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In vitro assessment of safety and functional probiotic properties of *Lactobacillus mucosae* strains isolated from Iranian native ruminants intestine

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ABSTRACT

The search for host isolated probiotic bacteria from animal intestine may discover new probiotic candidates with promising health properties. This study evaluated the safety and functional probiotic potential of the lactic acid bacteria (LAB) isolated from the Iranian native ruminants intestine under *in vitro* assays. The isolates were selected according to criteria including survivability in low pH, bile salts, pancreatic enzymes, different temperatures, NaCl concentrations, antibacterial activities, presence of adhesion genes and safety characteristics. The selected LAB were then identified to species level using *16S rRNA* gene sequencing. The results showed that out of one hundred and eighty-seven LAB isolates, only six strains (NABRII50, NABRII51, NABRII52, NABRII53, NABRII54 and NABRII55) were tolerant to low pH, bile salt, pancreatin enzyme, 45 °C temperature and 2% sodium chloride. The six selected isolates belonged to *Lactobacillus mucosae*. Two of the adhesion genes (*mub* and *map*) were detected in all strains except NABRII53. The virulence factors were observed in NABRII50, NABRII53 and NABRII55. The tetracycline resistance gene (*tet* (*S*)) was detected in NABRII55. This study was the first effort to select *Lb. mucosae* strains with the probiotic potential from the Iranian ruminants intestine. These results revealed that the ruminant intestinal ecosystem could be considered as a valuable origin of probiotic candidates and all the selected LAB strains except NABRII50, NABRII53 and NABRII55 could be considered as promising probiotics.

HIGHLIGHTS

- *Lactobacillus mucosae* strains isolated from Iranian native ruminants intestine including NABRII51, NABRII52 and NABRII54 showed probiotic potential under *in vitro* assays.
- The *Lb. mucosae* strains including NABRII51, NABRII52 and NABRII54 were able to survive in intestinal physiological conditions, and carried the adhesion genes, such as *mub* and *map*.
- *In vitro* biosafety assays confirmed that *Lb. mucosae* strains (NABRII51, NABRII52 and NABRII54) were safe to further studies.

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Introduction

Currently, there is a great interest in probiotics application for health and improve livestock performance (Li et al. 2020). The application of probiotic products can improve immune function, digestion and feed efficiency. Lactic acid bacteria (LAB) have been used as probiotics to modulate the composition of intestinal microbiota to promote the health of the host intestine (de Moraes et al. 2017). LAB isolates have been isolated from a variety of sources, including fermented and dairy products, plants, soil and various organs of poultry, cattle or fish (Kuppusamy et al. 2020). In addition, large amounts of LAB with vital function have been found in the ruminant's digestive tract (Timmerman et al. 2006).

Based on the selection criteria for probiotic strains, a bacterial strain should be able to withstand low gastric pH and bile salts of the intestine, adhere to the intestinal mucosa and exceed safety criteria, such as the absence of haemolytic activity and sensitivity to antimicrobials of human and veterinary importance (Musikasang et al. 2009; Iñiguez-Palomares et al. 2011). Besides, resistance to osmolytes and temperature is considered as notable features that lead to the

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successful performance of probiotic bacteria (Salas-Jara et al. 2016; Aleksandrzak-Piekarczyk et al. 2019) and those candidates which meet the established criteria for probiotic can be used to produce probiotic supplements.

The genus *Lactobacillus* is one of the beneficial LAB. Most of the studies have shown that *Lactobacillus* sp. are able to survive in harsh conditions of the gastrointestinal tract (GIT). The organic acids produced by LAB form an acidic environment that can inhibit the viability of pathogenic bacteria (Dunne et al. 2001; Bernardeau et al. 2008). Among LAB species, *Lactobacillus mucosae* is one of the highly mucosa-associated subpopulations closely related to the animal and human intestine and other mucosal niches (Etzold et al. 2014; Drobná et al. 2017). The results of various studies have shown that *Lb. mucosae* strains can promote host resistance against pathogens and improves mucosal immunity by increasing epithelial impermeability and barrier function, producing secondary metabolites and antimicrobial compounds (Pajarillo et al. 2017).

The indigenous livestock population of Iran is the unique reserves adapted to the various climatic conditions of this vast country. Adaptation to different climates has led to developing a specific microbial community in the GIT of ruminants (Ebrahimi et al. 2018; Naeemi et al. 2019). Given these points, this study aimed to investigate the functional probiotic properties and safety characteristics of autochthonous *Lb. mucosae* strains from the duodenal mucosal layer of native goat, sheep and cattle from west and north areas of Iran, using molecular and microbial approaches.

Material and methods

Initial isolation, purification and phenotypic characterisation

The LAB colonies were isolated from duodenum mucosal layer suspensions of cows ($n = 3$), sheeps ($n = 3$) and goats ($n = 3$) belonging to the North Providence, Guilan, and the west providences, Kermanshah and Hamedan of Iran. The samples were received from a livestock slaughterhouse located in the north of Iran (*Deylam Sanat Shargh Livestock Industrial Slaughterhouse CO-OP CO* (37°08′37.4″N 49°38′38.1″E)). The experimental protocols were carried out according to the principles of the Declaration of Helsinki (World Medical Association [WMA] 2008). The mucosal layer suspensions were spread on de Man, Rogosa and Sharpe (MRS) agar medium (Merck, Darmstadt, Germany), which were supplemented with 0.1% (w/v) L-cysteine (Merck, Darmstadt, Germany). After incubation at 37 °C for 48 h, the selected colonies were identified by Gram stain and catalase activity tests. The Gram-positive and catalase-negative isolates were stored in the MRS broth containing 10% (w/v) skimmed milk, and 60% (1:1) glycerol at −80 °C for further investigations.

Resistance to different acidic conditions, bile salts and pancreatin

Rapid preliminary screening for the acid tolerance of the LAB isolates was performed according to the method described by Ehrmann et al. (2002) with some modifications. Acid tolerance assay of each selected isolate from previous step was performed according to the method described by Yamazaki et al. (2012) and Grispoldi et al. (2020). Tolerance to the acidic conditions was determined by comparing number of bacterial colonies before (in neutralised PBS, as control) and after exposure to acidic conditions including pH = 2.5 and pH = 3 adjusted by 1 N HCl for 3 h.

The bacterial resistance to bile salts was performed according to the method described by Kumar and Kumar (2015) through measuring suspensions optical density at 630 nm (OD_{630}) before and after incubation in MRS broth containing 0.3% (w/v) Oxgall for 8 h. The suspensions without Oxgall were considered as control. At last, the LAB isolates which showed resistance to 0.3% (w/v) Oxgall more than 50% were considered as bile-resistant.

For evaluation of the bacterial cell resistance to pancreatin, 10 µL of each selected isolates suspension was inoculated into 170 µL MRS broth supplemented with 1.9 mg/mL of pancreatin (Sigma-Aldrich, St. Louis, MO), adjusted to pH = 8.0. Also, MRS broth (pH = 8.0) without pancreatin was considered as control. After incubating at 37 °C for 3 h, OD_{630} was recorded and the result was expressed based on growth rate (Hosseini et al. 2009; Sharifuzzaman et al. 2018).

Molecular identification

DNA extraction was performed with the Gram-positive bacteria DNA extraction kit (Cinaclone, Tehran, Iran) . The bacterial 16S ribosomal RNA-based polymerase chain reaction was carried out using universal primers 27f (5′AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′ TACGGYTACCTTGTTACGACTT-3′) (Plessas et al. 2017). After purification, PCR products were sequenced by the automated DNA sequencing system (Macrogen,

Seoul, Korea). The sequences were edited by Bioedit software version 7 (Hall 1999). A comparison of the sequence similarity was made by the Basic Local Alignment Search Tool (BLAST) of the National Centre of Biotechnology Information (NCBI). CLUSTALW program of the Bioedit software version 7 was used to sequences alignment. A phylogenetic tree was built using the neighbour-joining tree method based on the *16S rRNA* gene sequence analysis by MEGA software version 6 (Tamura et al. 2013). In the phylogenetic tree construction, nucleotide sequences, including six isolated LAB sequences derived from this study and five sequences of the *Lactobacillus* species derived from Genbank, were involved. The *Lactococcus lactis* strain NCDO 604 was used as an out group.

Resistance assay of Lb. mucosae strains under temperature stress and sodium chloride concentrations

The effects of different temperatures and sodium chloride concentrations on bacterial survivability were examined according to the procedure described by Mortezaei et al. (2020) and Aleksandrzak-Piekarczyk et al. (2019), respectively. During temperature treatments, each selected isolate was exposed to different temperatures (37, 45 and 50 °C) for 48 h and formation of bacterial colonies was evaluated. For salinity resistance test, each selected isolate was inoculated on MRS-agar plates supplemented with or without (control) NaCl (2, 4, 6 or 8% (w/v)). After incubation at the 37 °C for 48 h, presence and quality developed colonies were compared with control plates. The experiments were carried out in triplicate and the results were expressed by qualitative data.

Antimicrobial activity of Lb. mucosae strains

The inhibitory activity of the selected strains against four pathogenic bacteria (*Salmonella typhimurium* (ATCC 14028), *Salmonella enteritidis* (ATCC13076), *Escherichia coli* (o157) and *Staphylococcus aureus* (ATCC 25923)) was determined by the double agar layer method (Touré et al. 2003; Gaudana et al. 2010). Briefly, 2 µL of each selected *Lactobacillus* strain grown overnight culture was spotted onto MRS agar plates. Then plates incubated at 37 °C for 18 h in CO_2 incubator (5%). After colony development, the plates were overlaid with soft agar (containing 0.7% (w/v) agar and Trypticase Soy Agar (TSA) kept at 50 °C), seeded with 1% (v/v) of an active overnight culture of each pathogen, and incubated aerobically at 37 °C. After one night of incubation, the growth inhibition zones around LAB colonies were determined. The test was performed in triplicate.

Assessment of adhesion properties of Lb. mucosae strains

The adhesion properties of the selected *Lb. mucosae* strains were preliminarily determined by Congo red staining. Then, isolates were screened by PCR to investigate the presence of genes encoding adhesion proteins (*msa*, *map*, *mub* and *ef-tu*).

Congo red staining

The hydrophobicity of the *Lb. mucosae* strains was determined by Congo red staining (Leyva-Madrigal et al. 2011). The bacterial colonies were streaked on MRS agar plates containing 0.03% (w/v) Congo red (Merck, Darmstadt, Germany) and incubated at 37 °C for 24 h anaerobically. Subsequently, red colonies were considered as hydrophobic strains, and white or transparent colonies were considered as non-hydrophobic.

PCR detection of adhesion encoding genes

The selected *Lb. mucosae* strains were screened for adhesion encoding genes according to the PCR protocol described by de Moraes et al. (2017) after bacterial DNA extraction was done by the Gram-positive bacteria DNA extraction kit (Cinaclone, Iran). The primers were employed for the amplification of *msa*, *map*, *mub* and *ef-tu* presented in Table 1. The amplified products were then separated by electrophoresis in 1.0% (w/v) agarose gels.

Table 1. Primer sequences utilised in the investigation of adhesion properties of the selected isolates.

Target gene	Target protein/enzyme	Primers (5′–3′)	References
msa	Mannose-specific adhesin	GCTATTATGGGGATTACGTTG CTGTCTTGACAATAGCCATATA	Zago et al. (2011), de Moraes et al. (2017)
mub	Mucus-binding protein	GTAGTTACTCAGTGACGATCAATG TAATTGTAAAGGTATAATCGGAGG	Zago et al. (2011), de Moraes et al. (2017)
map	Mucus adhesion-promoting protein	TGGATTCTGCTTGAGGTAAG GACTAGTAATAACGCGACCG	Todorov et al. (2008), de Moraes et al. (2017)
ef-tu	Elongation factor	TTCTGGTCGTATCGATCGTG CCACGTAATAACGCACCAAC	Zago et al. (2011), de Moraes et al. (2017)

Safety assessments of Lb. mucosae strains

Haemolytic activity

The haemolytic activity of selected isolates was performed on blood agar (Quelab, Montréal, Canada), supplemented with 5% (v/v) of sheep blood. After 48 h incubation at 37 °C in CO_2 incubator (0.5%), the plates were then examined for the halo of haemolysis. The bacterial isolates without displaying the signs of β-haemolysis around the colonies were classified as non-haemolytic (without β-haemolysis) (Maragkoudakis et al. 2009).

Antibiotic susceptibility

The assay for antibiotic susceptibility of the six *Lb. mucosae* strains were performed in 96-well plates using the broth-microdilution method for the eight antibiotics of human and veterinary importance (ampicillin, clindamycin, gentamicin, streptomycin, tetracycline, erythromycin, kanamycin and chloramphenicol) according to the European Food Safety Authority (EFSA 2012). Antibiotic susceptibility was expressed as the minimum inhibitory concentration (MIC, μg/mL) necessary for inhibition bacteria visible growth and compared with the lactobacilli MIC breakpoint values recommended by EFSA (2012).

PCR detection of genes for tetracycline resistance, virulence factors and biogenic amines production

The presence of tetracycline resistance genes, virulence factors genes, such as *gelE* (gelatinase), *hyl* (hyaluronidase), *asa1* (aggregation substance), *esp* (enterococcal surface protein), *cylA* (cytolysin), *efaA* (endocarditis antigen), *ace* (adhesion of collagen) and genes encoding biogenic amines production, such as histidine decarboxylase (*hdc1*), histidine decarboxylase (*hdc2*), tyrosine decarboxylase (*tdc*) and ornithine decarboxylase (*odc*) were evaluated according to the PCR protocols (Muñoz et al. 2014; Perin et al. 2014; de Moraes et al. 2017). The primers employed for amplifying the encoding genes are presented in Table 2. Briefly, extracted DNA using the Gram-positive bacteria DNA extraction kit was used for the PCR amplification according to the PCR protocol described by Muñoz et al. (2014). The amplified products were then separated by electrophoresis in 0.8 to 2.0% (w/v) agarose gels in 0.5× TAE buffer.

Statistical analysis

The data were expressed as the mean values (or log values) ± standard error (SE). All *in vitro* assays were performed in triplicate. *p* Values of less than .05 were considered statistically significant. Significant differences

Table 2. Primer sequences utilised in the investigation of genes encoding for tetracycline resistance, virulence factors and biogenic amines.

Properties	Target gene	Target protein/enzyme	Primers (5′–3′)	References
Antibiotic resistance	*tet* (*L*)	Tetracycline resistance gene	CATTTGGTCTTATTGGATCG ATTACACTTCCGATTTCGG	Muñoz et al. (2014)
	tet (*M*)	Tetracycline resistance gene	GTTAAATAGTGTTCTTGGAG CTAAGATATGGCTCTAACAA	Muñoz et al. (2014)
	tet (*O*)	Tetracycline resistance gene	GATGGCATACAGGCACAGAC CAATATCACCAGAGCAGGCT	Muñoz et al. (2014)
	tet (*S*)	Tetracycline resistance gene	TGGAACGCCAGAGAGGTATT ACATAGACAAGCCGTTGACC	Muñoz et al. (2014)
	tet (*W*)	Tetracycline resistance gene	GAGAGCCTGCTATATGCCAGC GGGCGTATCCACAATGTTAAC	Muñoz et al. (2014)
Virulence factors	*gelE*	Gelatinase	TATGACAATGCTTTTTGGGAT AGATGCACCCGAAATAATATA	de Moraes et al. (2017)
	hyl	Hyaluronidase	ACAGAAGAGCTGCAGGAAATG GACTGACGTCCAAGTTTCCAA	de Moraes et al. (2017)
	asa1	Aggregation substance	GCACGCTATTACGAACTATGA TAAGAAAGAACATCACCACGA	de Moraes et al. 2017
	esp	Enterococcal surface protein	AGATTTCATCTTTGATTCTTG AATTGATTCTTTAGCATCTGG	de Moraes et al. (2017)
	cylA	Cytolysin	ACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTT	de Moraes et al. (2017)
	efaA	Endocarditis antigen	GCCAATTGGGACAGACCCTC CGCCTTCTGTTCCTTCTTTGGC	de Moraes et al. (2017)
	ace	Adhesion of collagen	GAATTGAGCAAAAGTTCAATCG GTCTGTCTTTTCACTTGTTTC	de Moraes et al. (2017)
Biogenic amines	*hdc1*	Histidine decarboxylase	AGATGGTATTGTTTCTTATG AGACCATACACCATAACCTT	Perin et al. (2014)
	hdc2	Histidine decarboxylase	AAYTCNTTYGAYTTYGARAARGARG ATNGGNGANCCDATCATYTTRTGNCC	Perin et al. (2014)
	tdc	Tyrosine decarboxylase	GAYATNATNGGNATNGGNYTNGAYCARG CCRTARTCNGGNATAGCRAARTCNGTRTG	Perin et al. (2014)
	odc	Ornithine decarboxylase	GTNTTYAAYGCNGAYAARCANTAYTTYGT ATNGARTTNAGTTCRCAYTTYTCNGG	Perin et al. (2014)

between means were determined by Duncan's multiple range tests after analysis of variance (ANOVA) with SPSS version 16.0 software (SPSS Inc., Chicago, IL).

Results and discussion

Bacterial initial isolation and phenotypic characterisation

The bacterial isolation and phenotypic characterisation resulted in isolation of one hundred and eighty-seven Gram-positive, non-spore-forming, catalase-negative and non-motile bacteria from the duodenum mucosal layer of native Iranian cows, sheep and goats. The LAB colonies were rod-shaped or coccoid form and the cells were shown in pairs or short chains.

Survival assays under acidic conditions, bile salts and pancreatin resistance

Based on the previous studies, *in vitro* assessments, such as acid, bile and pancreatic enzymes tolerance, and survey some physiological characteristics (such as optimum growth temperature and salt sensitivity) have been considered as a good indicator to evaluate the probiotic properties of a bacterial isolates (Kim et al. 2019; Kuppusamy et al. 2020). After preliminary growth assays under acidic condition, seventy LAB isolates exhibited good survivability to pH = 3 (data not shown).

In the small intestine, the presence of bile is the most crucial stress agent for ingested bacteria. According to the results, the six selected lactobacilli isolates showed an ability to grow over 50% at 0.3% Oxgall (Figure 1). Among them, NABRII52 and NABRII55 showed significantly higher (97.97%) and lower (58.88%) ability than other isolates in this regard, respectively ($p < .05$). 0.3% bile salt concentration is a critical concentration for evaluating the ability of LAB to tolerance bile salts, and those with resistance more than 50% at this range considered as bile resistant isolates (Sahadeva et al. 2011; Kumar and Kumar 2015).

In the ruminants, pH values can vary between 5.7 and 7.3 in the rumen and 2–3 in the abomasum (Gentile et al. 2004). The secretion of hydrochloric acid by the gastric cells is an important defence mechanism to protect the host body against the ingested pathogens (Smith 2003). The results of acid tolerance for all six *Lactobacillus* strains are shown in Table 3. All of the selected isolates showed a significant reduction in pH = 2.5 than control (0 h) ($p < .05$), But their survival rate remained more than 90% after exposure to pH = 2.5 for 3 h. In addition, all isolates' survivability in pH = 3 was similar to the control (0 h) expect NABRII55 ($p < .05$). Jensen et al. (2012) observed a reduction in cell viability of *Lb. reuteri* strains when incubated at pH = 3 for 3 h. Similarly, de Moraes et al. (2017) reported a reduction in the initial viability of *Lb. mucosae* strains population, after gastric simulation

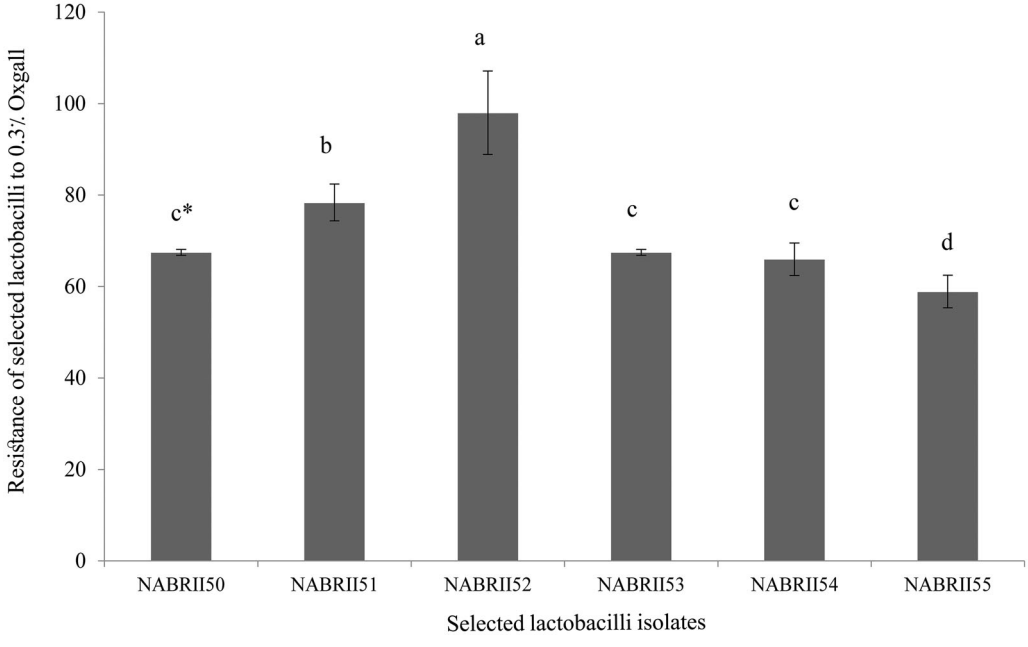

Figure 1. Bile tolerance for six selected lactobacilli exposure to 0.3% Oxgall. Data were presented as means ± SE, in three replicates. The lowercase letters show significant differences between values after 8 h ($p < .05$).

Table 3. Viability of six selected lactobacilli (log cfu/mL) after 3, h exposure to pH 2.5 and 3 after 3, h compared with control (0, h).

[A]Cell viability (log cfu/mL)						[B]Survivability (%)		
Selected lactobacilli	Before Exposure to acidic conditions (0, h)	Exposure to pH = 2.5	Exposure to pH = 3	SEM	*p* Value		After 3, h exposure to pH = 2.5	After 3, h exposure to pH = 3
NABRII50	9.08[a]	8.91[b]	9.13[a]	0.03	.01		98.34[a]	100.04[bc]
NABRII51	9.10[a]	8.21[c]	9.02[b]	0.14	.00		90.32[c]	99.12[c]
NABRII52	8.98[b]	8.17[c]	9.09[a]	0.14	.00		90.57[c]	101.26[b]
NABRII53	8.99[a]	8.59[b]	9.02[a]	0.07	.01		96.92[a]	100.07[bc]
NABRII54	8.64[a]	8.47[b]	8.65[a]	0.03	.01		96.56[a]	100.15[bc]
NABRII55	8.61[c]	8.71[b]	9.09[a]	0.07	.00		93.73[b]	105.0 [a]
						SEM	0.78	0.54
						p value	0.00	0.00

[A]Data were presented as means ± SE, in three replicates.
Different lowercase letters showing significant differences between the selected LAB colony counts in different pH values compared with control (0 h) ($p < .05$).
[B]Survivability: Different lowercase letters showing significant differences between survivability percentages of the selected LAB colony counts in each pH values (i.e. within column) ($p < .05$).

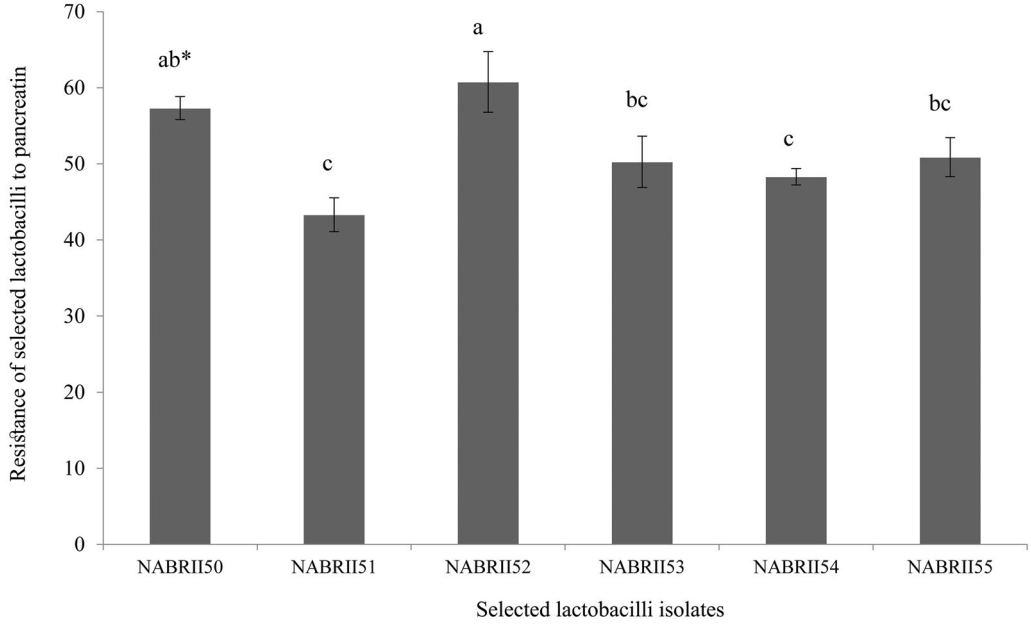

Figure 2. Tolerance of six selected lactobacilli to pancreatic enzymes. Data were presented as means ± SE, in three replicates. The lowercase letters show significant differences between values after 3 h ($p < .05$).

at pH = 2.5 for 3 h. Generally, the selected LAB showed different levels of resistance to acid and bile salt which probably could be due to the strain-dependent mechanisms (Li et al. 2020).

Furthermore, all six selected *Lactobacillus* isolates exhibited strong tolerance to pancreatic enzymes (Figure 2). Among them, NABRII52 showed significantly higher tolerance to 1.9 mg/mL of pancreatin (60.78%) after 3 h exposure in comparison to the other selected isolates ($p < .05$) except NABRII50. The results of tolerance to pancreatin are in agreement with the results obtained by Maragkoudakis et al. (2009) and Mahmoudi et al. (2016).

Molecular identification using 16S rRNA gene sequence

The results of comparative *16S rRNA* gene analysis showed that the six selected LAB belonged to the genus *Lactobacillus* and were 99–100% similar to *Lb. mucosae*. The *16S rRNA* gene sequences of the six *Lb. mucosae* strains were deposited in the GenBank database under the accession numbers MH595979.1 to MH595980.1 for isolates NABRII50 to NABRII55, respectively (Table 4). In this study, a phylogenetic tree (Figure 3) depicts the phylogenetic relationships between the six *Lb. mucosae* strains and five type

Table 4. Molecular identification of six selected isolates.

Selected isolates	Accession number	The nearest matched species from GenBank	Origins	Similarity (%)
NABRII50	MH595975.1	*Lactobacillus mucosae*	Goat intestine	100
NABRII51	MH595976.1	*Lactobacillus mucosae*	Goat intestine	100
NABRII52	MH595977.1	*Lactobacillus mucosae*	Sheep intestine	100
NABRII53	MH595978.1	*Lactobacillus mucosae*	Sheep intestine	100
NABRII54	MH595979.1	*Lactobacillus mucosae*	Goat intestine	99
NABRII55	MH595980.1	*Lactobacillus mucosae*	Cow intestine	100

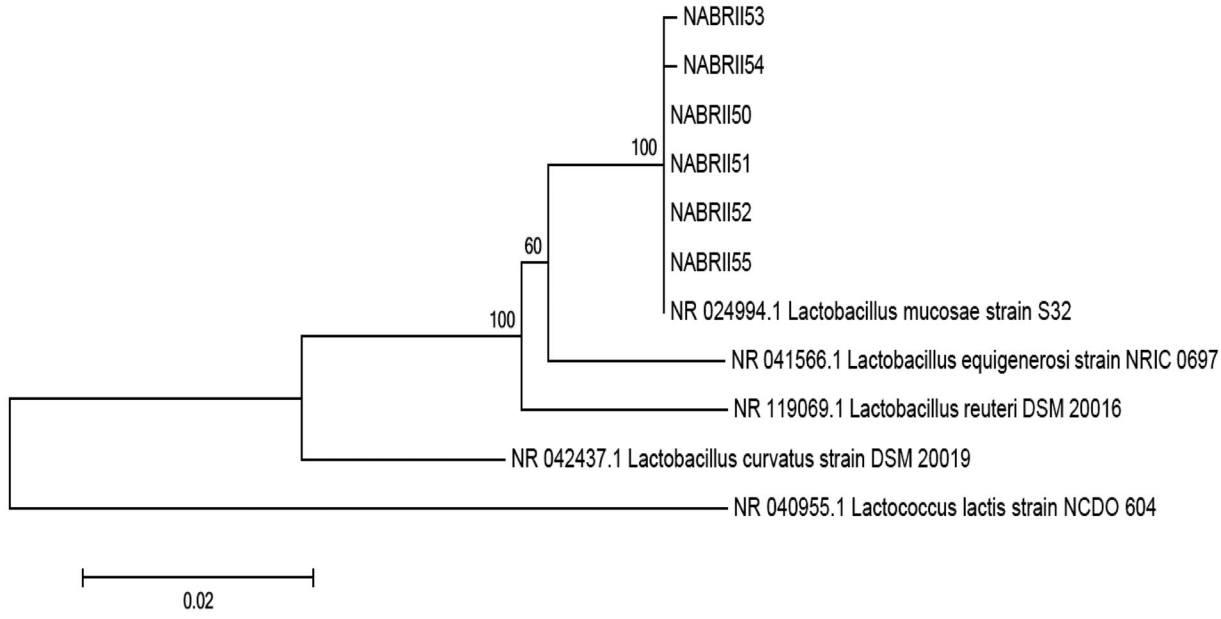

Figure 3. Phylogenetic tree based on the neighbour-joining method of *16S rRNA* gene sequences. Bootstrap values above 50% are indicated at the nodes of the tree. The scale bar represents 0.02-nucleotide substitutes per position.

strains obtained from the Genbank based on *16S rRNA* gene sequence analysis. *Lc. lactis* (NCDO 604) was used as the outgroup. The phylogenetic tree depicted that the six *Lb. mucosae* strains grouped into one leading group.

The *Lb. mucosae* was first isolated from the intestine of piglets as a new species (Iñiguez-Palomares et al. 2007). To our knowledge, the present research is the first report on the isolation of *Lb. mucosae* strains from the duodenum of Iranian sheep, goat and cow. Taxonomic studies have shown a close relation between *Lb. mucosae* and *Lb. reuteri* (Wang et al. 2016).

Resistance to temperatures and sodium chloride

Temperature also plays a vital role in LAB growth (Yang et al. 2018). The optimum growth temperature of lactobacilli lies between 30 and 40 °C, but depending on their species; they can grow at different temperatures from 5 to 53 °C (Ahmed et al. 2006). This study also revealed that a temperature of 50 °C has a negative effect on the survivability of the isolated *Lb. mucosae* strains, and their respective optimum temperatures were 37 and 45 °C (Table 5). Also, osmotic stress may be a significant inhibitor of bacterial growth and causes structural and functional damage to strains (Ge et al. 2011; Zhang et al. 2014). Furthermore, the *Lb. mucosae* strains in this study were salt sensitive and could not grow at more than 2% salt concentration (Table 5). Silva et al. (2019) found that *Lb. reuteri* strains had different growth rates towards 6% NaCl, while *Lb. mucosae* CRL 1508 was not resistant to the same NaCl concentration.

Antimicrobial activity of the selected Lb. mucosae strains

The Antimicrobial activity of the selected *Lb. mucosae* strains were evaluated using various Gram-positive (*S. aureus*) and Gram-negative (*S. typhimurium*, *S. enteritidis*, and *E. coli*) pathogenic bacteria (Table 6). Results of this study showed that NABRII52 and NABRII53 significantly inhibited the growth of the *S. typhimurium* (ATCC 14028) and *S. enteritidis*, respectively ($p < .05$). Our results are consistent with the results of the study

Table 5. The six selected isolates resistance to temperatures and sodium chloride.

		Selected isolates				
Characters	NABRII50	NABRII51	NABRII52	NABRII53	NABRII54	NABRII55
Temperature tolerance (°C)						
37	++	[a]++	++	++	++	++
45	++	++	[b]+	++	+	+
50	[c] –	–	–	–	–	–
Salinity tolerance (w/v)						
2, %	++	++	++	++	++	++
4, %	–	–	–	–	–	–
6, %	–	–	–	–	–	–
8, %	–	–	–	–	–	–

[a](++) Strong resistance.
[b](+) Moderate resistance.
[c](–) Susceptible.

Table 6. Antimicrobial activity of six selected lactobacilli.

	Inhibitory zone of the selected isolates (mm)							
Pathogens	NABRII50	NABRII51	NABRII52	NABRII53	NABRII54	NABRII55	SEM	*p* Value
S. typhimurium (ATCC 14028)	1.66^{c}	5.00^{b}	8.00^{a}	1.66^{c}	4.66^{b}	7.16^{a}	0.56	.00
S. enteritidis (ATCC13076)	3.83^{b}	4.66^{b}	4.00^{b}	7.33^{a}	4.66^{b}	4.33^{b}	0.37	.04
E. coli (o157)	2.00^{c}	3.66^{a}	3.16^{ab}	3.66^{a}	3.06^{ab}	2.16^{bc}	0.19	.01
S. aureus (ATCC25923)	1.16^{c}	1.16^{c}	2.50^{a}	1.66^{bc}	2.33^{ab}	Nd	0.22	.00

Data were presented as means ± SE, in three replicates. The lowercase letters show significant differences between values (i.e. within rows) ($p < .05$).
Nd: Not detected.

by Bian et al. (2011), which reported that *Lb. reuteri* DPC16 cell-free supernatants significantly inhibited the growth of selected Gram-negative food-borne pathogens (*S. Typhimurium* and *E. coli*) compared to Gram-positive pathogens (*Listeria monocytogenes* and *S. aureus*). Some previous studies have suggested that the production of bacteriocin-like metabolites by *Lb. mucosae* strain may be the reason (Maldonado et al. 2018).

Adhesion properties of the six selected Lb. mucosae strains

Adhesive ability and effective colonisation in GIT is a desirable feature for probiotic bacteria because it can inhibit pathogens' growth in the lumen through competitive exclusion (London et al. 2014). Various mechanisms, such as the presence of some adhesins, fimbriae, pili or cell surface proteins may be related to these phenomena (Devi and Halami 2017).

The hydrophobic nature of the outer surface may also play a role in the binding of bacteria to the host tissue. The evaluation hydrophobicity test using Congo red stain can confirm this nature (Leyva-Madrigal et al. 2011). A positive result indicates that the bacteria would not repel from the intestinal epithelium and have the ability to bind non-specifically to the intestinal epithelium by hydrophobic interactions (Leyva-Madrigal et al. 2011). In this study, all the *Lb. mucosae* strains had hydrophobic structures in the cell wall.

Previous studies have shown that mucus-targeting proteins or mucus-binding proteins (*mub*) and mucus adhesion-promoting protein (*map*), which were well characterised among *Lactobacillus* species, mediate the adherence of them to the intestinal mucosal layer (Buck et al. 2005; Devi and Halami 2017; Chatterjee et al. 2018). The presence of these genes strengthens the probiotic potential and mucus-binding ability of *Lb. mucosae* (Roos et al. 2000). Previously, it has been reported that this species usually carries the *mub* gene and can attach to the intestinal mucosal layer with this feature (Roos et al. 2000). The *mub* and *map* genes encode for extracellular mucus-binding proteins (*mub*) and a mucous adhesion-promoting protein (*map*), respectively (Buck et al. 2005). The presence of *mub* and *map* genes in the studied *Lb. mucosae* strains strengthens the probiotic potential of these strains. In this study, all the isolated *Lb. mucosae* strains except *Lb. mucosae* NABRII53, carried both genes (Table 7).

Safety assessments of the six selected Lb. mucosae strains

Haemolytic activity

The absence of haemolytic activity is one of the safety issues to select new potential probiotic strains because it indicates that the isolated bacteria do not have one of the virulence factors and potential possible negative effect on humans and animals (De Vuyst et al. 2003). The isolated strains displaying haemolytic activity (β or α-haemolysis) can produce toxins that induce

Table 7. Presence of genes associated with adhesion properties in six selected lactobacilli.

	Presence of genes in the selected isolates					
Adhesion genes	NABRII50	NABRII51	NABRII52	NABRII53	NABRII54	NABRII55
msa	[a]–	–	–	–	–	–
mub	[b]+	+	+	–	+	+
map	+	+	+	–	+	+
ef-tu	–	–	–	–	–	–

[a](–) Lack of genes encoding for adhesion properties.
[b](+) Presence of genes encoding for adhesion properties.

Table 8. Antibiotic susceptibility of six selected *Lb. mucosae* strains (MIC, μg/mL).

	Antibiotic susceptibility (MIC (μg/mL))							
Selected isolates	Am	Gm	km	Sm	Em	Cl	Te	Cm
NABRII50	0.25	4	8	32	0	0	8	4
NABRII51	0.25	1	4	16	0	0	8	4
NABRII52	0.25	8	32	32	0	0	8	4
NABRII53	0.25	2	16	32	0	0	16[aR]	4
NABRII54	0.25	1	4	16	0	0	8	4
NABRII55	0.25	2	2	16	0	0	32[R]	4

Values are provided by EFSA for obligate heterofermentative *Lactobacillus* strains; according to EFSA, susceptibility testing of obligate heterofermentative strains against vancomycin is not required. Am: Ampicillin, Gm: Gentamicin, Km: kanamycin, Sm: Streptomycin, Em: Erythromycin, Cl: Clindamycin, Te: Tetracycline, Cm: Chloramphenicol.
[a]R: Antibiotic resistant strain.

erythrocyte lysis. The results of the haemolytic activity in this study showed no harmful effects under *in vitro* assays. Our results confirm the findings of Repally et al. (2018) and Adetoye et al. (2018), which reported that other *Lb. mucosae* strains isolated from sheep milk and cattle faeces had no haemolytic activity.

Antibiotics susceptibility

Acquired antibiotic resistance remains a serious concern due to the high risk of horizontal spread of resistance genes when a viable microorganism is used as an active agent in probiotic supplements (EFSA 2012; Frieri et al. 2017). According to the EFSA (2012), when a bacterial strain is inhibited by a concentration of a specific antimicrobial agent equal to or lower than the specified cut-off values, considered as a susceptible strain and no further antibiotic resistance studies are required (EFSA 2012). In this study, we investigated the antibiotic susceptibility profiles of the six *Lb. mucosae* strains from the Iranian native cows, sheep and goats. The results of the antibiotic susceptibility profiles showed that all *Lb. mucosae* strains were sensitive to clinically relevant antibiotics such as ampicillin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin and chloramphenicol. However, only *Lb. mucosae* NABRII53 and *Lb. mucosae* NABRII55 were phenotypically resistant to tetracycline (MIC $>$ 8 μg/mL) (Table 8).

Table 9. Presence of genes associated with tetracycline resistance in *Lb. mucosae* strains.

	Presence of genes in tetracycline-resistant isolates	
Tetracycline resistance gene	NABRII53	NABRII55
tet (L)	[a]–	–
tet (M)	[b]–	–
tet (O)	–	–
tet (S)	–	+
tet (W)	–	–

[a](–) Lack of genes encoding for tetracycline resistance.
[b](+) Presence of genes encoding for tetracycline resistance.

PCR detection of genes encoding tetracycline resistance

The tetracycline-resistant strains were screened by PCR for the presence of tetracycline resistance genes (*tet* (*L*), *tet* (*M*), *tet* (*O*), *tet* (*S*) and *tet* (*w*)) for identifying the resistance determinants responsible for the tetracycline resistance (Table 9). PCR analysis showed that *Lb. mucosae* NABRII55 carried the *tet* (*S*) and neither the genes encoding ribosomal protection proteins *tet* (*M*), *tet* (*O*), *tet* (*S*) or *tet* (*W*) nor gene encoding the tetracycline efflux pump *tet* (*L*) were detected in *Lb. mucosae* NABRII53. However, the absence of resistance determinants *tet* (*M*), *tet* (*O*), *tet* (*S*), *tet* (*W*), *tet* (*L*) and *tet* (*K*) may suggest a new mechanism of resistance which can be due either to acquired genes or to the mutation of indigenous genes (EFSA 2012). Energy-dependent efflux of tetracycline from the cell, ribosome protection and enzymatic inactivation are three mechanisms of tetracycline resistance in microorganisms (Schaechter and Lederberg 2004). Studies have shown that genes conferring resistance to tetracycline are commonly found in the human gut microbiota, both in healthy adults and in breast-fed infants (Gueimonde et al. 2006). Similarly, these genes have been found in several lactobacilli isolated from dairy foods and it is vital to avoid their spread to pathogens through the consumption of fermented foods (Muñoz et al. 2014). Similar to this study, the absence of the resistance genes *tet* (*M*), *tet* (*O*), *tet* (*S*), *tet* (*W*), *tet* (*L*) and *tet* (*K*) was reported in *Lc. Pseudomesenteroides* from fermented table olive (Muñoz et al. 2014). Overall, *lactobacillus* resistance to antimicrobials is a

Table 10. Presence of genes associated with virulence factors in *Lb. mucosae* strains.

	Presence of genes in the selected isolates					
Virulence genes	NABRII50	NABRII51	NABRII52	NABRII53	NABRII54	NABRII55
gelE	[a]–	–	–	[b]+	–	–
hyl	–	–	–	+	–	–
asa1	–	–	–	+	–	–
esp	–	–	–	+	–	–
cylA	+	–	–	+	–	–
efaA	–	–	–	–	–	–
ace	–	–	–	+	–	+

[a](–) Lack of genes encoding for virulence factors.
[b](+) Presence of genes encoding for virulence factors.

relevant scientific topic and one of the crucial properties for identifying safety potential probiotics.

PCR detection of genes encoding virulence factors

Investigating the presence of virulence genes is another criterion for assessing the probiotic candidates' safety with potential applications in food products. The virulence factors are usually associated with competitive advantages of pathogenic strains, and their presence is more common in *Enterococcus* spp. and other clinical isolates (Semedo et al. 2003). The virulence factors usually locate in transferable plasmids. Therefore, due to the concern of transferring the mentioned genetic elements to the intestinal tract's pathogens, the detection of these genetic elements is inevitable (Eaton and Gasson 2001).

The *gelE*, *hyl*, *asa1*, *esp*, *cylA*, *efaA* and *ace* genes, which encode virulence factors, were screened in the genome of the *Lb. mucosae* strains (Table 10).

LAB species, such as *Lactococcus* spp. can carry different virulence genes, but their presence in the genome is not a definitive indicator of these species' pathogenesis because of the low capability of expressing these genes, which has been observed in different studies (Casalta and Montel 2008; Perin et al. 2014).

In this study, the *Lb. mucosae* NABRII53 and *Lb. mucosae* NABRII55 strains generated positive PCR results for the *ace* gene (adhesion of collagen protein). This protein facilitates the binding to collagen and may play a negative role during human infections (Girish and Kemparaju 2007); however, on the positive side, it can contribute to better adhesion and colonisation in the GIT (Todorov et al. 2017). dos Santos et al. (2015) observed positive results for the *ace* gene in the studied *Lb. rhamnosus* and *Lb. plantarum* strains. Furthermore, two of the investigated lactobacilli strains in this study, including *Lb. mucosae* NABRII53 and *Lb. mucosae* NABRII50, similar to the study conducted by de Moraes et al. (2017), carried the cytolysin gene. The cytolysin (*cylA*) is related to haemolytic activity among enterococci (Jiménez et al. 2013). Despite the presence of cytolysin gene, these strains (*Lb. mucosae* NABRII53 and *Lb. mucosae* NABRII50) were not able to exhibit haemolytic activity under *in vitro* assays. The cytolysin is a virulent substance due to its haemolytic potential, but it is also considered as an antibacterial bacteriocin, according to Cotter et al. (2005) classification. The presence of *cylA* is not enough to activate haemolytic activity by bacteria because the cytolysin expression requires the presence and functionality of eight genes (Perin et al. 2014). Additionally, *Lb. mucosae* NABRII53 showed positive results for the *hyl* gene. The *hyl* gene is involved in the production of hyaluronidase enzymes, which break down hyaluronic acid. The ability of bacteria to degrade hyaluronic acid may be a virulence factor and allow hyaluronidase-producing pathogens to penetrate hyaluronic acid-rich tissues (Aubin et al. 2017). However, the consequences of hyaluronidase activity among lactobacilli are not clear, as this has not been reported within the context of virulence and pathogenicity yet (Franz et al. 2005). The *gelE* gene, which encodes for the production of Gelatinase, was detected in *Lb. mucosae* NABRII53. The *gelE* is commonly found in *E. faecalis* (Munoz-Atienza et al. 2013). However, the presence of *gelE* gene is not enough for gelatinase activity since the complete *fsr* operon seems to be essential for its expression (Lopes et al. 2006).

Furthermore, the *esp* and *asa1* genes were detected in *Lb. mucosae* NABRII53 strain. These genes are relevant virulence factor, which contributes to intestinal adhesion through encodes the production of extracellular surface protein (Valenzuela et al. 2009; de Moraes et al. 2017). According to the results of this study, due to the presence of virulence genes in *Lb. mucosae* NABRII53, *Lb. mucosae* NABRII50 and *Lb. mucosae* NABRII55 these isolates are not recommended for food applications.

PCR detection of genes encoding biogenic amines

Examining the presence of genes encoding biogenic amines is another aspect of assessing the probiotic candidates' safety because their products could cause

Table 11. Presence of genes associated with biogenic amines production in *Lb. mucosae* strains.

Biogenic amine producing genes	Presence of genes in the selected isolates					
	NABRII50	NABRII51	NABRII52	NABRII53	NABRII54	NABRII55
hdc1	[a]–	–	–	–	–	[b]+
hdc2	–	–	–	–	–	–
tdc	–	–	–	–	–	–
odc	–	–	–	–	–	–

[a](–) Lack of genes encoding for biogenic amines.
[b](+) Presence of genes encoding for biogenic amines.

health problems. The *hdc1*, *hdc2*, *tdc* and *odc* genes that are involved in biogenic amine production were searched in the genome of the *Lb. mucosae* strains (Table 11). None of the genes associated with the production of histidine decarboxylase, tyrosine decarboxylase and ornithine decarboxylase was detected in the selected strains. In this study, only *hdc1*gene was detected in NABRII55.

Biogenic amines production is an intrinsic property (Franz et al. 2005; Lorenzo et al. 2010). *Lactobacillus* strains are usually considered safe organisms in this respect (Arena et al. 2002). de Moraes et al. (2017) found none of the genes associated with the production of histamine and cadaverine in the genome of the *Lb. mucosae* strains. Martín et al. (2005) reported that *Lb. gasseri* and *Lb. fermentum* cannot produce biogenic amines. However, the results of the biogenic amines production gene showed that *Lb. mucosae* NABRII55 isolated from cow carries the *hdc1* gene. The formation of biogenic amine from histidine is responsible for allergic reactions. Histamine is formed by the histidine decarboxylation activity of Gram-negative enteric bacteria. Furthermore, it is mainly produced by Gram-positive LAB in some fermented products. dos Santos et al. (2015) detected the *hdc1* gene in the genome of the All *Lb. rhamnosus* strains isolated from Artisanal Coalho cheeses except for *Lb. rhamnosus* EM1107. Therefore, all examined isolates in this study except for *Lb. mucosae* NABRII55 are safe in this regard and could be used as starter cultures or other food supplements.

Conclusions

In conclusion, the results of this study indicate that all the *Lb. mucosae* strains identified from native ruminant intestine of Iran except the *Lb. mucosae* NABRII50, *Lb. mucosae* NABRII53 and *Lb. mucosae* NABRII55 strains were considered as safe for antibiotic resistance, carried virulence factor, and biogenic amine genes. Moreover, they have probiotic properties including ability to survive under simulated gastric conditions, inhibiting bacterial pathogens, harbouring genes related to intestinal adhesion properties under *in vitro* assessment. Thus, the three *Lactobacillus* strains could be considered as candidate probiotic strains and should be further studied for their health benefits.

Ethical approval

The protocol followed in this study was approved by the the Ethics Committee recommendations.

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Disclosure statement

The authors are responsible for the content of this article and declare that there is no conflict of interest associated with the article.

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