Arab Universities Journal of Agricultural Sciences
(2023) 31 (1) 63-80
Website: http://ajs.journals.ekb.eg

Isolation and Characterization of Probiotics From Various Food Products as Potential Human Food Additives

Fekria M Ali[1*], Enas A Hassan[2], Ahmed AM Abdelhafez[1,2], Basma T Abd-Elhalim[2]
1- Faculty of Organic Agriculture, Heliopolis University for Sustainable Development, Bilbies, Cairo Desert Road, Cairo 11785, Egypt
2- Agricultural Microbiology Dept, Fac of Agric, Ain Shams Univ, P.O. Box 68, Hadayek Shoubra 11241, Cairo, Egypt

*Corresponding author: fekria.mohamed@hu.edu.eg

https://doi.org/10.21608/AJS.2023.173367.1503

Received 7 November 2022; Accepted 13 March 2023

Keywords:

Probiotics,
Lactic acid microorganisms,
Bacillus bingmayongesis,
Lactobacillus paracasei,
Pediococcus pentosaceus,
Saccharomyces Cerevisiae

Abstract: In this study, human-safe lactic acid microorganisms (LAM) were isolated from food samples to be used as potential additives for human food. Samples from various food sources (artisanal cheeses, fermented chickpeas, fermented rice, natural yogurt, pickles, and raw milk) were used to inoculate de Man, Rogosa, and Sharpe (MRS) and malt agar plates, which were incubated at 30°C for 48 h or 37°C for 72 h to isolate lactic acid bacteria and yeast, respectively. Out of 120 isolates (85 bacteria and 35 yeast), 75 isolates showed γ-hemolytic activity and were considered "Generally Recognized As Safe" (GRAS) isolates. After testing their growth rate under the gastrointestinal tract (GIT) conditions, including acidic pH and specific bile salt concentrations, we selected 30 isolates. Then, we evaluated the fermentative abilities of these 30 isolates on nine types of carbohydrates, their total acidity, and their antagonistic activity against five human pathogens. Based on the results of these tests, four isolates were selected for identification using the Biolog program and 16S rRNA sequencing for bacteria and 18S rRNA sequencing for yeast and found to be *Bacillus bingmayongesis* (FJAT-13831), *Lacticaseibacillus paracasei* (R094), *Pediococcus pentosaceus* (DSM 20336), and one yeast isolate as *Saccharomyces cerevisiae* (SaCe1 26S).

1 Introduction

The term "probiotic" comes from the Greek words "pro bios," which means "for life," as opposed to "antibiotic," which means "against life". The history of probiotics can be traced back to the consumption of fermented foods by the Greeks and Romans (Hill et al 2014). Probiotics include live microbes, which provide numerous health benefits to humans when consumed in sufficient quantities. The most significant benefits include enhancing or restoring the gut microbiota (FAO/WHO 2006). They contain viable mono or mixed cultures of bacteria and/or yeast, which, when consumed by humans, improves the qualities of the indigenous gut microbiota that colonize the entire human digestive system.

Probiotics comprise lactic acid microorganisms (LAM), including bacteria and yeasts, and are classified as "Generally Recognized as Safe" (GRAS) by the US Foods and Drug Administration (FDA), as previously reported by Hill et al (2014).

Lactic acid probiotics have several health-promoting effects, including reducing serum cholesterol, antibacterial, and anti-diabetic potential, and preventing hypertension and cancer progression (Shafi et al 2019). While selecting a probiotic strain, technical usability parameters should be considered, including excellent sensory properties, phage resistance, viability during technological treatment, and stability during production and storage, in addition to safety and other functional features (Miranda et al 2021).

The most common LAM probiotic bacterial genera are *Bacillus* sp., *Bifidobacterium* sp., *Enterococcus* sp., *Lactobacillus* sp., *Lactococcus* sp., *Leuconostoc* sp., *Pediococcus* sp., and *Streptococcus* sp. (Zheng et al 2020). According to Miranda et al (2021), several LAM strains have been granted "Qualified Presumption of Safety" (QPS) status by the European Union. Several *Lactobacilli sp.* and *Lactococci sp.* isolates have been granted GRAS status, including lactic acid *Streptococcus* sp. and other species. However, no *Enterococci* spp. have been granted GRAS/QPS status due to the possible existence of opportunistic pathogenic strains.

LAM yeast strains include *Saccharomyces bayanus, S. boulardii, S. cerevisiae, S. florentius, S. pastorianus, S. sake, S. unisporus*, and *Schizosaccharomyces* sp. (Tamang et al 2016, Amorim et al 2018). The most common sources of LAM isolates include the human gut microbiota (feces), breast milk, and fermented foods. These probiotic strains must be indigenous to the environment where they will be detected following consumption (Tarrah et al 2019).

The objectives of the current study were to isolate novel LAMs with potential probiotic properties from various traditional food sources and to select the potent bacterial and yeast isolates based on their functional physiochemical properties, which enable them to tolerate human GIT conditions.

2 Materials and methods

2.1 Samples collection

Samples of artisanal cheeses, fermented chickpeas, fermented rice, natural yogurt, pickles, and raw milk were collected from the local market in sterile plastic bags and bottles. The samples were transported to the microbiology laboratory in an ice box, where they were used to isolate the probiotic LAMs.

2.2 Chemicals

All chemicals were purchased from Oxoid Ltd., Basingstoke, UK, de Man, Rogosa, and Sharpe (MRS) broth medium was used as a selective medium for isolation and cultivation of the lactic acid bacteria (LAB) (De Man et al 1960), while malt agar medium was used to culture and maintain yeast (Difco Manual 1984). Nutritional blood agar medium was used to detect blood hemolysis in the LAM isolates (Difco Manual 1984). The total acidity of the isolates was determined using a bromo cresol purple indicator solution (Lee and Lee 2008).

2.3 Isolation of the LAMs

In order to recover lactic acid microorganisms (LAM) and use the serial dilution plate method (Benson 2002). Ten g or 10 mL of each sample (based on sample type) were mixed with 90 mL of sterile distilled water and vortexed for 10 min to homogenize. Plates of the LABs and yeasts were incubated for 48 and 72 h at 30°C and 37°C, respectively. Following incubation, yeast colonies were observed growing on the surface of the isolation plates, while LAB colonies were detected below the agar layer. The selected and purified stock cultures of bacteria and yeasts were preserved on slants at 4°C.

2.4 Standard inoculum

The standard inoculum of each isolate was prepared according to Benson (2002). A single colony of the tested isolates was inoculated into a conical flask (250 mL) containing 50 mL of MRS or malt agar medium. Then, aliquots of each culture containing 0.6 - 2 $x10^9$ colony forming units (CFUs)/mL were used as standard inoculum unless otherwise mentioned. Then, they were statically incubated at 30 and 37°C for 48 and 72 h for LAB and yeast isolates, respectively.

2.5 Hemolytic activity of the LAM isolates

To determine the hemolytic ability of the LAM isolates, the nutritional blood agar plates were streaked with a single colony of each culture and incubated at 37°C for 48 h. After incubation, the hemolytic activity of the isolates was evaluated and classified based on the results of red blood cell lysis in the inoculated medium. A green zone around the growing colony indicates α-hemolysis, a clear zone indicates β-hemolysis, while no clear zone implies γ-hemolysis. Isolates with γ-hemolysis were selected as safe isolates (Mangia et al 2019).

2.6 Morphological and biochemical characteristics of the LAM isolates

Morphological characteristics and Gram and spore staining were carried out for the selected LAM as described below.

2.6.1 pH tolerance

Approximately 5 mL of MRS and malt broth media were adjusted to different pH values (2.0, 3.0, 3.5, 4.0, and 6.5) using NaOH (1.0 N) or HCl (1.0 N) and inoculated aseptically with 1 mL of each isolate's standard inoculum then incubated as previously mentioned. The optical density (O.D) of the LAM isolates was measured at 600 nm using a spectrophotometer and compared with those of the un-inoculated controls (Turgay and Erbilir 2006). All experiments were conducted in triplicate.

2.6.2 Bile salt tolerance

A modified approach developed by Gilliland et al (1984) was used to test the ability of the LAM isolates to withstand bile salts. Tubes of 5 mL of MRS or malt broth containing 0.2, 0.3, 0.4, 0.8, and 1.5% bile salts with 1 mL of standard inoculum from each isolate, and then incubated as previously described. To monitor the growth of the LAM isolates, absorbance was measured at 600 nm using a spectrophotometer (Unico S2100 series UV/Vis). Un-inoculated bile salt-free MRS and malt media were used as controls in this assay. The isolates that grew under low pH and high bile salt concentrations were selected for further assays. All experiments were conducted in triplicates.

2.6.3 Carbohydrate fermentation profiles

To determine carbohydrate fermentation profiles of the selected LAM isolates, MRS (without carbon source) and malt basal media were supplemented with each of the following carbohydrates as a sole carbon source of carbon; hexose monosaccharides (fructose, glucose, mannitol), pentose monosaccharides (arabinose, ribose), disaccharides (sucrose, lactose, maltose), and a trisaccharide (raffinose) along with bromo cresol purple indicator. The testing media were inoculated with the standard inoculum and incubated as previously described. Change in the color of the incubated media from purple to yellow indicated a positive result, while no color change was considered negative. The negative control was prepared by inoculating the cultures in both media without the carbohydrates (Gupta et al 1996). All experiments were conducted in triplicates.

2.6.4 Titrated acidity and pH values of the LAM growth media

The titrated acidity and pH values in the growth media were determined at the end of the fermentation period. Individual aliquots (10 mL) from each broth culture were transferred to a beaker containing phenolphthalein indicator and then titrated with 0.1 M NaOH until a consistent pink color was observed (Parmar 2003). The sample's acidity was measured using a pH meter.

2.6.5 Antimicrobial activity of the LAM isolates against several human pathogens

Using the agar-well diffusion assay, the antimicrobial activity of the selected LAM isolates was tested against certain human pathogens as previously described by Ashraf et al (2009). Five bacterial and fungal strains, including *Bacillus cereus* (ATCC 9634), *Escherichia coli* (O157: H7) (ATCC 8739), *Salmonella typhi* (ATCC 14028), *Listeria monocytogenes* (ATCC 7644), and *Candida albicans* (ATCC 24433), were obtained from the Microbiological Resources Center (Cairo MIRCEN), Faculty of Agriculture, Ain Shams University, Egypt. A 50 µL aliquot of a cell-free supernatant was prepared from each LAM isolate and placed aseptically in a 7 mm-diameter well on a nutrient agar plate mixed with the tested pathogen. The plates were incubated at 37°C for 24 h, then the clear inhibition zones were measured. The experiment was performed in triplicate.

2.7 Characterization of the potent LAM isolates

LAM isolates showing the highest abilities to ferment most fermentable carbohydrates and tolerate GIT conditions were selected for identification using phenotypic and genotypic identification techniques.

2.7.1 Phenotypic characterization using Biolog microplate system

Biolog microplate system (Biolog, Inc., 3938 Trust Way, Hayward, CA 94545, USA) was employed to identify the selected isolates based on their abilities to utilize a preselected panel of different carbon sources and amino acids. The test yields a characteristic pat-

tern of purple wells, which represents the metabolic fingerprint of each inoculated microorganism (Harris-Baldwin and Gudmestad 1996). Tetrazolium violet was used as a redox dye for colorimetric assays to study the utilization of the carbon sources. Then, 16S rRNA and 18S rRNA sequencing were performed for the LAB and yeast isolates, respectively.

2.7.2 Genotypic characterization by 16S rDNA and 18S rDNA sequencing

Pure cultures of the selected LAB and yeast isolates were grown on MRS and malt agar, respectively (Yadav et al 2009). After incubation, DNA was extracted from the samples by the Sigma Scientific Services Co. using the GeneJet genomic DNA purification Kit (Thermo Fisher, USA). The extracted DNA was amplified using PCR, and the PCR products were cleaned using the Gene JETTM PCR Purification Kit (Thermo Fisher Scientific). The PCR amplicons of the 16S rRNA and 18S rRNA genes were sequenced using an ABI 3730xl DNA sequencer with forward and reverse bacterial and yeast primers (GATC Company) (Sarker et al 2014).

2.7.3. Phylogenetic analysis

16S rRNA and 18S rRNA sequencing data were aligned and examined to identify the selected isolates using the Basic Local Alignment Search Tool (BLAST) tool. The unknown query of 16S rRNA and 18S rRNA nucleotide sequences were compared with the nucleotide databases maintained by the National Center for Biotechnology Information (NCBI 2022), and then retrieved from the GenBank database. The algorithm describes the alignment of multiple sequences built for homologous sequences using the CLUSTAL Omega software, which uses seeded guide trees and HMM profile-profile techniques to generate alignments between three or more sequences. Finally, the Neighbor-joining trees were constructed.

2.8 Statistical analysis

The assay data was statistically analyzed using Duncan's Multiple Range Test and significance was set at $P \leq 0.05$ (Duncan 1955).

3 Results and Discussion

2.7 Isolation of the LAMs

Several food products from local markets were collected and used as sources of lactic acid microorganisms (LAM), to obtain potential probiotics for human consumption. Results, presented in **Table 1** and demonstrated in **Fig 1,** revealed that a total of 120 bacteria and yeast isolates were recovered from these samples. Results showed that the bacterial isolates outnumbered the yeast. Furthermore, the number of LAM isolates obtained from the artisanal cheese and pickles was higher than that obtained from other food sources because these food products contain diverse ingredients that support a wide variety of microorganisms (Ramírez-Rivera et al 2018). Similarly, previous studies indicated that LAMs were isolated from fermented rice, yogurt, artisanal cheeses, fermented chickpeas, pickles, and raw milk (Zhang et al 2022).

2.8Hemolytic activity of the LAM isolates

To identify the LAM isolates that are safe for human consumption, blood agar plates were inoculated with each test isolate and incubated at 37°C for 48 h before detecting the presence of a lytic zone around the colony. The results showed that 32 (26 %) of the LAM isolates produced α -hemolysis, 13 (11 %) were β -hemolytic, and 75 (63 %) were γ -hemolytic **Fig 2**. Based on this, 75 LAM isolates (19 yeast and 56 bacterial isolates) were considered GRAS and selected for further characterization and identification. Previous studies have shown that most isolated *Lactococci* and *Lactobacilli* were γ -hemolytic (Wang et al 2022). Fernández-Pacheco et al (2021) showed that none of the 20 strains of yeast possessed this activity in TSA-blood medium after 48 h, despite normal growth.

3.3 Morphological characterization of the selected LAM isolates

The physical properties of the selected 75 LAM isolates were investigated. The bacterial colonies on the MRS plates were smooth, convex, and transparent, with a milky-yellow color **Fig 3** and **Table 2**. All the bacterial isolates were Gram-positive either bacilli or cocci, present as single colonies or in chains and motile or non-motile. They grew on the agar surface, indicating microaerophilia. Based on cell morphology, 38 bacterial isolates were rod-shaped, and 18 were spherical. The colonies of the 19 yeast isolates recovered from malt agar were smooth and creamy, elongated, and/or spherical to oval.

Table 1. Total LAM isolates recovered from different food products

Food Sources	No. of bacterial isolates	No. of yeast isolates	Total no. of isolates	Prevalence percentage %
artisanal cheese	19	5	24	20
Fermented chickpeas	15	6	21	17
Fermented rice	6	4	10	8
Natural yogurt	11	8	19	16
Pickles	19	7	26	22
Raw milk	15	5	20	17
Total isolates	85	35	120	100

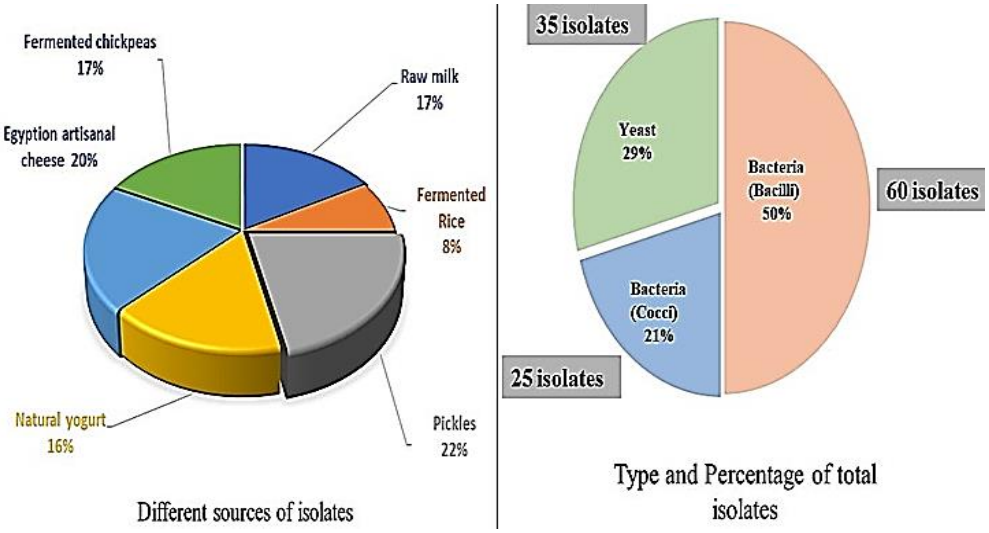


Fig 1. Distribution of total LAM isolates recovered from different food products.

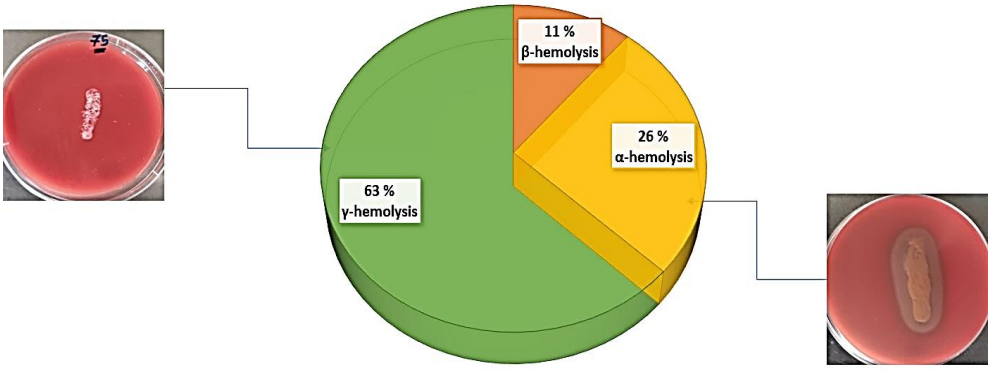


Fig 2. Probiotic LAM isolates selection based on hemolytic activity

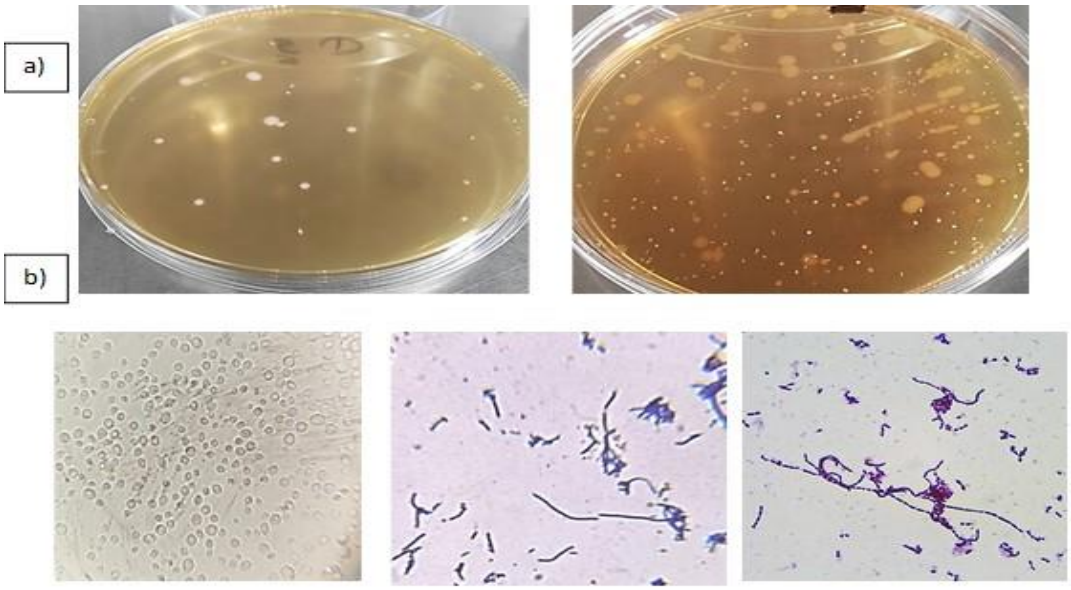


Fig 3. a) Growth of the LAM isolates on MRS medium (left) and Malt medium (right), b) Gram stain and cell morphology of some of the LAM isolates

Table 2. Morphological characterization of the yeast and bacterial LAM isolates

Microbial Group	Isolates code	Morphological shape	Microbial Group	Isolates code	Morphological shape
Yeast	P Y1	Spherical	Bacteria	P B2	Bacilli
	P Y2	Spherical		P B3	Bacilli
	P Y3	Oval		P B4	Bacilli
	P Y4	Elongate		P B5	Bacilli
	P Y5	Spherical		P B7	Bacilli
	P Y6	Oval		P B8	Bacilli
	P Y7	Spherical		Yo B1	Bacilli
	P Y8	Spherical		Yo B2	Bacilli
	Yo Y1	Elongate		Yo B3	Bacilli
	Yo Y4	Elongate		Yo B4	Bacilli
	Yo Y5	Oval		Yo B5	Bacilli
	Ch Y1	Spherical		Ch B1	Bacilli
	Ch Y4	Oval		Ch B2	Bacilli
	Ch Y5	Elongate		Ch B3	Bacilli
	FR Y1	Spherical		Ch B4	Bacilli
	FR Y2	Spherical		Ch B5	Bacilli
	FK Y1	Elongate		Ch B6	Bacilli
	FK Y2	Oval		Ch B7	Bacilli
	FK Y3	Oval		Ch B8	Bacilli
Bacteria	P C1	Cocci		Ch B9	Bacilli
	P C2	Cocci		Ch B10	Bacilli
	P C3	Cocci		Ch B11	Bacilli
	P C4	Cocci		Ch B12	Bacilli
	P C5	Cocci		M B2	Bacilli
	Yo C1	Cocci		M B4	Bacilli
	Yo C2	Cocci		M B6	Bacilli
	Ch C1	Cocci		M B8	Bacilli
	Ch C4	Cocci		FR B1	Bacilli
	M C1	Cocci		FR B2	Bacilli
	M C2	Cocci		FR B3	Bacilli
	M C3	Cocci		FK B1	Bacilli
	FR C1	Cocci		FK B2	Bacilli
	FK C2	Cocci		FK B3	Bacilli
	FK C3	Cocci		FK B4	Bacilli
	FK C4	Cocci		FK B5	Bacilli
	FK C5	Cocci		FK B6	Bacilli
	FK C6	Cocci		FK B7	Bacilli
	P B1	Bacilli			

3.4 Physiological characteristics of the LAM isolates

3.4.1 pH tolerance

To select potential probiotic isolates with human health benefits, the isolates' abilities to withstand the GIT conditions, one of which is low pH, were investigated (Montoro et al 2016). The selected 75 isolates were cultivated in MRS and malt broth media with pH of 2.0, 3.0, 3.5, 4.0 and 6.5, incubated and their growth was measured spectrophotometrically, as previously described. Results in **Tables 3 and 4** show that low pH drastically affected the growth (represented by OD at 600 nm) of all isolates. All of the 75 isolates except the FKB5 isolate, variably tolerated the acidic condition. At pH 2, 50 bacterial and yeast isolates showed OD_{600} > 0.2, while the other 25 showed much lower growth. Reports have shown that the acid tolerance of LAMs is due to their ability to convert amino acids (lysine, cysteine, aspartic acid, tyrosine, valine, leucine, arginine, and glutamine) into biogenic amines e.g. histamine, tyramine, cadaverine, and putrescine (Arena et al 2011). Several LAM isolates have been shown to survive under acidic conditions (Wang et al 2020).

3.4.2 Bile salt tolerance

Bile salt stress exhibits a stronger inhibitory effect on *Lactobacillus* sp. than acid stress (Saini and Tomar 2017). According to Dunne et al (2001), the typical bile levels in the GIT are between 0.2 to 2%. To ensure that the selected 75 LAM isolates can tolerate the bile salts in the GIT, they were cultured in 5 mL of MRS and malt broths containing 0.2, 0.3, 0.4, 0.8, and 1.5% bile salts and incubated as previously described. The results shown in **Tables 5 and 6** revealed that the 75 tested LAB isolates had varying degrees of resistance to bile salt (0.3%–1.5%). Of these, 45 isolates (60%) could grow at 1.5% bile salt (OD_{600} = 0.86–2.6), while 25 isolates (40%) showed weak growth (OD_{600} < 0.7).

The bile tolerance of LAM might be linked to the presence of several proteins. Five proteins, including α-small heat shock protein 1 (Hsp1), bile salt hydrolase 1 (Bsh1), glucose-6-phosphate 1-dehydrogenase (Gpd), GroEL chaperonin (GroEL), and ATP synthase subunit (AtpH)) were abundant in the highly resistant strains. Whereas three proteins (glycine/betaine/carnitine/choline ABC transporter (OpuA), glutathione reductase 1 (GshR1), and ATP-dependent Clp protease proteolytic subunit) were present in similar quantities in both resistant and intermediate strains, but in the sensitive ones (Hamon et al 2011).

Based on the results of pH and bile salt tolerance, 30 isolates were selected for the following experiments based on their ability to grow at pH 2.0 and high bile salt concentration **(**up to 1.5%).

3.4.3 Carbohydrates fermentation

The selected 30 LAM isolates were tested for their abilities to ferment nine different carbohydrates (arabinose, glucose, fructose, maltose, mannitol, lactose, ribose, sucrose, and raffinose), which indicate their potential beneficial influence on human health. Results showed that 15 isolates could ferment all tested carbohydrates, three isolates fermented eight sugars, and one isolate fermented seven sugars. All of the isolates could ferment lactose **Table 7**.

A related study showed that the LAM isolates, including *Weissella soli* and *Lactobacillus pentosus*, were able to efficiently metabolize and ferment most of the monosaccharides, such as arabinose, ribose, galactose, glucose, and fructose, and disaccharides, such as maltose and lactose, and some complex substrates, such as starch. However, *L. pentosus* and *L. paracasei* subsp. *tolerans* can ferment only one or two sugars (Montero-Zamora et al 2022).

3.4.4 Total acidity and pH of the growth media

Due to carbohydrate fermentation by the LAB, numerous organic acids are produced, lowering the pH of the medium (Wu et al 2011). Furthermore, the organic acids found in fermented dairy products indicate the metabolic activity of other bacterial cultures. Furthermore, these acids act as natural preservatives and contribute to the product's sensory qualities (Adhikari et al 2002). **Fig 4** illustrates that the pH of the supernatant of all LAM isolates varied from 3.20 to 6.03, with a mean value of 4.6. Moreover, 10 isolates (33.3%) exhibited a total acidity of more than 150 meq/mL.

Cho et al (2013) and Melia et al (2022) showed that most LAB have comparatively greater acidification rates as the titratable acidity is elevated by the lactic acid generated as a preliminary metabolite. Moreover, (Melia et al 2022) discovered that lactic acid buildup during fermentation might enhance the titratable acidity. Owade et al (2021) and Nahaisi et al (2021) revealed the low pH tolerance and high total acidity levels of these microbes.

Table 3. Tolerance of the LAM bacterial isolates to different pH values, after cultivation in MRS medium after 48 h of incubation at 37°C

Isolate code	Growth (O.D 600 nm) pH level					Isolate code	Growth (O.D 600 nm) pH level				
	2.0	3.0	3.5	4.0	6.5		2.0	3.0	3.5	4.0	6.5
PC1	0.326^{g}	0.323^{h}	0.506^{g}	1.022^{e}	1.940^{c}	YoB4	0.154^{h}	0.957^{f}	1.124^{e}	1.233^{e}	2.051^{b}
PC2	0.359^{g}	0.466^{g}	0.444^{g}	1.791^{c}	1.975^{c}	YoB5	0.224^{h}	0.394^{h}	0.966^{f}	1.233^{e}	2.185^{a}
PC3	0.216^{h}	0.344^{h}	0.651^{g}	1.223^{e}	1.208^{e}	ChB1	0.261^{h}	0.391^{h}	0.978^{f}	1.254^{e}	2.158^{a}
PC4	0.249^{h}	0.452^{g}	0.500^{g}	0.981^{f}	1.955^{c}	ChB2	0.247^{h}	0.281^{h}	0.792^{f}	1.236^{e}	2.206^{a}
PC5	0.138^{h}	0.182^{h}	0.755^{f}	1.200^{e}	1.613^{e}	ChB3	0.218^{h}	0.250^{h}	0.921^{f}	1.240^{e}	2.260^{a}
YoC1	0.408^{g}	0.587^{g}	0.800^{f}	1.185^{e}	2.108^{a}	ChB4	0.280^{h}	0.371^{h}	0.970^{f}	1.250^{e}	2.176^{a}
YoC2	0.208^{h}	0.341^{h}	0.474^{g}	1.173^{e}	2.115^{a}	ChB5	0.156^{h}	1.018^{e}	1.121^{e}	1.236^{e}	2.036^{b}
ChC1	0.358^{h}	0.386^{h}	1.699^{d}	2.277^{a}	2.480^{a}	ChB6	0.386^{h}	0.518^{g}	0.728^{f}	1.079^{e}	2.037^{b}
ChC4	0.314^{h}	0.311^{h}	1.472^{d}	2.003^{b}	2.429^{a}	ChB7	0.196^{h}	0.207^{h}	0.181^{h}	0.844	2.036^{b}
MC1	0.281^{h}	0.151^{h}	0.198^{h}	1.451^{d}	2.386^{a}	ChB8	0.187^{h}	0.285^{h}	0.796^{f}	1.210^{e}	2.106^{a}
MC2	0.438^{g}	0.640^{g}	2.103^{a}	2.319^{a}	2.335^{a}	ChB9	0.149^{h}	0.176^{h}	0.429^{g}	1.179^{e}	2.015^{b}
MC3	0.327^{h}	0.501^{g}	1.976^{c}	2.288^{a}	2.367^{a}	ChB10	0.231^{h}	0.316^{h}	0.961^{f}	1.214^{e}	2.111^{a}
FRC1	0.118^{h}	0.156^{h}	0.225^{h}	0.452^{g}	2.138^{a}	ChB11	0.298^{h}	0.466^{g}	1.618^{c}	2.180^{a}	2.462^{a}
FKC2	0.326^{h}	0.405^{g}	0.732^{f}	1.208^{e}	1.651^{e}	ChB12	0.191^{h}	0.699^{g}	0.733^{f}	0.681^{g}	0.232^{h}
FKC3	0.276^{h}	0.460^{g}	0.865^{f}	1.176^{e}	1.371^{e}	MB2	0.321^{h}	0.225^{h}	0.454^{g}	1.413^{e}	2.292^{a}
FKC4	0.347^{h}	0.632^{g}	0.884^{f}	1.247^{e}	1.580^{e}	MB4	0.341^{h}	0.401^{g}	1.455^{d}	2.143^{a}	2.356^{a}
FKC5	0.292^{h}	0.312^{h}	0.642^{g}	1.032^{e}	1.603^{e}	MB6	0.263^{h}	0.156^{h}	0.224^{h}	1.353^{e}	2.305^{a}
FKC6	0.015^{i}	0.006^{j}	0.015^{i}	0.019^{i}	0.740	MB8	0.150^{h}	0.175^{h}	0.343^{h}	2.059^{b}	2.362^{a}
PB1	0.241^{h}	0.510^{g}	0.910^{f}	1.268^{e}	2.066^{b}	FRB1	0.051^{i}	0.098^{i}	0.044^{i}	0.177^{h}	1.160^{e}
PB2	0.353^{h}	0.340^{h}	0.776^{f}	1.205^{e}	1.540^{e}	FRB2	0.056^{i}	0.053^{i}	0.088^{i}	0.166^{h}	1.895^{c}
PB3	0.355^{h}	0.566^{g}	0.995^{f}	1.265^{e}	2.075^{b}	FRB3	0.080^{i}	0.079^{i}	0.040^{i}	0.154^{h}	1.800^{c}
PB4	0.183^{h}	0.350^{h}	0.926^{f}	1.290^{e}	2.090^{b}	FKB1	0.286^{h}	0.439^{g}	0.621^{g}	0.893^{f}	1.276^{e}
PB5	0.289^{h}	0.427^{g}	0.769^{f}	0.814^{f}	1.985^{c}	FKB2	0.424^{g}	0.552^{g}	0.864^{f}	1.059^{e}	1.350^{e}
PB7	0.319^{h}	0.333^{h}	0.479^{g}	0.950^{f}	1.956^{c}	FKB3	0.033^{i}	0.038^{i}	0.042^{i}	0.057^{i}	0.797^{f}
PB8	0.233^{h}	0.249^{h}	0.220^{h}	0.890^{f}	1.969^{c}	FKB4	0.032^{i}	0.039^{i}	0.040^{i}	0.046^{i}	0.679^{g}
YoB1	0.255^{h}	0.457^{g}	0.955^{f}	1.242^{e}	2.100^{a}	FKB5	0.000^{j}	0.027^{i}	0.026^{i}	0.026^{i}	0.832^{f}
YoB2	0.460^{g}	0.563^{g}	0.950^{f}	1.238^{e}	2.037^{b}	FKB6	0.235^{h}	0.365^{h}	0.512^{g}	0.827^{f}	1.224^{e}
YoB3	0.168^{h}	1.058^{e}	1.130^{e}	1.233^{e}	2.047^{b}	FKB7	0.465^{g}	0.631^{g}	0.865^{f}	1.144^{e}	1.973^{c}

Values represent the means of three replicates. Values with different letters in the same column indicate significant differences at $P \leq 0.05$, as analyzed by Duncan's Multiple Range Test.

Table 4. Tolerance of the LAM yeast isolates to different pH values cultivated in malt medium for 72 h at 30°C. Growth was measured by O.D at 600 nm

Isolate Code	pH level				
	2.0	3.0	3.5	4.0	6.5
P Y1	0.227^{h}	0.480^{g}	0.896^{e}	1.802^{c}	1.884^{c}
P Y2	0.083^{i}	0.444^{g}	0.398^{g}	0.543^{f}	1.086^{d}
P Y3	0.262^{h}	0.369^{g}	0.473^{g}	1.144^{d}	1.973^{c}
P Y4	0.163^{h}	0.239^{h}	0.220^{h}	1.170^{d}	1.947^{c}
P Y5	0.093^{i}	0.142^{h}	0.140^{h}	0.649^{f}	1.928^{c}
P Y6	0.161^{h}	0.191^{h}	0.096^{i}	0.775^{e}	1.958^{c}
P Y7	0.258^{h}	0.400^{g}	0.669^{f}	1.123^{d}	2.030^{b}
P Y8	0.312^{g}	0.462^{g}	0.952^{e}	1.801^{c}	1.857^{c}
Yo Y1	0.155^{h}	0.247^{h}	0.210^{h}	0.947^{e}	2.042^{b}
Yo Y4	0.205^{h}	0.279^{h}	0.408^{g}	1.150^{d}	2.139^{b}
Yo Y5	0.286^{h}	0.433^{g}	0.989^{e}	1.254^{d}	2.118^{a}
Ch Y1	0.276^{h}	0.227^{h}	0.601^{f}	1.766^{c}	2.292^{a}
Ch Y4	0.306^{g}	0.210^{h}	0.791^{e}	1.777^{c}	2.340^{a}
Ch Y5	0.333^{g}	0.308^{g}	0.355^{g}	1.768^{c}	2.346^{a}
FR Y1	0.272^{h}	0.131^{h}	0.222^{h}	1.580^{d}	2.179^{a}
FR Y2	0.078^{i}	0.155^{h}	0.148^{h}	1.815^{c}	2.411^{a}
FK Y1	0.306^{g}	0.210^{h}	0.521^{f}	1.777^{c}	2.340^{a}
FK Y2	0.286^{h}	0.403^{g}	0.653^{f}	1.815^{c}	2.411^{a}
FK Y3	0.165^{h}	0.242^{h}	0.511^{f}	1.032^{d}	1.603^{c}

Growth of yeast isolates was measured by O.D at 600 nm, and values represent the mean of three replicates. Values with different letters in the same column indicate significant difference at $P \leq 0.05$, as analyzed by Duncan's Multiple Range Test.

Table 5. Tolerance of tested LAM bacterial isolates to different bile salt concentrations tested, cultivated in MRS medium for 48 h at 37°C

Isolate code	Growth (O.D 600 nm) Bile salts concentration %					Isolate code	Growth (O.D 600 nm) Bile salts concentration %				
	0.2	0.3	0.4	0.8	1.5		0.2	0.3	0.4	0.8	1.5
PC1	1.823^{c}	1.912^{c}	1.866^{c}	1.589^{d}	1.794^{c}	YoB4	0.570^{f}	0.472^{g}	0.384^{g}	0.201^{g}	0.144^{g}
PC2	2.561^{a}	2.462^{a}	2.364^{a}	2.307^{a}	2.084^{b}	YoB5	1.290^{e}	1.494^{d}	1.021^{e}	1.067^{e}	1.113^{e}
PC3	1.896^{c}	1.114^{e}	1.570^{d}	1.286^{e}	1.051^{e}	ChB1	0.633^{f}	0.613^{f}	0.935^{f}	0.465^{g}	0.525^{f}
PC4	2.233^{a}	2.308^{a}	2.310^{a}	2.439^{a}	2.559^{a}	ChB2	0.655^{f}	0.420^{g}	0.390^{g}	0.424^{g}	0.558^{f}
PC5	0.060^{i}	0.028^{i}	0.028^{i}	0.032^{i}	0.051^{i}	ChB3	0.124^{h}	0.199^{g}	0.250^{g}	0.230^{g}	0.110^{h}
YoC1	2.245^{a}	2.112^{b}	1.954^{c}	1.839^{c}	1.598^{d}	ChB4	0.508^{g}	0.309^{g}	0.418^{g}	0.159^{h}	0.132^{h}
YoC2	2.228^{a}	2.170^{b}	2.055^{b}	1.797^{c}	1.412^{d}	ChB5	1.070^{e}	0.718^{f}	0.842^{f}	0.951^{f}	0.567^{f}
ChC1	1.852^{c}	1.653^{c}	1.705^{c}	1.554^{d}	1.568^{d}	ChB6	2.164^{b}	2.100^{b}	2.087^{b}	2.072^{b}	2.069^{b}
ChC4	2.339^{a}	2.339^{a}	2.057^{b}	1.554^{d}	1.248^{e}	ChB7	0.950^{f}	0.893^{f}	2.048^{b}	0.203^{g}	0.229^{g}
MC1	0.108^{g}	0.148^{g}	0.101^{g}	0.136^{g}	0.169^{h}	ChB8	2.165^{b}	2.157^{b}	2.130^{b}	2.123^{b}	2.043^{b}
MC2	1.214^{e}	0.950^{f}	0.989^{f}	0.631^{f}	0.241^{g}	ChB9	1.161^{e}	0.999^{f}	1.406^{d}	1.579^{d}	1.512^{d}
MC3	2.450^{a}	2.456^{a}	1.660^{d}	1.569^{d}	0.944^{f}	ChB10	2.192^{b}	1.690^{d}	1.489^{d}	1.235^{e}	1.081^{e}
FRC1	1.252^{e}	1.222^{e}	1.427^{d}	1.115^{e}	0.994^{f}	ChB11	1.494^{d}	1.627^{d}	1.349^{d}	1.587^{d}	1.263^{e}
FKC2	1.617^{d}	1.551^{d}	1.523^{d}	1.584^{d}	1.546^{d}	ChB12	0.961^{f}	1.130^{e}	1.136^{e}	1.191^{e}	1.056^{e}
FKC3	1.050^{e}	1.033^{e}	1.133^{e}	1.012^{e}	1.188^{e}	MB2	1.550^{d}	0.936^{f}	1.089^{e}	0.528^{f}	0.139^{h}
FKC4	1.538^{d}	1.232^{e}	1.371^{d}	1.478^{d}	1.350^{e}	MB4	1.613^{d}	1.125^{e}	1.121^{e}	0.343^{g}	0.435^{g}
FKC5	1.419^{d}	1.386^{e}	1.429^{d}	1.347^{e}	1.200^{e}	MB6	1.040^{e}	0.793^{f}	0.670^{f}	0.031^{i}	0.179^{h}
FKC6	0.864^{f}	0.683^{f}	0.771^{f}	0.776^{f}	0.798^{f}	MB8	0.177^{h}	0.144^{h}	0.122^{h}	0.018^{i}	0.091^{i}
PB1	1.743^{c}	1.775c	1.783^{c}	1.811^{c}	1.994^{c}	FRB1	1.262^{e}	1.396^{e}	1.438^{d}	1.287^{e}	1.107^{e}
PB2	2.183^{b}	2.341^{a}	2.669^{a}	2.448^{a}	2.558^{a}	FRB2	2.420^{a}	1.478^{d}	2.322^{a}	2.252^{b}	1.180^{e}
PB3	1.552^{d}	1.257^{e}	1.132^{e}	1.309^{e}	1.503^{d}	FRB3	1.268^{e}	1.478^{d}	2.452^{a}	2.210^{b}	1.154^{e}
PB4	2.307^{a}	2.310^{a}	1.990^{c}	1.789^{c}	1.884^{c}	FKB1	1.059^{e}	1.038^{e}	1.016^{e}	1.015^{e}	0.941^{f}
PB5	2.441^{a}	2.319^{a}	2.264^{a}	1.979^{c}	1.160^{e}	FKB2	1.661^{d}	1.432^{d}	1.391^{d}	1.319^{e}	1.146^{e}
PB7	1.511^{d}	1.332^{e}	1.233^{e}	1.017^{e}	1.055^{e}	FKB3	0.785^{f}	0.882^{f}	0.831^{f}	0.772^{f}	0.761^{f}
PB8	2.193^{a}	2.371^{a}	1.959^{c}	2.477^{a}	2.588^{a}	FKB4	0.803^{f}	0.944^{f}	0.795^{f}	0.421^{g}	0.403^{g}
YoB1	1.116^{e}	1.151^{e}	1.153^{e}	1.052^{e}	1.090^{e}	FKB5	0.734^{f}	0.796^{f}	0.682^{f}	0.682^{f}	0.700^{f}
YoB2	2.328^{a}	2.126^{b}	2.038^{b}	1.985^{c}	1.927^{c}	FKB6	1.205^{e}	1.195^{e}	1.202^{e}	1.438^{d}	1.042^{e}
YoB3	1.370^{e}	1.333^{e}	1.368^{e}	1.253^{e}	1.075^{e}	FKB7	1.265^{e}	1.232^{d}	1.211^{e}	1.123^{e}	1.042^{e}

Values represent the means of three replicates. Values with different letters in the same column indicate significant differences at $P \leq 0.05$, as analyzed by Duncan's Multiple Range Test.

Table 6. Tolerance of LAM yeast isolates to different bile salt concentrations, cultivated in malt medium for 72 h at 30°C

Isolate Code	Growth (O.D 600 nm) Bile salts concentration %				
	0.2	0.3	0.4	0.8	1.5
P Y1	1.966^{b}	1.895^{c}	1.832^{c}	1.780^{c}	1.561^{c}
P Y2	0.018^{j}	0.042^{i}	0.010^{k}	0.024^{j}	0.023^{j}
P Y3	1.894^{c}	1.810^{c}	1.723^{c}	1.887^{c}	1.532^{c}
P Y4	0.659^{f}	1.454^{d}	0.627^{f}	0.038^{i}	0.037^{i}
P Y5	1.728^{c}	1.699^{c}	1.808^{c}	1.759^{c}	1.916^{b}
P Y6	1.701^{c}	1.386^{d}	1.336^{d}	0.827^{e}	0.912^{e}
P Y7	1.210^{d}	1.133^{e}	0.923^{e}	0.886^{e}	0.801^{e}
P Y8	1.254^{d}	1.204^{d}	1.091^{e}	0.899^{e}	0.809^{e}
Yo Y1	0.366^{f}	0.117^{g}	0.227^{g}	0.173^{g}	0.077^{h}
Yo Y4	0.200^{f}	0.450^{f}	0.102^{g}	0.078^{h}	0.131^{g}
Yo Y5	1.390^{d}	1.353^{d}	1.352^{d}	1.169^{d}	0.988^{e}
Ch Y1	0.093^{h}	0.138^{g}	0.115^{g}	0.044^{i}	0.105^{g}
Ch Y4	0.048^{i}	0.123^{g}	0.103^{g}	0.002^{k}	0.070^{h}
Ch Y5	1.371^{d}	0.900^{e}	0.978^{f}	0.474^{f}	0.140^{g}
FR Y1	0.079^{h}	0.140^{g}	0.063^{h}	0.047^{i}	0.130^{g}
FR Y2	0.961^{e}	0.918^{e}	0.767^{e}	0.767^{f}	1.777^{c}
FK Y1	2.420^{a}	1.478^{d}	2.322^{a}	2.252^{a}	1.180^{c}
FK Y2	2.193^{b}	2.171^{b}	1.959^{b}	1.820^{c}	1.798^{c}
FK Y3	1.312^{d}	1.153^{e}	1.148^{e}	1.210^{e}	0.987^{e}

Values represent the means of three replicates. Values with different letters in the same column indicate significant differences at $P \leq 0.05$, as analyzed by Duncan's Multiple Range Test.

Table 7. Fermentation profile of 30 LAM isolates, cultivated in media supplemented with the test sugars as the sole sources of carbon for the LAB and yeast isolates

Type of LAM	Isolates code	Sugar source									
		Arabinose	Glucose	Fructose	Maltose	Mannitol	Lactose	Ribose	Sucrose	Raffinose	No. of fermented sugars
Bacteria	P C1	+	+	+	+	+	+	-	+	+	8
	P C2	+	+	+	+	+	+	+	+	+	9
	P C3	+	+	+	+	+	+	-	+	+	8
	P C4	+	+	+	+	+	+	+	+	+	9
	Yo C1	+	+	+	+	+	+	+	+	+	9
	Ch C1	-	+	+	-	-	+	+	+	-	5
	Ch C4	-	+	+	-	-	-	-	-	-	2
	FK C2	+	+	+	+	+	+	+	+	+	9
	FK C3	+	+	+	+	+	+	+	+	+	9
	FK C4	+	-	+	+	+	+	-	+	-	6
	FK C5	+	+	+	+	+	+	+	+	+	9
	P B1	+	+	+	+	+	+	+	+	+	9
	P B3	-	+	+	-	-	+	-	+	-	4
	P B5	+	+	-	+	+	+	-	+	-	6
	P B7	+	+	+	+	+	+	+	+	+	9
	Yo B2	+	+	+	+	+	+	+	+	+	9
	Yo B5	+	+	+	+	+	+	+	+	+	9
	Ch B5	-	+	+	+	+	+	-	+	-	6
	Ch B6	-	+	-	+	+	+	-	+	-	5
	Ch B11	+	+	+	+	+	+	-	+	-	7
	M B4	+	+	+	+	+	+	+	+	+	9
	FK B1	+	+	+	+	+	+	+	+	+	9
	FK B2	+	+	+	-	-	+	-	+	-	5
	FK B6	+	+	+	+	+	+	+	+	+	9
	FK B7	-	+	+	+	+	-	-	+	+	6
Yeast	P Y1	-	+	+	+	+	-	-	+	+	6
	P Y3	+	-	+	+	+	-	-	+	+	6
	P Y7	+	+	+	+	+	+	-	+	+	8
	P Y8	+	+	+	+	+	+	+	+	+	9
	FK Y3	-	+	+	+	+	-	-	+	+	6
No. of positive LAM isolates		23	28	28	26	26	26	16	29	21	

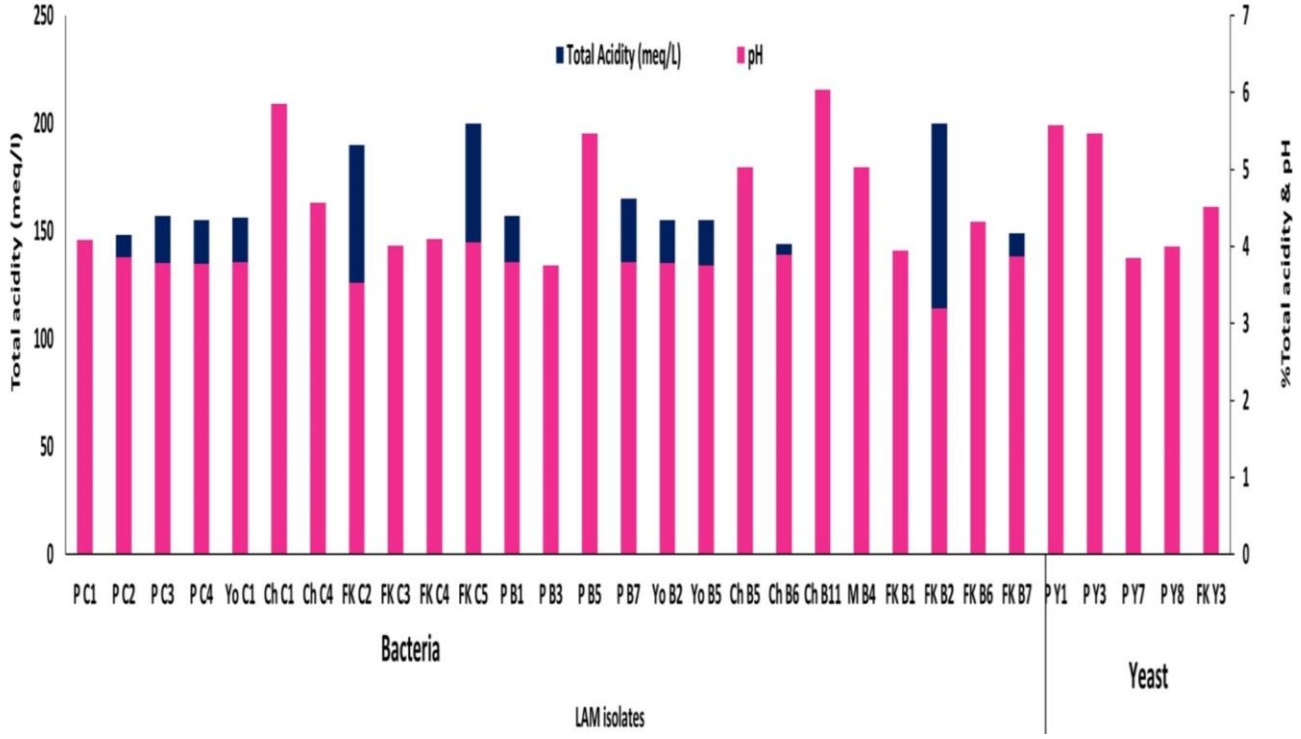


Fig 4. Total acidity and pH of LAM isolates on media for the bacteria and yeasts, respectively

3.5 Antagonistic activity of LAM isolates against several human pathogens

The antimicrobial activity of the selected 30 LAM isolates was tested against five common human pathogens; *Bacillus cereus, Escherichia coli* O157:H7, *Salmonella typhi, Listeria monocytogenes,* and *Candida albicans*, using agar well diffusion method. All 30 isolates showed antagonistic effects against all tested microorganisms with inhibition zones ranging from 6 to 25 mm. Among all the tested isolates, isolates FKC5, FKB7, and FKB2 produced inhibition zones in the following ranges: 15–25 mm, 15–23 mm, and 14–22 mm, respectively, against all five pathogens **Table 8 and Fig 5.**

Similarly, Mangia et al (2019) reported that *Lactobacillus paracasei* FS103 isolated from sheep cheese showed an antagonistic effect against *Listeria monocytogenes* (DSMZ 20600), *Salmonella enterica* (DSMZ 13772), *Staphylococcus aureus* (DSMZ 20231), *and Escherichia coli* (DSMZ 30083).

Choi et al (2018) isolated four LAB strains, namely *Lactobacillus curvatus, Leuconostoc mesenteroides, Weissella cibaria, and W. koreensis* from plant-based fermented food and showed that these strains could completely inhibit the growth of four foodborne pathogenic bacteria, including *E. coli O157:H7, Salmonella enteritidis, Salmonella typhimurium, and S. aureus.*

3.6 Phenotypic and genotypic identification of the potent LAB isolates

Currently, 4 isolates were selected, based on their high bile salt tolerance, for phenotypic and genotypic identification, fermentative ability, total acidity, and antagonistic activity. After appropriate identification and evaluation, these probiotic organisms might be considered safe and beneficial for human use (Mohania et al 2008).

3.6.1 Phenotypic identification

As several LABs have similar nutritional and growth requirements, biochemical methodologies for identification might not prove conclusive. The Biolog program (Biolog, Hayward, CA) is widely used for such identifications as it is based on the results of the fermentation patterns performed by the test microorganisms. Biolog is a unique technique that analyzes the fermentation of 96 carbohydrates (Moraes et al 2013), and the first microtube without any active carbohydrate substrate serves as the negative control.

Results showed that the selected 4 LAM isolates are able to ferment glucose, fructose, sucrose, maltose, mannitol, and lactose, indicated by the change of color from purple to pale yellow. However, their fermentation pattern for other substrates was different. These selected LAM isolates PC2, PB1, PB7, and PY8 were identified as *Lacticaseibacillus* sp., *Bacillus* sp., *Pediococcus* sp. and *Scaccharomyces cerevisiae*, respectively.

3.6.2 Genotypic identification

The physiological and metabolic characteristics of the probiotics can be determined by their genus, species, and strain. Genotype biochemical information, such as the 16s rDNA and 18s rDNA sequences, are used to identify the bacterial and yeast isolates, respectively, at the species level (De Melo Pereira et al 2018). All the obtained sequences were compared to various sequenced bacteria in the National Center for Biotechnology Information (NCBI), GenBank, and the Ribosomal Database Project (RDP) databases, which revealed a percentage of similarity between the extracted sequences and few sequences belonging to the 16S and 18S small subunit rDNA of other bacteria and yeast, respectively.

The PC2, PB1, PB7, and PY8 isolates showed similarity percentage of 99%, 99%, 98%, and 99 % to *Lacticaseibacillus paracasei* R094, *B. bingmayongesis* FJAT-13831, *Pediococcus pentosaceus* ATCC 25745, and *S. cerevisiae* SaCe1 26S strains, respectively. Following the BLAST analysis, the phylogenetic trees were built using the BLAST search sequences. The genetic trees were created in the FASTA format and were constructed using the phylogenetic distance analysis technique of neighbor-joining **Fig 6**.

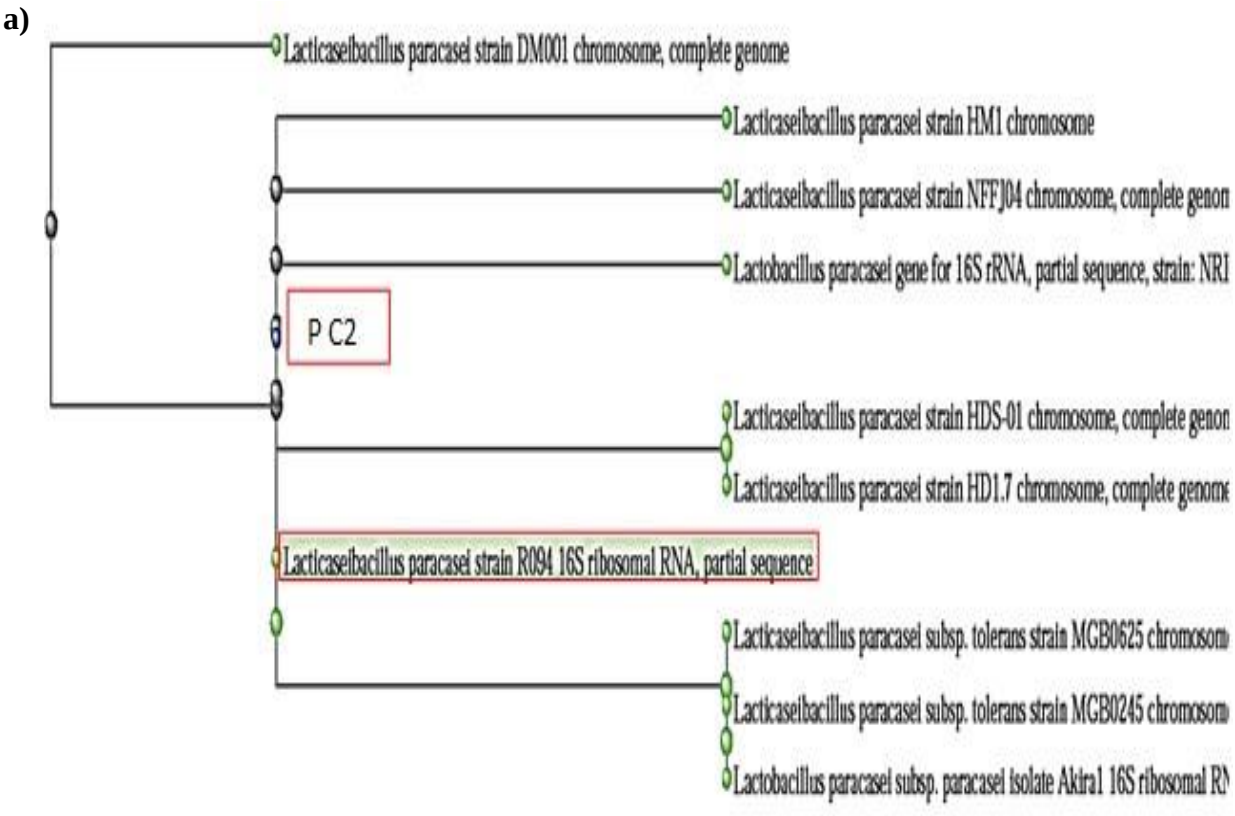
Table 8. Antagonistic activities of 30 LAM isolates against five human pathogens

Isolate code	Diameter of inhibition zone (mm) Human Pathogen					Isolate code	Diameter of inhibition zone (mm) Human Pathogen				
	S. typhi	***E. Coli***	***L. monocytogenes***	***B. cereus***	***C. albicans***		***S. typhi***	***E. coli***	***L. monocytogenes***	***B. cereus***	***C. albicans***
Bacterial isolates											
PC1	13	18	11	22	14	PB5	13	14	9	13	10
PC2	12	14	10	16	13	PB7	19	14	17	17	19
PC3	11	23	9	15	12	YoB2	15	11	11	8	14
PC4	15	19	14	18	12	YoB5	18	10	12	13	14
YoC1	6	15	17	11	16	ChB5	10	14	18	12	13
ChC1	15	10	18	16	11	ChB6	13	11	11	15	17
ChC4	11	19	13	20	15	ChB11	20	12	15	14	10
FKC2	23	18	19	15	15	MB4	12	15	14	11	17
FKC3	15	17	16	10	12	FKB1	14	20	14	16	17
FKC4	13	15	20	17	16	FKB2	22	17	14	20	15
FKC5	15	22	25	14	20	FKB6	14	13	16	11	12
PB1	13	18	11	22	14	FKB7	19	15	17	23	15
PB3	14	7	14	18	13						
Yeast isolates											
P Y1	14	10	14	8	11	P Y8	15	20	9	10	14
P Y3	10	8	13	15	12	FK Y3	10	14	10	12	13
P Y7	11	10	8	13	5						

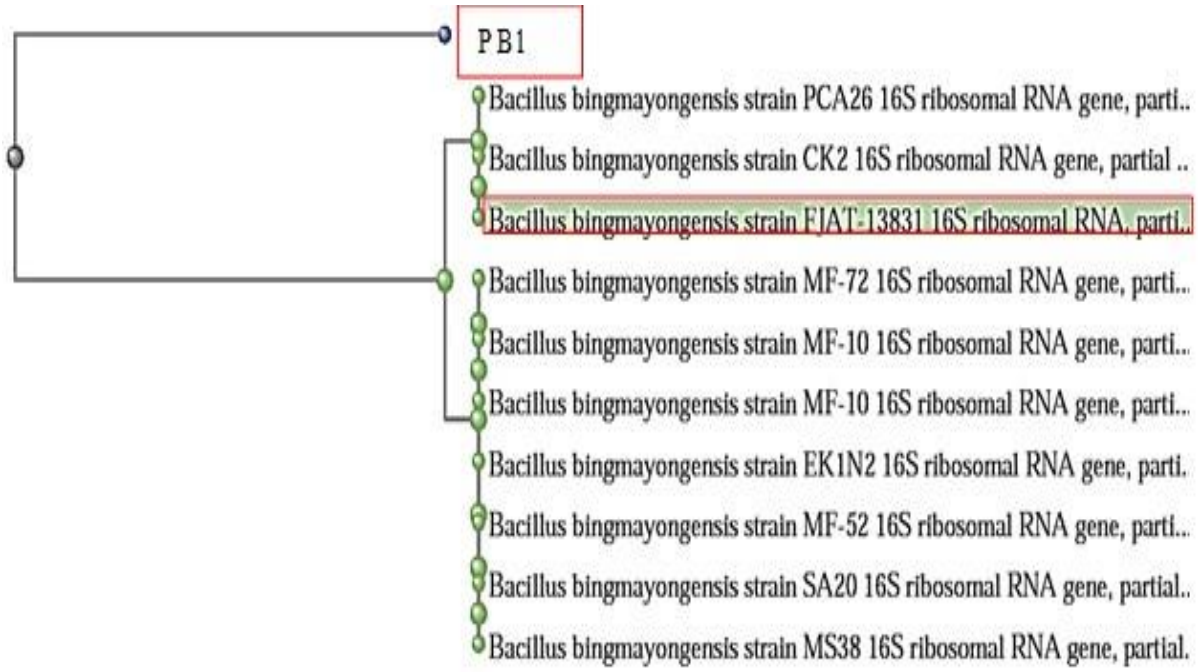
a) **b)** **c)**

Fig 5. Antagonistic effects of (a): isolate PB1 against *E. coli*, (b): isolate FKC5 against *L. monocytogenes*, and (c): isolate YoC1 against *S. typhi*

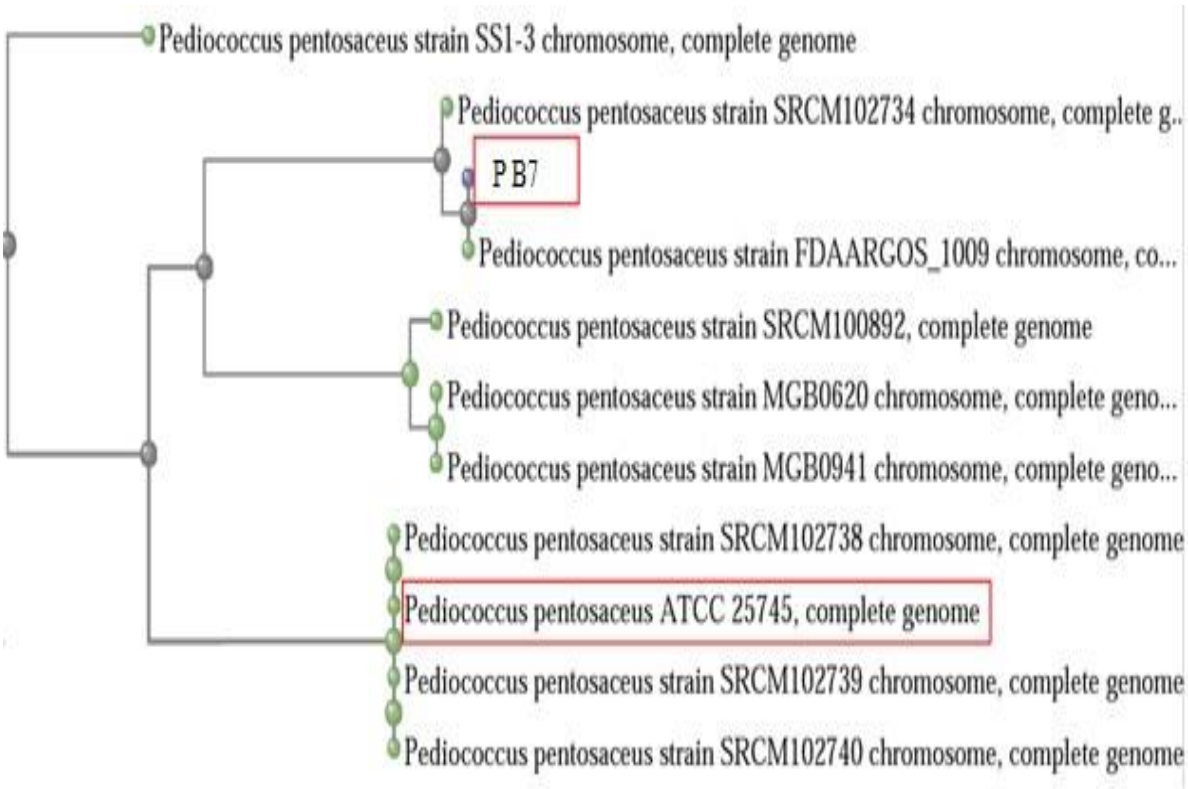
a)



b)


PB1
Bacillus bingmayongensis strain PCA26 16S ribosomal RNA gene, parti..
Bacillus bingmayongensis strain CK2 16S ribosomal RNA gene, partial ..
Bacillus bingmayongensis strain FJAT-13831 16S ribosomal RNA, parti..
Bacillus bingmayongensis strain MF-72 16S ribosomal RNA gene, parti...
Bacillus bingmayongensis strain MF-10 16S ribosomal RNA gene, parti...
Bacillus bingmayongensis strain MF-10 16S ribosomal RNA gene, parti...
Bacillus bingmayongensis strain EK1N2 16S ribosomal RNA gene, parti.
Bacillus bingmayongensis strain MF-52 16S ribosomal RNA gene, parti...
Bacillus bingmayongensis strain SA20 16S ribosomal RNA gene, partial..
Bacillus bingmayongensis strain MS38 16S ribosomal RNA gene, partial.

c)


Pediococcus pentosaceus strain SS1-3 chromosome, complete genome
Pediococcus pentosaceus strain SRCM102734 chromosome, complete g..
PB7
Pediococcus pentosaceus strain FDAARGOS_1009 chromosome, co...
Pediococcus pentosaceus strain SRCM100892, complete genome
Pediococcus pentosaceus strain MGB0620 chromosome, complete geno...
Pediococcus pentosaceus strain MGB0941 chromosome, complete geno...
Pediococcus pentosaceus strain SRCM102738 chromosome, complete genome
Pediococcus pentosaceus ATCC 25745, complete genome
Pediococcus pentosaceus strain SRCM102739 chromosome, complete genome
Pediococcus pentosaceus strain SRCM102740 chromosome, complete genome

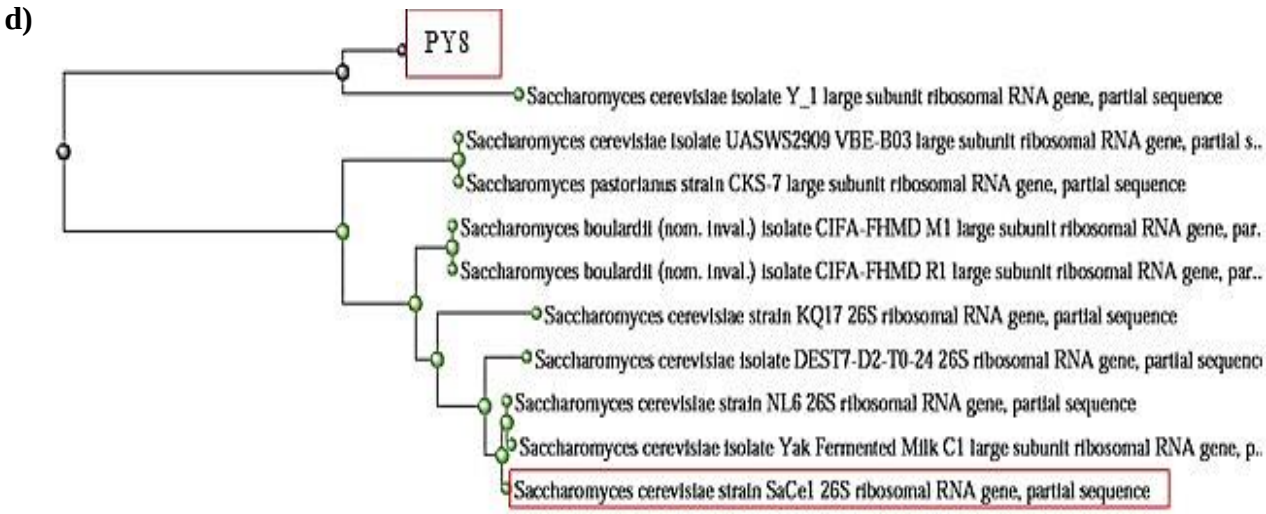


Fig 6. Neighbor-joining trees based on 16S rRNA sequences of the LAM isolates obtained from BLAST search, identified as *Lacticaseibacillus paracasei*, *B. bingmayongesis*, *Pediococcus pentosaceus*, and *S. cerevisiae* strain

4 Conclusion

In this study, the probiotic properties of LAMs isolated from different traditional food sources were investigated. LAM isolates comparatively show high growth under the GIT conditions (i.e. low pH and high bile salts content), antagonistic activity, and non-hemolytic nature. Based on this, four isolates were selected for identification and further studies. Using Biolog and 16S rRNA (for bacteria) and 18S rRNA (for yeast), these four isolates were identified as *Lacticaseibacillus paracasei* (R094), *B. bingmayongesis* (FJAT-13831), *Pediococcus pentosaceus* (ATCC 25745), and *S. cerevisiae* (SaCe1 26S) with 99%, 99%, 98%, and 99% similarity, respectively. These strains need to be further evaluated based on certain properties, including (1) as starters in the fermentation of various foods; (2) as probiotics to improve the human body health, such as modulating the balance of gut microbiota, lowering blood cholesterol, and lowering blood pressure, (3) in cosmetics products; and (4) in animal feeds to improve their productivity.

Acknowledgment

The authors would like to thank the management team of the Microbiology laboratory at Heliopolis University for their assistance and for providing all materials and facilities for the microbiological tests.

References

Adhikari K, Grün IU, Mustapha A, et al (2002) Changes in the profile of organic acidity in the plain set and stirred yogurts during manufacture and refrigerated storage. *Journal of Food Quality* 25, 435-451. https://doi.org/10.1111/j.1745-4557.2002.tb01038.x

Amorim JC, Piccoli RH, Duarte WF (2018) Probiotic potential of yeasts isolated from pineapple and their use in the elaboration of potentially functional fermented beverages. *Food Research International* 107, 518–527. https://doi.org/10.1016/j.foodres.2018.02.054

Arena MP, Russo P, Capozzi V, et al (2011) Effect of abiotic stress conditions on expression of the *Lactobacillus brevis* IOEB 9809 tyrosine decarboxylase and agmatine deiminase genes. *Annals of Microbiology* 61, 179–183. https://doi.org/10.1007/s13213-010-0089-1

Ashraf M, Arshad M, Siddique M, et al (2009) In vitro screening of locally isolated *Lactobacillus* species for probiotic properties. *Pakistan Veterinary Journal* 29, 186-190. http://www.pvj.com.pk/abstract/29_4/8.htm

Benson HJ (2002) Microbiological Applications: Laboratory Manual in General Microbiology, 8th ed. Mc Graw Hill Companies, Boston, Massachusetts New York.

Cho YH, Hong SM, Kim CH (2013) Isolation and characterization of lactic acidity bacteria from kimchi, korean traditional fermented food to apply into fermented dairy products. *Food Science of Animal Resources* 33, 75-82. https://doi.org/10.5851/kosfa.2013.33.1.75

Choi AR, Patra JK, Kim WJ, et al (2018) Antagonistic activities and probiotic potential of lactic acid bacteria derived from a plant-based fermented food. *Frontiers in Microbiology* 9, 1963. https://doi.org/10.3389/fmicb.2018.01963

De Man JC, Rogosa M, Sharpe ME (1960) A medium for the cultivation of *lactobacilli*. *Journal Applied Bacteriology* 23, 130-135. https://doi.org/10.1111/j.1365-2672.1960.tb00188.x

De Melo Pereira GV, de Oliveira BC, Júnior AM, et al (2018) How to select a probiotic? A review and update of methods and criteria. *Biotechnology Advances* 36, 2060–2076. https://doi.org/10.1016/j.biotechadv.2018.09.003

Difco Manual (1984) Dehydrated Culture Media and Reagents for Microbiology. 10th Ed. Difco Laboratories, Detroit, M. USA.

Duncan DB (1955) Multiple ranges and multiple f test. *Biometric* 11, 1–42. https://doi.org/10.2307/3001478

Dunne C, O'Mahony L, Murphy L, et al (2001) In vitro selection criteria for probiotic bacteria of human origin: Correlation with in vivo findings. *The American Journal of Clinical Nutrition* 73, 386S-392S. https://doi.org/10.1093/ajcn/73.2.386s

FAO/WHO (2006) Probiotics in food health and nutritional properties and guidelines for evaluation: Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria, Cordoba, Argentina, and Report of a Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food, London, Ontario, Canada. FAO food and nutrition paper 85, 1–56. https://nla.gov.au/nla.cat-vn3788914

Fernández-Pacheco P, Monge IMR, Fernández-González M, et al (2021) Safety evaluation of yeasts with probiotic potential. *Frontiers In Nutrition* 8, 659328. https://doi.org/10.3389/fnut.2021.659328

Gilliland SE, Staley TE, Bush LJ (1984) Importance of bile tolerance of *Lactobacillus acidophilus* used as dietary adjunct. *Journal of Dairy Science* 67, 3045–3051. https://doi.org/10.3168/jds.S0022-0302(84)81670-7

Gupta S, Goswami A, Sadhukhan AK, et al (1996) Comparative study of lactate removal in short-term massage of extremities, active recovery and a passive recovery period after supramaximal exercise sessions. *International Journal of Sports Medicine* 17, 106-110. https://doi.org/10.1055/s-2007-972816

Hamon E, Horvatovich P, Izquierdo E, et al (2011) Comparative proteomic analysis of *Lactobacillus plantarum* for the identification of key proteins in bile tolerance. *BMC Microbiology* 11, 63. https://doi.org/10.1186/1471-2180-11-63

Harris-Baldwin A, Gudmestad NC (1996) Identification of phytopathogenic coryneform bacteria using the biolog automated microbial identification system. *Plant Disease Journal* 80, 874-878. https://doi.org/10.1094/PD-80-0874

Hill C, Guarner F, Reid G, et al (2014) The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature Reviews Gastroenterology and Hepatology* 11, 506–514. https://doi.org/10.1038/nrgastro.2014.66

Lee HM, Lee Y (2008) A differential medium for lactic acid-producing bacteria in a mixed culture. *Letters in Applied Microbiology Journal* 46, 676–681. https://doi.org/10.1111/j.1472-765X.2008.02371.x

Mangia NP, Saliba L, Deiana P (2019) Functional and safety characterization of autochthonous *Lactobacillus paracasei* FS103 isolated from sheep cheese and its survival in sheep and cow fermented milks during cold storage. *Annals of Microbiology* 69, 161–170. https://doi.org/10.1007/s13213-018-1416-1

Melia S, Juliyarsi I, Kurnia YF, et al (2022) Examination of titratable acidity, pH, total lactic acid bacteria and sensory properties in whey fermented with probiotic *Pediococcus acidilactic* BK01. *Advances in Animal and Veterinary Sciences* 10, 114-119. DOI:10.17582/journal.aavs/2022/10.1.114.119

Miranda C, Contente D, Igrejas G, et al (2021) Role of exposure to lactic acid bacteria from foods of animal origin in human health. *Foods* 10, 2092. https://doi.org/10.3390/foods10092092

Mohania D, Nagapal R, Kumar M, et al (2008) Molecular approaches for identification and characterization of lactic acid bacteria. *Journal of Digestive Diseases* 9, 190-198. https://doi.org/10.1111/j.1751-2980.2008.00345.x

Montero-Zamora J, Fernández-Fernández S, Redondo-Solano M, et al (2022) Assessment of different lactic acid bacteria isolated from agro-industrial residues: first report of the potential role of *Weissella soli* for lactic acid production from milk whey. *Applied Microbiology* 2, 626–635. https://doi.org/10.3390/applmicrobiol2030048

Montoro BP, Benomar N, Lerma LL, et al (2016) Fermented Aloreña table olives as a source of potential probiotic *Lactobacillus pentosus* strains. *Frontiers in Microbiology* 7, 1583. https://doi.org/10.3389/fmicb.2016.01583

Moraes PM, Perin LM, Silva Júnior A, et al (2013) Comparison of phenotypic and molecular tests to identify lactic acid bacteria. *Brazilian Journal of Microbiology* 44, 109-112. https://doi.org/10.1590/S1517-83822013000100015

Nahaisi M, Almaroum N, Ziyaina MA (2021) Isolation and characterization of lactic acid bacteria from Libyan traditional fermented Milk "laban". *Preprints* 1, 030576. https://doi.org/10.20944/PREPRINTS202103.0576.V1

NCBI (2022) http://www.ncbi.nlm.nih.gov/BLAST/

Owade JO, Abong GO, Okoth MW, et al (2021) Comparative profiling of lactic acid bacteria isolates in optimized and spontaneous fermentation of cowpea leaves. *Food Science and Nutrition* 9, 1651–1664. https://doi.org/10.1002/fsn3.2140

Parmar R (2003) Incorporation of acid whey powders in probiotic yogurt. M. Sc. *Dissertations*, Major in Biological Sciences, Specialization in Dairy, South Dakota State University, U.S.A. 1497 p. https://openprairie.sdstate.edu/etd/1497/

Ramírez-Rivera E, Ramón-Canul LG, Torres-Hernández G, et al (2018) Typing aged goat cheeses produced in the mountainous central region of the state of Veracruz, Mexico. *Agrociencia* 52, 15-34. https://www.scielo.org.mx/scielo.php?pid=S1405-31952018000100015&script=sci_arttext&tlng=en

Saini K, Tomar SK (2017) In vitro evaluation of probiotic potential of *Lactobacillus* cultures of human origin capable of selenium bioaccumulation. *LWT-Food Science Technology* 84, 497–504. https://doi.org/10.1016/j.lwt.2017.05.034

Sarker AH, Chatterjee A, Williams M, et al (2014) NEIL2 protects against oxidative DNA damage induced by side stream smoke in human cells. *PLoS ONE* 9, e90261. https://doi.org/10.1371/journal.pone.0090261

Shafi A, Raja NH, Farooq U, et al (2019) Antimicrobial and antidiabetic potential of synbiotic fermented milk: A functional dairy product. *International Journal of Dairy Technology* 72, 15-22. http://doi.org/10.1111/1471-0307.12555

Tamang JP, Shin DH, Jung SJ, et al (2016) Functional properties of microorganisms in fermented foods. *Frontiers in Microbiology* 7, 578. https://doi.org/10.3389/fmicb.2016.00578

Tarrah A, Duarte VDS, de Castilho J, et al (2019) Probiotic potential and biofilm inhibitory activity of *Lactobacillus c*asei group strains isolated from infant feces. *Journal of Functional Foods* 54, 489–497. https://doi.org/10.1016/j.jff.2019.02.004

Turgay Ö, Erbilir F (2006) Isolation and characterization of *Lactobacillus bulgaricus* and *Lactobacillus casei* from various foods. *Turkish Journal of Biology* 30, 39-44. https://journals.tubitak.gov.tr/biology/vol30/iss1/7

Wang Q, Liu J, Cai J, et al (2022) Functional characteristics of lactic acid bacteria in vitro isolated from spontaneously fermented sour porridge with broomcorn millet in northwestern Shanxi province of China. *Foods* 11, 2353. https://doi.org/10.3390/foods11152353

Wang Y, Wu Y, Sailike J, et al (2020) Fourteen composite probiotics alleviate type 2 diabetes through modulating gut microbiota and modifying M1/M2 phenotype macrophage in db/db mice. *Pharmacological Research* 161, 105150. https://doi.org/10.1016/j.phrs.2020.105150

Wu SC, Su YS, Cheng HY (2011) Antioxidant properties of *Lactobacillus*-fermented and non-fermented *Graptopetalum paraguayense* E. Walther at different stages of maturity. *Food Chemistry* 129, 804-809. https://doi.org/10.1016/j.foodchem.2011.05.025

Yadav V, Prakash S, Srivastava S, et al (2009) Identification of *Comamonas* species using 16S rRNA gene sequence. *Bioinformation* 3, 381-383. https://doi.org/10.6026/97320630003381

Zhang W, Lai Sh, Zhou Z, et al (2022) Screening and evaluation of lactic acid bacteria with probiotic potential from local Holstein raw milk. *Frontiers in Microbiology* 13, 918774. https://doi.org/10.3389/fmicb.2022.918774

Zheng J, Wittouck S, Salvetti E, et al (2020) A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus beijerinck* 1901 and union of *Lactobacillaceae* and *Leuconostocaceae*. *International Journal of Systematic and Evolutionary Microbiology* 70, 2782–2858. https://doi.org/10.1099/ijsem.0.004107