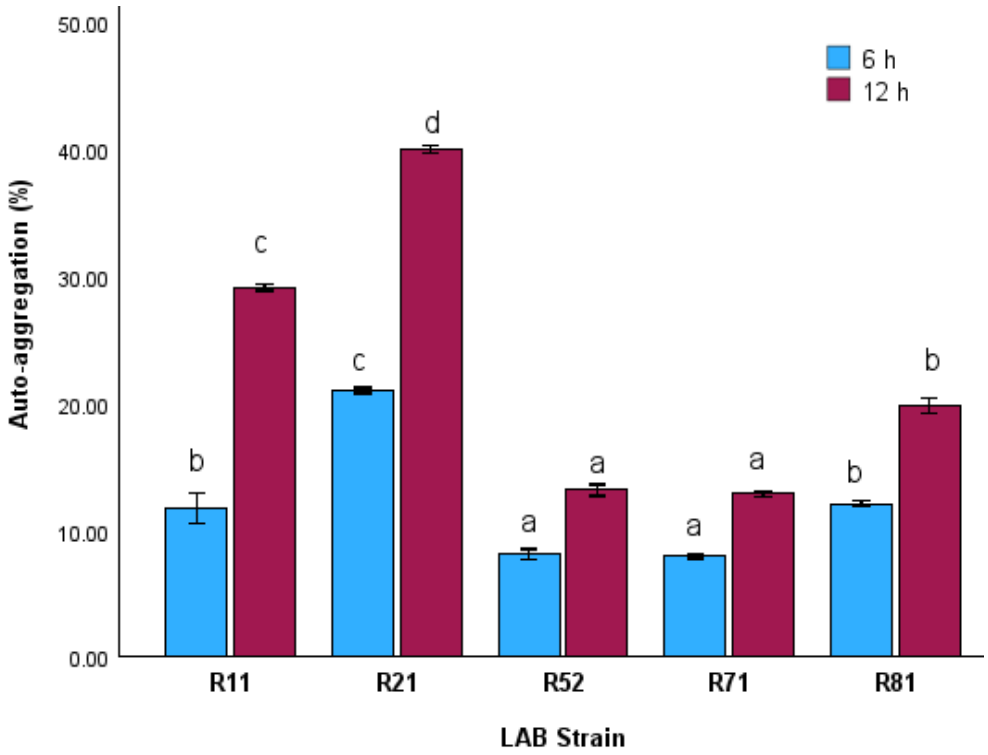

Figure 1. Auto-aggregation of the LAB strains after 6 and 12 h of incubation. Means with different superscript letters are significantly different (p<0.05), letter "a"being the lowest mean.

for 6 h, enduring the challenges posed by the low pH, digestive enzymes, and bile salt concentration (Table 1). Among the strains, R11 and R71 had the highest (87.41%) and lowest (76.51%) survival rates, respectively.

Auto-aggregation

The auto-aggregation ability of the lactic acid bacteria strains was assessed at 6 and 12 h after incubation. All five LAB strains were able to auto-aggregate at different rates after 6 and 12 h of incubation, and the auto-aggregation rates increased with the incubation time. Strain R21 exhibited the highest auto-aggregation rate (21%) after 6 h of incubation, which increased to 40% after 12 h. The lowest auto-aggregation rates were observed in R52 and R71 (Figure 1).

Antimicrobial activity

The antimicrobial activity of the cell-free supernatant from the lactic acid bacteria strains was assessed against four indicator pathogens, and the results were presented as the diameters of zones of inhibition. All strains exhibited inhibitory activity against all indicator pathogens to varying

Table 2. Antimicrobial activity of Lactic acid bacteria isolates.

Strain	Zone of inhibition (mm) against indicator pathogens			
	***E. coli* ATCC 25922**	***S. aureus* ATCC 43300**	***P. aeruginosa* ATCC 27853**	***C. albicans* ATCC 64124**
R11	20.33±0.58^{a}	16.33± 0.58^{b}	11.00±0.00^{c}	9.33 ± 0.58^{c}
R21	16.67±0.58^{b}	14.33 ±0.58^{c}	12.00±0.00^{b}	11.00 ± 0.58^{b}
R52	18.00±0.00^{b}	23.33± 0.58^{a}	9.33± 0.58^{d}	10.33 ± 0.58^{bc}
R71	20.33±0.58^{a}	15.33± 0.58^{bc}	13.00±0.00^{a}	11.33 ± 0.58^{b}
R81	14.67±0.58^{c}	15.33± 0.58^{bc}	11.33±0.58^{bc}	13.67 ± 0.58^{a}

Low activity:>7.8 mm; moderate activity: ≥ 13 mm; strong activity: ≥ 20 mm. Data are expressed as mean value ± SD. Means with different superscripts in a column are significantly different ($p<0.05$).

Table 3. Antibiotic sensitivity of Lactic acid bacteria isolates.

Strain	CIP	AK	CAZ	AMP	E	GEN	VAN	CRO	AMX	TE
R11	S	**R**	I	S	I	**R**	S	S	S	S
R21	S	**R**	**R**	S	S	**R**	S	S	S	S
R52	S	**R**	S	S	S	I	S	S	S	**R**
R71	S	**R**	S	S	S	S	S	S	S	**R**
R81	S	**R**	I	S	I	**R**	S	S	S	S

CIP= Ciprofloxacin 30 µg, AK= Amikacin 30 µg, CAZ= Ceftazidime 30 µg, AMP= Ampicillin 10 µg, E= Erythromycin 15 µg, GEN= Gentamicin 10 µg, VAN= Vancomycin 30 µg, CRO= Ceftriaxone 30 µg, AMX= Amoxycillin 30 µg, TE= Tetracycline 30 µg, S= Sensitive, R= Resistant, and I= Intermediate sensitivity.

extents (Table 2). Notably, the neutral supernatants of R11 and R71 showed strong inhibitory activity against *E.* coli, while R2 showed strong inhibition against *S. aureus*. Four strains (80%) showed low inhibitory activity against *C. albicans*.

Antibiotic susceptibility

The susceptibility of the lactic acid bacteria strains to ten different antibiotics was assessed using the disc diffusion method. All five strains were sensitive to Ciprofloxacin, Ampicillin, Vancomycin, Ceftriaxone, and Amoxicillin (Table 3). However, all strains were resistant to Amikacin, and three strains (R11, R21, and R81) were also resistant to Gentamicin. Additionally, two strains (R52 and R71) were found resistant to Tetracycline.

Haemolytic activity

All five lactic acid bacterial isolates did not exhibit haemolytic activity, in contrast to the positive control, which showed a haemolytic reaction (Figure 2).

Molecular identification and phylogenetic analysis

BLAST analysis was performed for sequence similarity search. The isolates R11 and R52 showed 99% similarity with *Enterococcus faecalis* 12YGD (OQ123535) and *Lactococcus garvieae* J (MT640284), respectively. Isolates R21, R71, and R81 showed 100% similarity with *Enterococcus hirae* 1104 (MT626063), *L. garvieae* F (MT640282), and *E. faecalis* 2623 (MT611645), respectively. The five strains were part of three major clades, including *E. faecalis*, *E. hirae*, and *L.garvieae*, as indicated by the phylogenetic analysis (Figure 3). The sequences generated were submitted to GenBank and assigned accession numbers are indicated in Table 4.

DISCUSSION

Five types of lactic acid bacteria were found to be possible probiotic strains in this study. They are *E. faecalis* R11, *E. faecalis* R81, *E. hirae* R21, *L. garvieae* R52, and *L. garvieae* R71. Previous research has suggested that lactic acid bacteria such as *Enterococcu*s strains isolated from human faeces and saliva (Bazireh et al., 2020), *L. garvieae* from healthy piglets (Zhang et al., 2016), *Enterococcus hirae* from healthy Chinese infants (Wei et al., 2020), and *E. faecalis* from Iranian fermented dairy product, Kashk (Saboori et al., 2022) are promising probiotic candidates. *Enterococcus* strains, naturally present in the gut of both humans and animals, can survive, compete, and adhere to host cells in the gastrointestinal tract (GIT). This is crucial for their effective utilisation as probiotics (Hanchi et al., 2018).

High survival rate during gastrointestinal transit is a

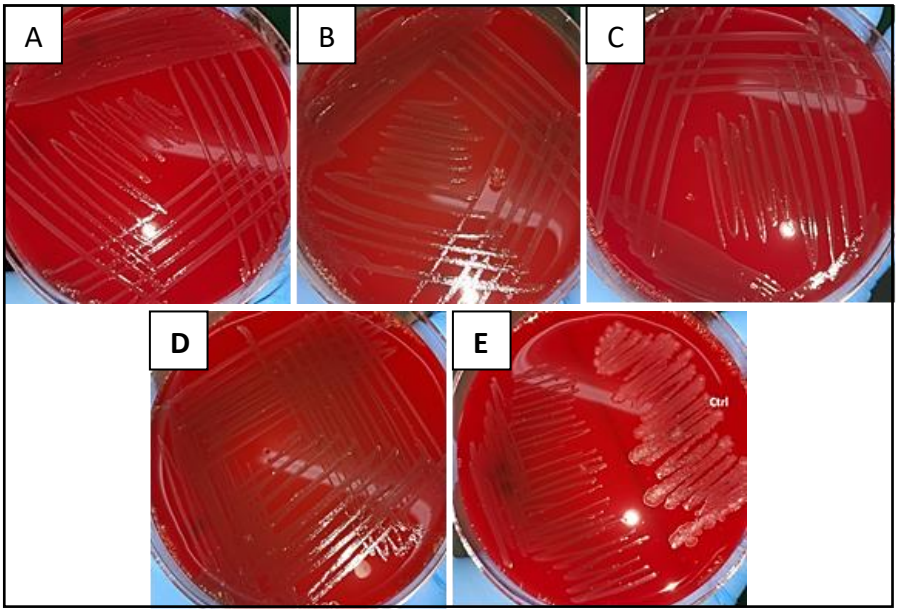

Figure 2. Haemolytic activity of LAB isolates. A: strain R11; B: strain R21; C: strain R52; D: strain R71; E: strain R81; and Ctrl= positive control (*Listeria monocytogenes*).

crucial feature as it indicates the potential of a probiotic bacterial strain. This means that the probiotic strains should be able to withstand the acidity and bile concentrations in the colon to effectively provide its beneficial health effects to the consumer (Celiberto et al., 2018). Factors such as low pH levels can impede metabolism and reduce the growth and viability of LAB. Gastric cells release highly acidic gastric secretions, establishing stringent conditions for the viability of bacteria passing through the stomach (Saboori et al., 2022). In our study, all identified LAB strains exhibited high survival rates in simulated gastric and intestinal juices, with *E. faecalis* R11 showing the significantly highest survival rate (Table 1). Our experimental results demonstrated that *Lactococcus* and *Enterococcus* strains isolated from rat faeces could withstand extreme acidic pH (pH 2.5), basic pH (pH 8), and survived in high concentrations of bile salt (1%). These findings align with previous studies where *Enterococcus* strains (Baccouri et al., 2019) and *L. garvieae* strains (Patel et al., 2020) were viable in acidic pH and high bile salt concentrations. Our results suggest that these lactic acid bacteria isolates can survive in the human and animal gastrointestinal tract and likely withstand passage through the stomach and intestines.

Auto-aggregation is another essential criterion to consider when selecting potential probiotic candidates. The ability of microorganisms to aggregate is directly related to their ability to adhere to and colonise the gastrointestinal tract of the host (Byakika et al., 2019). In the present study, the auto-aggregation rate of all the strains increased with the incubation time. The highest auto-aggregation rates were observed in *E. hirae* R21 (40%), followed by *E. faecalis* R11 (29.11%), and *E. faecalis* R81 (19.81%). The *L. garvieae* strains showed the lowest autoaggregation rates. The *Enterococcus* and *L. garvieae* strains isolated in this study exhibited higher auto-aggregation rates after a 12-h incubation period than *Lactobacilli* strains isolated from rat faeces by Jena et al. (2013). These strains have the ability to impact the immune system, outcompete pathogens for binding to the intestinal epithelial receptor cells, and reduce the presence of harmful intestinal microorganisms (Nascimento et al., 2019).

Antimicrobial activity is one of the key features to consider when evaluating the probiotic potential of microorganisms (Byakika et al., 2019). The production of organic acids, hydrogen peroxide, phenols, diacetyl, proteins, and probiotic development itself may contribute to probiotics' antimicrobial action. These metabolites, along with a competitive exclusion mechanism, help probiotics eliminate and prevent pathogenic microbes from colonising the body by competing with them for adhesion and resources (Aditya et al., 2020). In this study, the neutral cell-free supernatants of the strains exhibited varying degrees of inhibitory activity against all the indicator pathogens. Our results align with those of Jena et al. (2013) in which all the lactic acid isolated from rat faeces inhibited the growth of all the pathogens to different extents. *E. hirae* R52 showed the strongest inhibitory activity against methicillin-resistant *S. aureus* (MRSA) ATCC43300. These findings emphasize the importance of the isolated *Enterococcus* and *Lactococcus* strains in this study, as they have a broad spectrum of

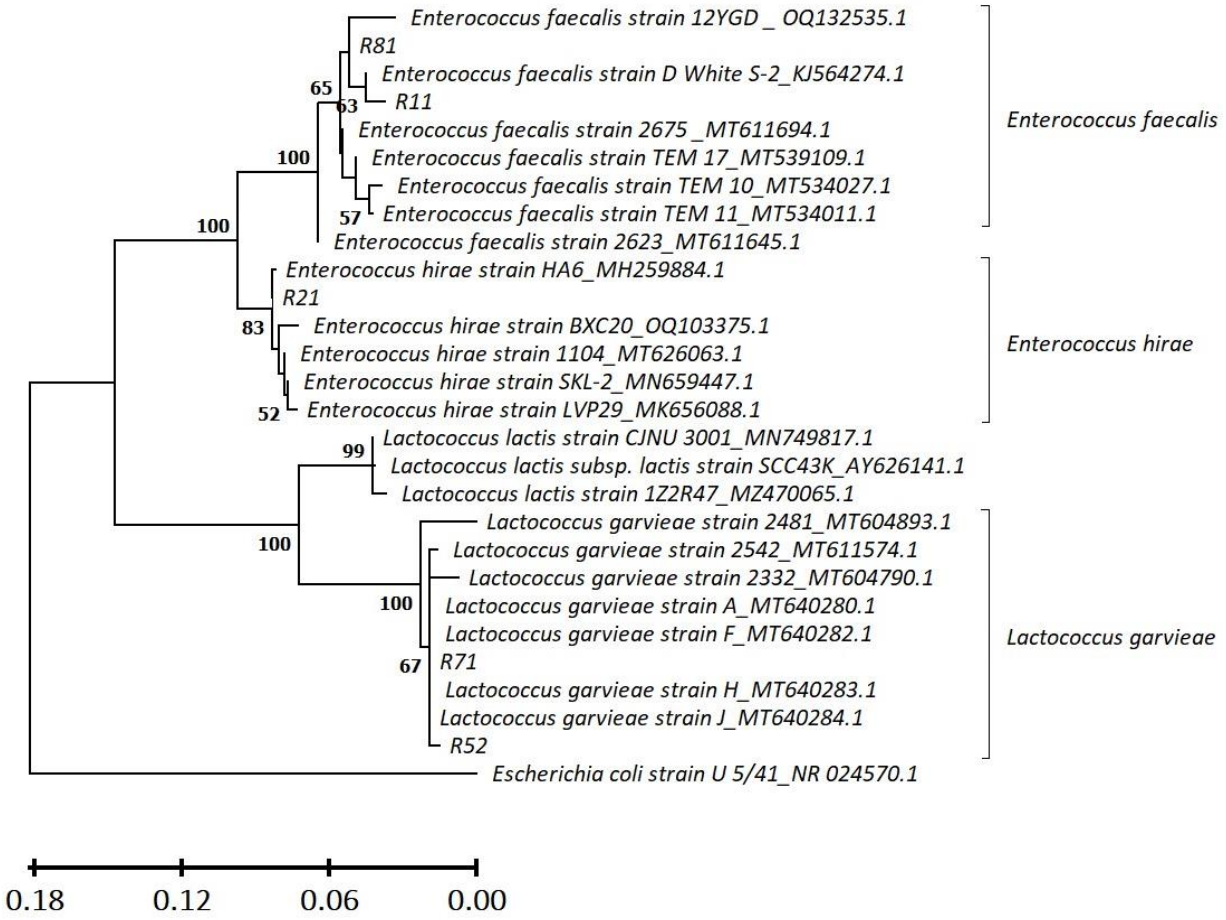

Figure 3. Maximum-Likelihood phylogenetic trees showing the taxa related to the isolated *Enterococcus* and *Lactococcus* strains. *Escherichia coli* U 5/41 was used as an outgroup.

Table 4. Molecular identification of the isolated LAB strains.

Strain	Species	NCBA Accession No.
R11	*Enterococcus faecalis*	OR921269
R21	*Enterococcus hirae*	OR921270
R52	*Lactococcus garvieae*	OR921272
R71	*Lactococcus garvieae*	OR921273
R81	*Enterococcus faecalis*	OR921274

antimicrobial activity, particularly against MRSA and the fungus *C. albicans*.

Evaluating the antibiotic susceptibility is crucial to ensuring the safety of a potential probiotic candidate. The potential transfer of antibiotic resistance from probiotic strains to pathogenic members of the microbiota, either directly or through intermediary microorganisms, is a worrisome issue as it may lead to the development of antibiotic-resistant pathogens that are difficult to treat (Roe et al., 2022). Based on the guidelines of the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2020), all strains in this study were susceptible to ciprofloxacin, ampicillin, vancomycin, ceftriaxone, and amoxicillin. In this study, *Enterococcus* strains were resistant to gentamicin and sensitive to vancomycin, which is consistent with the findings of Baccouri et al. (2019). The sensitivity to vancomycin was attributed to the absence of vancomycin resistance genes in the genomic analysis of *Enterococcus* strains. Resistance to gentamicin was previously described as intrinsic (Baccouri et al., 2019) and therefore cannot be transmitted to other bacteria.

Haemolysis is one of the two main virulence factors of pathogenic bacteria (Halder et al., 2017). Strains with haemolytic activity have the ability to cause anaemia, bacteraemia, and oedema, posing a significant risk to the consumer's health (Qin et al., 2023). Therefore, it is crucial to ensure that a microorganism intended for use as a probiotic does not exhibit haemolytic activity. In this study, none of strains exhibited haemolytic activity. The absence of haemolytic activity is another necessary safety criterion when selecting a probiotic strain since it indicates that the bacteria are not harmful, and the absence of haemolysin prevents the emergence of opportunistic virulence among the strains (Casarotti et al., 2017).

Conclusion

In this study, five strains of lactic acid bacteria with potential probiotic properties were successfully isolated from rat faecal samples, identified, and characterised. These strains, *E. faecalis* R11, *E. hirae* R21, *L. garvieae* R52, *L. garvieae* R71, and *E. faecalis* R81, had good probiotic traits such as being able to survive in simulated gastrointestinal fluids, clumping together on their own, and inhibiting the growth of indicator pathogens. The strains were also susceptible to a range of antibiotics and non-haemolytic, making them safe for use not only in studies involving rat models but also in industries and human as well as animal health. However, further *in vivo* studies are required to assess their safety and putative health benefits.

ACKNOWLEDGMENT

The authors greatly acknowledge the African Union Commission for funding this study through the Pan African University Scholarship. The authors thank Mr. Patrick Koech and Mrs. Jennifer Wambugu, technician in the Laboratory of Molecular Biology and Biotechnology, Pan African University for Basic Sciences, Technology and Innovation (PAUSTI) and manager of the Laboratory of Food Microbiology in JKUAT, respectively, for their technical support during the laboratory experiments.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Aditya A, Peng M, Young A, Biswa D (2020). Antagonistic Mechanism of Metabolites Produced by Lactobacillus casei on Lysis of Enterohemorrhagic *Escherichia coli*. Frontiers in Microbiology 11(11):1-13.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990). Basic local alignment search tool. Journal of Molecular Biology 215(3):403-410.

Ayivi RD, Gyawali R, Krastanov A, Aljaloud SO, Worku M, Tahergorabi R, Silva RC, Ibrahim SA (2020). Lactic Acid Bacteria: Food Safety and Human Health Applications. Dairy 1(3):202-232.

Baccouri O, Boukerb AM, Farhat LB, Zébré A, Zimmermann K, Domann E, Cambronel M, Barreau M, Maillot O, Rincé I, Muller C, Marzouki MN, Feuilloley M, Abidi F, Connil N (2019). Probiotic Potential and Safety Evaluation of Enterococcus faecalis OB14 and OB15, Isolated from Traditional Tunisian Testouri Cheese and Rigouta, Using Physiological and Genomic Analysis. Frontiers in Microbiology 10(4):1-15.

Barcenilla C, Ducic M, López M, Prieto M, Álvarez-Ordóñez A (2022). Application of lactic acid bacteria for the biopreservation of meat products: A systematic review. Meat Science 183 p. https://doi.org/10.1016/j.meatsci.2021.108661

Barzegar H, Alizadeh Behbahani B, Falah F (2021). Safety, probiotic properties, antimicrobial activity, and technological performance of Lactobacillus strains isolated from Iranian raw milk cheeses. Food Science and Nutrition 9(8):4094-4107.

Bazireh H, Shariati P, Azimzadeh Jamalkandi S, Ahmadi A, Boroumand MA (2020). Isolation of Novel Probiotic Lactobacillus and Enterococcus Strains from Human Salivary and Fecal Sources. Frontiers in Microbiology 11(12):1-12.

Byakika S, Mukisa IM, Byaruhanga YB, Muyanja C (2019). A Review of Criteria and Methods for Evaluating the Probiotic Potential of Microorganisms. Food Reviews International 35(5):427-466).

Casarotti SN, Carneiro BM, Todorov SD, Nero LA, Rahal P, Penna ALB (2017). In vitro assessment of safety and probiotic potential characteristics of Lactobacillus strains isolated from water buffalo mozzarella cheese. Annals of Microbiology 67(4):289-301.

Celiberto LS, Pinto RA, Rossi EA, Vallance BA, Cavallini DCU (2018). Isolation and characterization of potentially probiotic bacterial strains from mice: proof of concept for personalized probiotics. Nutrients 10(11).

Clinical and Laboratory Standards Institute (CLSI) (2020). Performance Standards for Antimicrobial Susceptibility Testing. In M100 (Ed.),Clsi (30th Ed., 40:1).

Edgar RC (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32(5):1792-1797.

Fan S, XueT, Bai B, Bo T, Zhang J (2022). Probiotic Properties Including the Antioxidant and Hypoglycemic Ability of Lactic Acid Bacteria from Fermented Grains of Chinese Baijiu. Foods 11(21).

Gil-Rodríguez AM, Beresford T (2021). Bile salt hydrolase and lipase inhibitory activity in reconstituted skim milk fermented with lactic acid bacteria. Journal of Functional Foods 77:104342.

Gopal V, Dhanasekaran D (2021). Probiotics as a Growth Promotant for Livestock and Poultry Production. In Advances in Probiotics (pp. 349–364). Academic Press.

Gupta M, Raut R, Manandhar S, Chaudhary A, Shrestha U, Dangol S, Sudarshan GC, Budha KR, Karki G, Díaz-Sánchez S, Gortazar C, de la Fuente J, Rajbhandari P, Manandhar P, Napit R, Karmacharya D (2023). Identification and characterization of probiotics isolated from indigenous chicken (*Gallus domesticus*) of Nepal. PLoS ONE 18(1 January):1-12.

Halder D, Mandal M, Chatterjee SS, Pal NK, Mandal S (2017). Indigenous probiotic Lactobacillus isolates presenting antibiotic like activity against human pathogenic bacteria. Biomedicines 5(2):1-11.

Hall TA (1999). BioEdit: a user friendly biological seque. In Nucleid Acids Symposium Series 41:95-98.

Hanchi H, Mottawea W, Sebei K, Hammami R (2018). The genus Enterococcus: Between probiotic potential and safety concerns-an update. Frontiers in Microbiology *9*(AUG), 1-16.

Jena PK, Trivedi D, Thakore K, Chaudhary H, Giri SS, Seshadri S (2013). Isolation and characterization of probiotic properties of lactobacilli isolated from rat fecal microbiota. Microbiology and Immunology 57(6):407-416.

Kimura M (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16(2):111-120.

Kort R (2014). Personalized therapy with probiotics from the host by

TripleA. Trends in Biotechnology 32(6):291-293.
Lei W, Liu C, Pan L, Peng C, Wang J, Zhou H (2021). Screening of probiotic Lactobacilli with potential anti-allergic activity based on hyaluronidase inhibition and degranulation of RBL-2H3 cells in vitro. Lwt 140(1):110707.
Nascimento LCS, Casarotti SN,Todorov SD, Penna ALB (2019). Probiotic potential and safety of enterococci strains. Annals of Microbiology 69(3):241-252.
Ngene AC, Onwuakor CE, Aguiyi JC, Ifeanyi VO, Ohaegbu CG, Okwuchukwu CP, Kim EG, Egbere JO (2019). Screening of Some Lactic Acid Bacteria Isolated from Selected Nigerian Fermented Foods for Vitamin Production. Advances in Microbiology 09(11):943-955.
Paiva IHR, Duarte-Silva E, Peixoto CA (2020). The role of prebiotics in cognition, anxiety, and depression. European Neuropsychopharmacology 34:1-18.
Patel P, Patel B, Amaresan N, Joshi B, Shah R, Krishnamurthy R (2020). Isolation and characterization of *Lactococcus garvieae* from the fish gut for in vitro fermentation with carbohydrates from agro-industrial waste. Biotechnology Reports 28:e00555.
Pessoa WFB, Melgaço ACC, De Almeida ME, Ramos LP, Rezende RP, Romano CC (2017). In Vitro Activity of Lactobacilli with Probiotic Potential Isolated from Cocoa Fermentation against Gardnerella vaginalis. BioMed Research International. https://doi.org/10.1155/2017/3264194
Qin S, Du H, Zeng W, Bai A, Liu J, Chen F, Ma L, Qin S, Zhu P, Wu J (2023). Identification and Characterisation of Potential Probiotic Lactic Acid Bacteria Extracted from Pig Faeces. Journal of Pure and Applied Microbiology 17(2):788-798.
Quinto EJ, Jiménez P, Caro I, Tejero J, Mateo J, Girbés T (2014). Probiotic Lactic Acid Bacteria: A Review. Food and Nutrition Sciences 05(18):1765-1775.
Rittiphairoj T, Pongpirul K, Janchot K, Mueller NT, Li T (2021). Probiotics Contribute to Glycemic Control in Patients with Type 2 Diabetes Mellitus: A Systematic Review and Meta-Analysis. Advances in Nutrition 12(3):722-734.
Roe AL, Boyte ME, Elkins CA, Goldman VS, Heimbach J, Madden E, Oketch-Rabah H, Sanders ME, Sirois J, Smith A (2022). Considerations for determining safety of probiotics: A USP perspective. Regulatory Toxicology and Pharmacology 136:1-36.
Saboori B, Shahidi F, Hedayati S, Javadmanesh A (2022). Investigating the Probiotic Properties and Antimicrobial Activity of Lactic Acid Bacteria Isolated from an Iranian Fermented Dairy Product, Kashk. Foods 11(23).
Shahbazi R, Sharifzad F, Bagheri R, Alsadi N, Yasavoli-Sharahi H, Matar C (2021). Anti-inflammatory and immunomodulatory properties of fermented plant foods. Nutrients 13(5):1-20.
Shehata MG, El Sohaimy SA, El-Sahn MA, Youssef MM (2016). Screening of isolated potential probiotic lactic acid bacteria for cholesterol lowering property and bile salt hydrolase activity. Annals of Agricultural Sciences 61(1):65-75.
Sornplang P, Piyadeatsoontorn S (2016). Probiotic isolates from unconventional sources: a review. Journal of Animal Science and Technology 58:1-11.
Tamura K, Stecher G, Kumar S (2021). MEGA11: Molecular Evolutionary Genetics Analysis Version 11. Molecular Biology and Evolution *38*(7):3022-3027.
Wang G, Si Q, Yang S, Jiao T, Zhu H, Tian P, Wang L, Li X, Gong L, Zhao J, Zhang H, Chen W (2020). Lactic acid bacteria reduce diabetes symptoms in mice by alleviating gut microbiota dysbiosis and inflammation in different manners. Food and Function 11(7):5898-5914.
Wei M, Gu E, Luo J, Zhang Z, Xu D, Tao X, Shah NP, Wei H (2020). Enterococcus hirae WEHI01 isolated from a healthy Chinese infant ameliorates the symptoms of type 2 diabetes by elevating the abundance of Lactobacillales in rats. Journal of Dairy Science 103(4):2969-2981.
Zhang T, Xie J, Zhang M, Fu N, Zhang Y (2016). Effect of a potential probiotics *Lactococcus garvieae* B301 on the growth performance, immune parameters and caecum microflora of broiler chickens. Journal of Animal Physiology and Animal Nutrition 100(3):413-421.