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Full Paper

Cholesterol-lowering effects of lactic acid bacteria isolated from *Musa sapientum* Linn.

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Abstract: Hypercholesterolemia is generally recognised as a risk factor for cardiovascular disease. *Musa sapientum* Linn. or Kluai Namwa (banana; ABB genome type) provides a functional nutrient in health promotion products and is a source of various probiotics. Lactic acid bacteria (LAB) isolated from several sources are reported with cholesterol-lowering potential. However, the effects of LAB isolated from bananas on total cholesterol are still unknown. The aim of this study is to investigate the cholesterol-removing ability of 5 LAB strains isolated from raw *M. sapientum*. All tested strains show a cholesterol-lowering property in species- and strain-dependent manner, with *Weissella paramesenteroides* NR27 being the most effective probiotic in reducing cholesterol concentration ($P < 0.001$). The removal of cholesterol by LAB is partially due to the production of bile salt hydrolase. All tested isolates exhibit more than 75% and 50% survival rates after challenging with simulated gastric and intestinal fluids respectively. Of these, *W. paramesenteroides* NR27 has a significantly higher survival rate (80%) in gastrointestinal conditions ($P < 0.001$), compared with all tested strains. In addition, antibiotic-resistant genes are not present in all drugs tested on this isolate. Therefore, *W. paramesenteroides* NR27 could be a promising probiotic candidate for reducing total cholesterol.

Keywords: lactic acid bacteria, cholesterol-lowering property, *Musa sapientum* Linn.

Maejo Int. J. Sci. Technol. **2022**, *16*(03), 249-261

INTRODUCTION

Musa sapientum Linn. (ABB genome type; a banana locally known as Kluai Namwa), an herbaceous plant in the Musaceae family, is one of the most important tropical fruits in many countries including Thailand [1-3]. It is the most consumed fruit with many nutritional and medicinal benefits [1, 4]. Importantly, the unripe or raw *M. sapientum* contains a high level of resistant starch which is beneficial for digestive functions [2] and acts as a prebiotic that stimulates and supports the growth of probiotic microorganisms [1].

Recently, raw bananas have drawn attention as a source of probiotic organisms [5, 6]. Lactic acid bacteria (LAB) are a major group of probiotic bacteria [7]. Its several health benefits to the host have been revealed [8, 9]. Production of antimicrobial substances such as bacteriocins, hydrogen peroxide and lactic acid by LAB against the growth of pathogenic bacteria has been reported [9]. Furthermore, the adhesion of LAB to the intestinal epithelium to modulate the host immune response was demonstrated in both *in vitro* and *in vivo* preclinical studies [10, 11].

Hypercholesterolemia is a major risk factor for cardiovascular disease [12]. Recently, public health programmes in most developed countries have attempted to reduce serum cholesterol levels, which might be one strategy to minimise cardiovascular disease incidence rates [13]. Although the cholesterol-lowering ability of certain drugs has been proven, they also have unwanted side effects such as gastrointestinal discomfort, bloating, flatulence and cramping [14]. With the recent increased interest in the cholesterol-lowering effect of LAB, several groups have attempted to isolate LAB as a source of probiotic organisms to investigate their biomedical utility [15-17]. Several LAB strains have been isolated from bananas, such as *Lactobacillus plantarum*, *Weissella cibaria* and *W. paramesenteroides* [5, 6]. Many studies have reported that LAB strains display a cholesterol-lowering effect in both *in vivo* (e.g. *L. casei, L. plantarum* and *Streptococcus thermophilus*) [15, 18] and *in vitro* (e.g. *L. brevis, L. fermentum* and *L. plantarum*) [17, 19] studies. Cholesterol-lowering effects of LAB from various sources have been studied, e.g. corn silage [17], pickle [19], fermented milk [15], fermented sausages [20] and human intestine [21].

Although studies regarding removal of cholesterol deposits by LAB isolated from multiple sources have been reported, the effects of LAB isolated from banana on the cholesterol level have not been revealed. In the present study we firstly investigated the cholesterol-lowering effect of LAB strains isolated from raw banana (*M. sapientum*, ABB genome type). Next, the resistance to simulated gastrointestinal conditions (gastric and intestinal fluids), bacterial adhesion abilities and antibiotic-resistant genes of the LAB strains were also determined.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Bacterial strains in this study, viz. *Lactococcus lactis* NR22, *L. lactis* NR24, *L. lactis* NR38, *Weissella paramesenteroides* NR27 and *W. paramesenteroides* NR28, were kindly provided by Microbiology Program, Faculty of Science and Technology, Pibulsongkram Rajabhat University. All bacterial strains were previously isolated from raw banana (*M. sapientum*, ABB genome type) [22]. Each bacterial strain was primarily cultured on De Man, Rogosa and Sharpe (MRS) agar (HiMedia®, India) at 37°C for 24 hr. A single colony of each isolate was cultured anaerobically in MRS broth at 37°C in a shaker incubator for 18 hr. The bacterial cells were harvested by centrifugation at 7,000×*g* for 10 min. The cells were washed twice using a sterile phosphate-buffered saline, pH 7.2.

Maejo Int. J. Sci. Technol. **2022**, *16*(03), 249-261

Cell Surface Hydrophobicity

The hydrophobicity of LAB has been suggested as an indicator of the adhesion ability on the cell surface for colonisation [7, 23]. Bacterial adhesion to hydrocarbons (n-hexadecane) was measured according to Rosenberg et al [24]. Briefly, the bacterial cells were adjusted to a cell density of 10^8 CFU/ml (OD_{600}; A1). Then n-hexadecane (Merck, Germany) was added to the bacterial suspension and the mixture was vortexed for 2 min. Subsequently, the mixture was left for phase separation at room temperature for 20 min. The optical density of the aqueous phase was measured (OD_{600}; A2). Each experiment was performed independently three times in duplicate. The hydrophobicity index (HPBI) was calculated:

$$\% \text{ HPBI} = [(A1-A2)/A1] \times 100$$

The interaction was defined as high hydrophobicity when HPBI $\geq$ 70%, moderate hydrophobicity when 50% $\geq$ HPBI < 70% and low hydrophobicity when HPBI < 50%.

Resistance of LAB under Simulated Gastrointestinal Conditions

Resistance to gastric and intestinal fluids is essential for predicting the survival rate of the potential probiotic strains in gastrointestinal conditions [19]. Survival of LAB under simulated gastrointestinal conditions (gastric and intestinal fluids) was performed as previously described with some modifications [19, 25]. For simulated gastric fluid, each bacterial suspension (10^8 CFU/ml) was inoculated into MRS broth (pH 2.0) with 3 mg/ml of pepsin (Sigma-Aldrich, USA). Aliquots of samples were taken at 0, 30, 60, 90 and 120 min. after anaerobic incubation at 37°C, then serially diluted and cultivated on MRS agar at 37°C for 48 hr to allow colony counting.

Subsequently, the remaining bacteria were then used for testing under simulated intestinal juice. The bacteria suspension was centrifugated at 11,000×*g* at 4°C for 10 min. Afterwards, the collected pellet was then inoculated into MRS broth (pH 8.0) in the presence of 1 mg/ml of pancreatin (Sigma-Aldrich) and 0.3% of bile salts (Sigma-Aldrich). Samples were then taken at 150, 180, 210 and 240 min. and cultivated on MRS agar to observe the bacterial survival. Each experiment was performed independently three times in duplicate. The percentage of bacterial survival was calculated:

% Bacterial survival = log CFU of viable cells survived/ log CFU of initially viable cells inoculated

In Vitro Cholesterol-Lowering Property

The cholesterol-lowering activity was determined as previously described with some modifications [17, 21]. Briefly, water-soluble cholesterol (cholesteryl-polyethylene glycol 600 sebacate) (Sigma-Aldrich) was filtered using 0.45-µm Minisart® Syringe Filters (Sartorius, Germany) and added to MRS broth supplemented with 0.3% (w/v) oxgall (Sigma-Aldrich) to reach a final concentration of 100 µg/ml. The bacterial cells were inoculated into the prepared broth at an inoculum size of 1% (v/v) and incubated anaerobically at 37°C for 24 hr. Culture media with cholesterol was used as a negative control. After 24 hr of incubation, the cells were centrifuged at 11,000×*g* for 10 min. Supernatants were taken to determine cholesterol concentrations by a total cholesterol colourimetric assay kit (Elabscience Biotechnology Inc., USA) according to the manufacturer's protocol. The amount of cholesterol was determined using a standard curve. Each experiment was performed independently three times in duplicate. The percentage of cholesterol removed by LAB compared to control was given by the following equation:

Maejo Int. J. Sci. Technol. **2022**, *16*(03), 249-261

% Cholesterol reduction = [1- (residual cholesterol in cell-free broth/cholesterol of control broth)] x 100

Bile Salt Hydrolase Activity

Deconjugation of bile salts by bile salt hydrolase (BSH) has been hypothesised that it might reduce serum cholesterol levels [26]. In this study the quantification of BSH activity in all tested LAB was performed as previously described with some modifications [21]. Briefly, overnight cultures of LAB strain (10 µl) were spotted onto MRS agar supplemented with 0.5% (w/v) taurodeoxycholic acid (Sigma-Aldrich) and 0.37 g/l $CaCl_2$ (Merck). The plates were anaerobically incubated at 37°C for 72 hr. The precipitated bile acid around the colonies (opaque or halo) was considered a positive result. Each experiment was performed independently three times in duplicate.

Antibiotic Susceptibility Testing

In this study the assessment of bacterial susceptibility to antibiotics was carried out according to European Food Safety Authority with some modifications [27, 28]. The test was performed using the BD BBL™ Sensi-Disc™ antimicrobial susceptibility test disc (BD, USA). Briefly, the bacterial suspension was prepared at a turbidity equivalent of 0.5 McFarland turbidity standard. Bacterial cells on MRS agar were incubated with antibiotics: ampicillin (10 µg/disc), chloramphenicol (30 µg/disc), clindamycin (2 µg/disc), erythromycin (15 µg/disc), gentamicin (120 µg/disc), kanamycin (30 µg/disc), streptomycin (30 µg/disc) and tetracycline (30 µg/disc), at 37°C for 24 hr. *Escherichia coli* ATCC 25922 (ATCC, USA) was also included in this experiment as a reference strain. The results were interpreted using BD BBL™ Sensi-Disc™ antimicrobial susceptibility test discs according to the manufacturer's instructions.

Detection of Antibiotic-Resistant Genes

Safety concerns have been raised regarding the use of LAB strains [29]; thus, distributions of antimicrobial-resistant genes in the tested LAB strains were investigated using polymerase chain reaction (PCR) with specific primers for antimicrobial-resistant determinants (Table 1) as previously described with some modifications [30].

Briefly, bacterial genomic DNA was extracted using DNAzol™ Reagent (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. PCR master mixes were prepared using Quick Taq™ HS DyeMix (TOYOBO Inc., Japan). PCR amplifications were performed by SimpliAmp™ Thermocycler (Thermo Fisher Scientific) using the following conditions: initial denaturation at 94°C for 5 min., 28 cycles of 94°C for 1 min., 48–60°C according to annealing temperature for the individual primers (Table 1) and 72°C for 1.5 min., and a final extension step at 72°C for 5 min. The PCR amplicons were separated by electrophoresis on 1% (w/v) agarose in 1X TAE buffer at 75 V for 1 hr and stained with ethidium bromide (Amresco®, USA) for 15 min. Gels were visualised using GelDoc2000 (Bio-Rad, USA).

Statistical Analysis

Data are presented as means ± standard deviation (SD). Statistically significant differences were analysed using one-way ANOVA by Tukey's multiple comparison test. All statistics were performed using GraphPad Prizm software (version 9.0).

Maejo Int. J. Sci. Technol. **2022**, *16*(03), 249-261

Table 1. PCR primers used in this study

Target gene (antibiotic)	Primer sequence (5′-3′)	Amplicon size (bp)	Annealing temperature (°C)	Reference
bla (AMP)	F: CATARTTCCGATAATASMGCC R: CGTSTTTAACTAAGTATSGY	297	48	[31]
cat (CHL)	F: TTAGGTTATTGGGATAAGTTA R: GCATGRTAACCATCACAWAC	300	50	[31]
tetM (TET)	F: GTGGACAAAGGTACAACGAG R: CGGTAAAGTTCGTCACACAC	406	57	[32]
strA (STM)	F: CTTGGTGATAACGGCAATTC R: CCAATCGCAGATAGAAGGC	548	60	[33]

Note: AMP = ampicillin, CHL = chloramphenicol, STM = streptomycin, TET = tetracycline

RESULTS AND DISCUSSION

Bacterial Cell Surface Hydrophobicity

One of the basic requirements of probiotic bacteria is the ability to adhere the intestinal epithelium [34, 35]. The attachment ability of each LAB isolate is observed as shown in Figure 1. The highest HPBI value is observed in *W. paramesenteroides* NR28 (52.6 ± 2.1%) followed by *W. paramesenteroides* NR27 (52.2 ± 0.8%). The adhesion ability of both isolates (NR27 and NR28) is classified as moderate hydrophobicity. In comparison, all *L. lactis* isolates show low hydrophobicity. The HPBI values of *L. lactis* NR22, NR24 and NR38 are 32.1 ± 2.5%, 31.6 ± 3.7% and 36.5 ± 1.3% respectively. *W. paramesenteroides* NR27 and NR28 significantly exhibit higher HPBI values ($P < 0.001$) than those of the three isolates of *L. lactis*. Our findings are consistent with other reports in that the adhesion capacity of LAB isolates is strain-dependent [36].

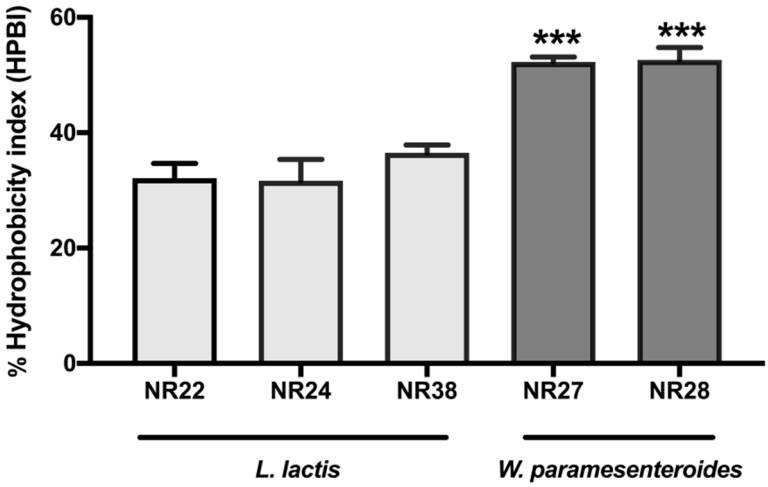

Figure 1. Cell surface hydrophobicity of tested LAB strains. Results are presented as mean values ± SD of three independent experiments carried out in duplicate. (***$P < 0.001$; comparison of either *W. paramesenteroides* NR27 or NR28 to all *L. lactis* strains)

The adhesion ability is crucial in considering a probiotic for gastrointestinal tract colonisation [35]. Our data indicate that both *W. paramesenteroides* NR27 and NR28 have a high

capacity for adhesion, which indicates an advantage for bacterial maintenance on the human intestinal mucosal surface.

Survival of LAB under Simulated Gastrointestinal Conditions

After oral administration, a range of physico-chemical factors may impact the viability of LAB strains, including stomach pH and pancreatic fluid. Therefore, resistance to the gastrointestinal tract is considered a critical functional requirement for probiotic bacteria, which enables them to survive in the gastrointestinal condition [8].

The survival of the LAB strains under simulated gastrointestinal conditions is shown in Figure 2. After 120 min. of incubation, bacterial viability values under gastric condition are 75.9 ± 5.6%, 77.3 ± 2.5%, 81.8 ± 1.2%, 89.8 ± 0.6% and 86.1 ± 0.8% for NR22, NR24, NR38, NR27 and NR28 respectively (Figure 2a). The highest survival rate is observed in *W. paramesenteroides* NR27 ($P < 0.01$) relative to all tested isolates. Subsequently, all tested strains also show >50% survival rates under simulated intestinal fluid (Figure 2b). Noticeably, *W. paramesenteroides* NR27 has a significantly higher survival rate (80.5 ± 3.7%; $P < 0.001$) than all tested strains. These results indicate that *W. paramesenteroides* NR27 exhibits great probiotic properties in resisting gastrointestinal conditions.

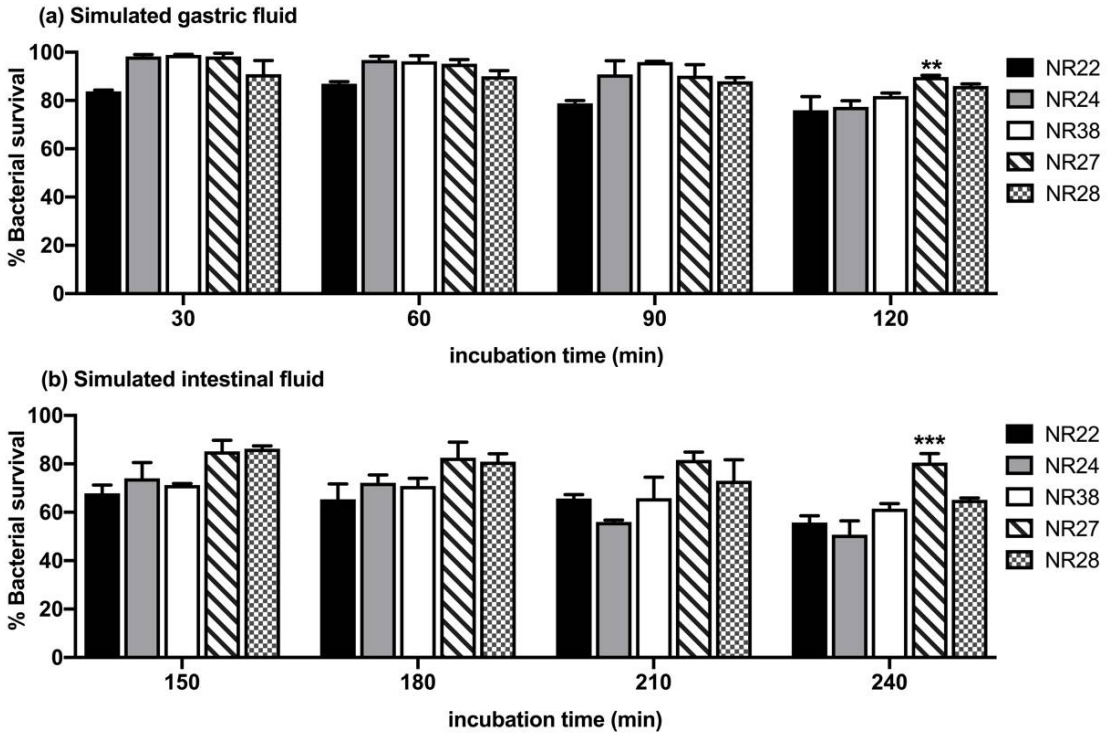

Figure 2. Bacterial viability under simulated gastric (a) and intestinal (b) conditions. Results are presented as mean values ± SD of three independent experiments carried out in duplicate. (**$P < 0.01$, ***$P < 0.001$; comparison between NR27 and all tested isolates)

Yadav and Shukla [37] reported that growing *W. paramesenteroides* at low pH significantly affected its viability. In our study 75% to 89% of the tested LAB strains could survive under pH 2.0 (simulated gastric condition), particularly *W. paramesenteroides* NR27. The conflicting results emphasise that *W. paramesenteroides* NR27 may partially survive and adapt to the stress environments during passage through the gastrointestinal tract.

Maejo Int. J. Sci. Technol. **2022**, *16*(03), 249-261

Removal of Total Cholesterol Level

Cholesterol-lowering effects of all tested LAB are shown in Figure 3. Among *L. lactis* strains, NR24 (25.4 ± 3.1%) shows higher cholesterol-reducing activity than NR22 (10.2 ± 1.2%) and NR38 (2.57 ± 0.56%). For *W. paramesenteroides* strains, NR27 (62.5 ± 3.7%) is more efficient in reducing cholesterol content than NR28 (35.6 ± 15.3%). These results indicate that *W. paramesenteroides* NR27 has the greatest cholesterol-lowering activity, serving as one of the most promising candidate for cholesterol-lowering probiotics. Thus, although cholesterol removal by *L. lactis* has been studied [38], we have provided the first report of cholesterol reduction by *W. paramesenteroides*.

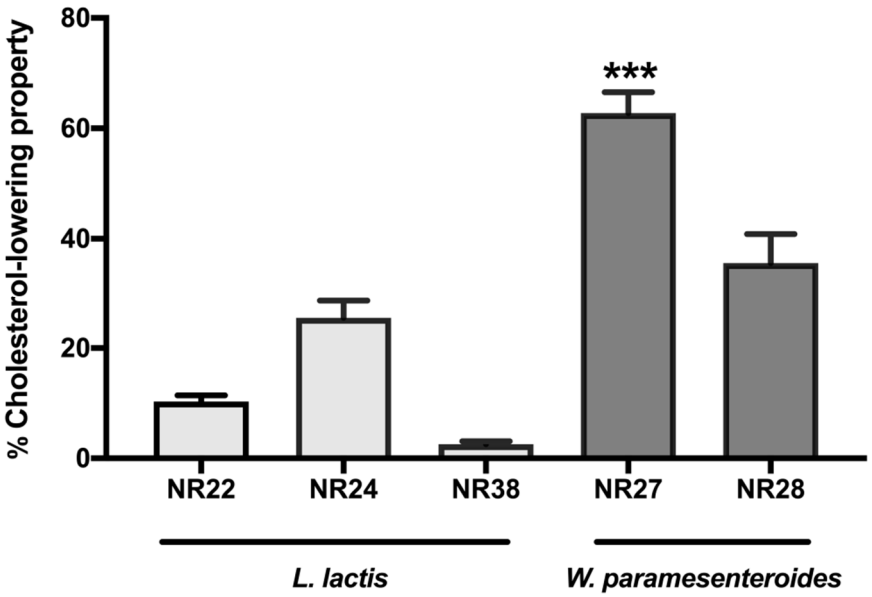

Figure 3. *In vitro* cholesterol-reducing activities of tested LAB strains. Results are presented as mean values ± SD of three independent experiments carried out in duplicate. (***$P < 0.001$; comparison between NR27 and all tested isolates)

Production of BSH

The BSH activity of probiotic bacteria has been associated with the ability to lower serum cholesterol levels in hypercholesterolemic conditions [16, 39]. The BSH is an enzyme that catalyses the hydrolysis of conjugated bile salts, resulting in free bile acid [39]. Consequently, deconjugated bile salts are less reabsorbed through the intestines compared with their conjugated forms; thus, the replacement of new bile salts from cholesterol as a precursor leads to decreased serum cholesterol levels [39, 40]. Therefore, the cholesterol-lowering property of LAB may be confirmed by determining the BSH production.

In the present study we observed BSH activities in *L. lactis* NR24, *W. paramesenteroides* NR27 and NR28 (Figure 4). The highest white precipitate around colonies is observed in *W. paramesenteroides* NR27 (Figure 4b). Such observation is consistent with cholesterol-lowering results. The results indicate that three LAB strains, particularly *W. paramesenteroides* NR27, exhibit BSH activity (bile salt hydrolysis), which could reduce cholesterol levels. Although BSH activities exhibited by *L. lactis* NR22 and NR38 were not observed (data not shown), reduced cholesterol could still be seen. It was found that probiotics could remove cholesterol via assimilation into cell membranes and conversion to coprostanol [41].

Maejo Int. J. Sci. Technol. **2022**, *16*(03), 249-261

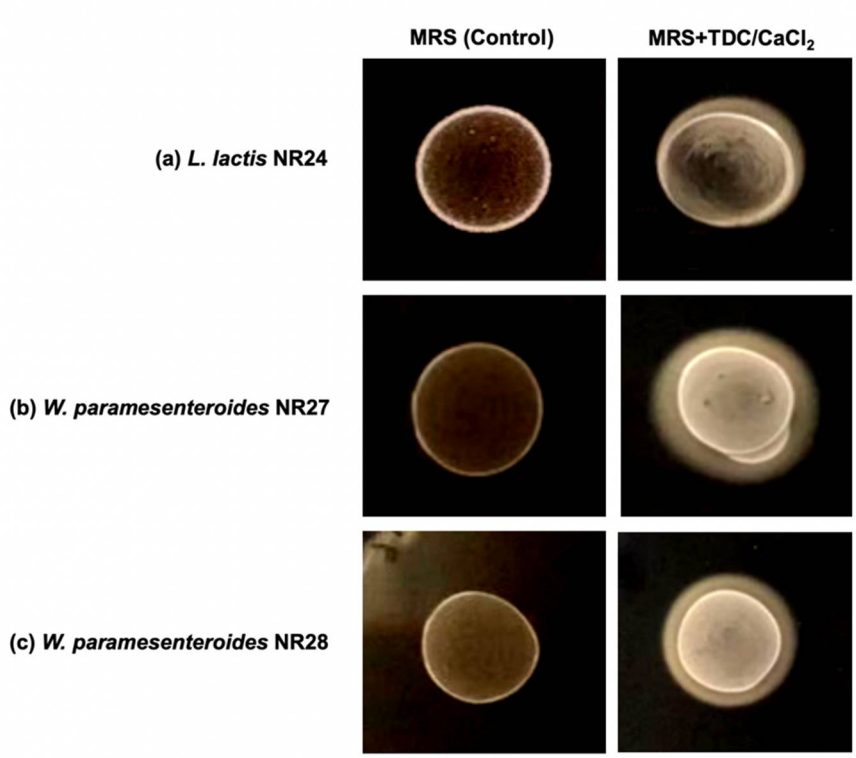

Figure 4. BSH activities of *L. lactis* NR24 (a), *W. paramesenteroides* NR27 (b) and *W. paramesenteroides* NR28 (c). White precipitates around colonies indicate BSH activity.
Note: TDC = taurodeoxycholic acid

Phenotypic Antibiotic Susceptibility Testing

Probiotic strains intended for human consumption should not harbour intrinsic and mobile genetic elements that render antibiotics resistant [42]. For these reasons, the safety trait of isolated LAB strains was investigated and results are shown in Table 2. Only *L. lactis* NR22 is resistant to streptomycin. Both *L. lactis* NR24 and NR38 display intermediate phenotype to streptomycin. These findings are consistent with the previous studies [43, 44]. In addition, *L. lactis* NR22 also shows an intermediate level of resistance to ampicillin and tetracycline, while *W. paramesenteroides* NR27 and NR28 exhibit weak drug resistance (intermediate) to chloramphenicol. Therefore, these groups of antibiotics (ampicillin, chloramphenicol, streptomycin and tetracycline) were selected to determine genes encoding for antimicrobial resistance in further experiments.

The results also show that *W. paramesenteroides* (NR27 and NR28) seem more sensitive to tested antibiotics than *L. lactis* (NR22, NR24 and NR38). Although resistance to aminoglycosides (gentamicin and kanamycin) has been found for LAB strains [30, 45], there is no resistance to these antibiotics in the present study. Moreover, all tested LAB strains are sensitive to clindamycin and erythromycin. Thus, these results indicate that LAB strains isolated from banana tend to be sensitive to antibiotics.

Maejo Int. J. Sci. Technol. **2022**, *16*(03), 249-261

Table 2. Antimicrobial susceptibility of tested LAB strains

Bacterial strain	Antibiotic							
	AMP	**CHL**	**CLN**	**ERY**	**GEN**	**KAN**	**STM**	**TET**
L. lactis NR22	I	S	S	S	S	S	R	I
L. lactis NR24	S	S	S	S	S	S	I	S
L. lactis NR38	S	S	S	S	S	S	I	S
W. paramesenteroides NR27	S	I	S	S	S	S	S	S
W. paramesenteroides NR28	S	I	S	S	S	S	S	S
E. coli ATCC25922 (control)	S	S	ND	ND	ND	S	ND	S

Note: ND = Not determined, S = Susceptible, I = Intermediate, R = Resistant, AMP = ampicillin, CHL = chloramphenicol, CLN = clindamycin, ERY = erythromycin, GEN = gentamicin, KAN = kanamycin, STM = streptomycin, TET = tetracycline

Antibiotic-Resistant Genes

The evaluation of antibiotic-resistant genes of LAB has been raised because of their potential to spread resistance by horizontal gene transfer, transposons and integrons [30]. Thus, possible drug-resistant genes of LAB strains were determined. Four antibiotics–ampicillin, chloramphenicol, streptomycin and tetracycline–were selected according to European Food Safety Authority [27] and the previous results. The findings show that most LAB strains isolated from banana do not contain drug-resistant genes involved in conventional antibiotics (Table 3); only *L. lactis* NR22 proves to be resistant to streptomycin, which is consistent with the previous work [43].

Previously, tetracycline resistance by *W. paramesenteroides* isolated from customary dairy products was reported [37]. Contrary to the aforementioned study, we did not detect the tetracycline resistance gene in *W. paramesenteroides* NR27 and NR28. Interestingly, *W. paramesenteroides* NR27, a potent cholesterol-reducing probiotic strain, was not found to carry an antibiotic-resistant gene for all tested antibiotics.

Table 3. Detection of antibiotic-resistant genes in tested LAB strains using PCR

Bacterial strain	Gene (antibiotic)			
	***bla* (AMP)**	***cat* (CHL)**	***strA* (STM)**	***tetM* (TET)**
L. lactis NR22	-	-	+	-
L. lactis NR24	-	-	-	-
L. lactis NR38	-	-	-	-
W. paramesenteroides NR27	-	-	-	-
W. paramesenteroides NR28	-	-	-	-

Note: AMP = ampicillin, CHL = chloramphenicol, STM = streptomycin, TET = tetracycline

CONCLUSIONS

The cholesterol-lowering property in LAB isolated from raw banana (*M. sapientum* or Kluai Namwa; ABB genome type) has been investigated. Our study suggests that banana could be considered a novel bioactive health-promoting source of LAB strains. In particular, *Weissella*

paramesenteroides NR27 as a probiotic nutritional supplement might be helpful in reducing the total cholesterol and minimising the risk of cardiovascular disease.

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Maejo Int. J. Sci. Technol. **2022**, *16*(03), 249-261

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