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FOOD MICROBIOLOGY - RESEARCH PAPER

Comparison of probiotic *Lactobacillus* strains isolated from dairy and Iranian traditional food products with those from human source on intestinal microbiota using BALB/C mice model

Samaneh Hatami[1] · Masoud Yavarmanesh[1] · Mojtaba Sankian[2] · Seyed Ali Issazadeh[3]

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Abstract

This study compares the probiotic *Lactobacillus* strains isolated from dairy and Iranian traditional food products with those from human sources on intestinal microbiota using BALB/C mice model. First, *Lactiplantibacillus plantarum* (M11), *Limosilactobacillus fermentum* (19SH), *Lactobacillus acidophilus* (AC2), and *Lactobacillus gasseri* (52b) strains, isolated from either Iranian traditionally fermented products or human (healthy woman vaginal secretions), identified with molecular methods and selected based on the surface hydrophobicity, auto- and co-aggregation, were investigated for their probiotic properties and compared with their standard probiotic strains in vitro. The native strains and their mixtures (MIX) were then orally fed to five groups of female inbred BALB/C mice over the course of 38 days by gavage at 0.5 and 4 McFarland, respectively, equal to 1.5×10^8 and 1×10^9 cfu/ml. Feeding paused for 6 days to test the bacteria's adhesion in vivo. According to the findings, the probiotic *Lactobacillus* strain isolated from human source (52b) exhibited the best in vitro and in vivo adhesion ability. Probiotic *Lactobacillus* strains isolated from Iranian traditional food products (19SH and AC2) had the most co-aggregation with *Listeria monocytogenes* (ATTC 7644), *Salmonella enterica* subsp. *enterica* (ATCC 13,076), and *Escherichia coli* (NCTC 12,900 O157:H7) in vitro. These strains produced the most profound decreasing effect on the mice intestinal microbiota and pathogens in vivo. The difference in the strains and their probiotic potential is related to the sources from which they are isolated as well as their cell walls. The results suggest that (19SH and 52b strains) are the best candidates to investigate the cell wall and its effect on the host immune system.

Keywords Native probiotic bacteria · BALB/C mice intestinal microbiota · Probiotic properties · Adhesion · Co-aggregation

Introduction

Lactic acid bacteria (LAB) are a category of Gram-positive bacteria which naturally occur in the GI tract of mammals including humans. They have the highest population density in the region between duodenum and the ileum terminal of human digestive system and in the urogenital tract of women. Conversely, in rodents like mouse, a large number of LAB are present in the upper gut [1]. LAB, including *Lactobacillus* species, produce lactic acid (above 50% of sugar carbon) as the major final product of the metabolism of carbohydrates [2].

Gram-negative pathogenic bacteria such as *Escherichia coli* O157:H7, *Salmonella*, *L. monocytogenes*, and *Campylobacter* are another group of microorganisms which exist in the large intestine of animals including mouse. If such bacteria are transmitted to food products or animal feed, they will cause diseases like diarrhea and even death. Diarrhea is the second common cause of the mortality of under-5 children. As a result, these bacteria are not only a serious risk to human health but also an enormous cause of economic losses [3].

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✉ Masoud Yavarmanesh
yavarmanesh@um.ac.ir

[1] Department of Food Science and Technology, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran

[2] Immunology Research Center, Medical School, Mashhad University of Medical Sciences, Mashhad, Iran

[3] Department of Food Science and Technology, Quchan Branch, Islamic Azad University, Quchan, Iran

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The intentional consumption of some specific types of bacteria can directly or indirectly influence the microbiota and act as a preventive factor in controlling diseases [4]. The literature has demonstrated that the consumption of such bacteria, known as probiotic bacteria, plays a key role in promoting the human and animal health [5]. According to the instruction given by food and agriculture organization and world health organization, international scientific organization has issued a statement to introduce probiotic and prebiotic bacteria, whereby probiotic bacteria are defined as the live microorganisms which will produce positive effects on the host body health if consumed in sufficient quantities [6].

Probiotic bacteria (isolated from different sources) could be an appropriate choice for controlling and inhibiting foodborne diseases, as the results of numerous studies have proven the efficiency and effectiveness of such bacteria in reducing the pathogenicity of the pathogens present in food products. At certain amounts, probiotic bacteria, especially those isolated from human sources, can have beneficial impacts on the host immune system and body health [7].

De Waard et al. [8] investigated the influence of the viable *Lactobacillus casei* strain Shirota (isolated from human feces) on the mice infected by *L. monocytogenes* through oral administration. The mice were infected by *L. monocytogenes* at 10^9 cfu/ml. Three days before infection, they were fed with *L. casei* at 10^9 cfu/ml. It was shown that the oral administration of *L. casei* significantly reduced the numbers of *L. monocytogenes* in stomach, caecum, feces, spleen, and liver 2 days after *L. monocytogenes* infection. It was also revealed that *L. casei* was able to increase cellular immunity significantly as determined with the delayed-type hypersensitivity response to heat-killed *L. monocytogenes*. Furthermore, it was concluded that the enhancement of this anti-*Listeria* activity might be, at least partly, due to the increased cell-mediated immunity.

Another study demonstrated that supplementation of the mice infected with *E. coli* O157:H7 by *Lactobacillus rhamnosus* and *Lactobacillus plantarum* 299 V (isolated from human source) led to a decrease in the colonization of *E. coli* and its pathogenicity caused by shiga toxin. It also increased the anti-*E. coli* IgA response as well as the activity of white blood cells, thus preventing the host body from being infected [9]. Owing to their antimicrobial metabolites, several probiotics including *Bifidobacterium longum* and *L. casei* restrain the transfer and spread of shiga toxin produced by *E. coli*. This is probably due to the created immune response [10].

Shu and Gill [11] examined the effect of *L. rhamnosus* (strain HN001), isolated from yoghurt, on the BALB/C mice orally infected with *Salmonella typhimurium.* The control mice, which had not been fed with the probiotic bacteria, died 3–4 days after being infected. However, mortality markedly decreased in the mice fed with *L. rhamnosus* (strain HN001). Additionally, the blood and peritoneal leucocytes obtained from HN001-fed mice exhibited a significantly higher ex vivo phagocytic capacity compared with the control mice. Consequently, they found out that supplementation of the mice by probiotic bacteria like *L. rhamnosus* (strain HN001) could prevent the intestinal infectious diseases caused by *S. typhimurium*.

Probiotics produce positive effects on the host immune system by inhibiting the adhesion of pathogens to mucosal membranes which retain the balance between lymph cells and the intestine microflora. Moreover, they can reinforce the host innate immune system through the host receptor recognition [12].

Probiotic bacteria should reach the intestine through growth, biofilm formation, or aggregation in order to influence the host immune system and body health. The difference in the strains and their probiotic potential is related to the sources from which they are isolated as well as their cell walls [13].

Therefore, the objective of the present research is to evaluate and compare probiotic *Lactobacillus* strains isolated from dairy and Iranian traditional food products with those from human source on intestinal microbiota using BALB/C mice model.

Materials and methods

Bacterial strains

The native strains of *L. gasseri* (52b; accession number: KP090115), *L. plantarum* (M11; accession number: KP212405), *L. acidophilus* (AC2; accession number: LC155899.1), and *L. fermentum* (19SH; accession number: [14]) (respectively, isolated from human (healthy woman vagina), milk, sour dough, and a fermented seed named Horreh), the standard strains (*L. gasseri* ATCC 33,323, *L. plantarum* subsp. *plantarum* ATCC 14,917, *L. fermentum* ATCC 9338 and *L. acidophilus* ATCC 4356), and the pathogens (*E. coli* NCTC 12,900 O157:H7, *S. enterica* subsp. *enterica* ATCC 13,076, and *L. monocytogenes* ATCC 7644) were all supplied from the microbial collection of the department of food science and technology, faculty of agriculture, Ferdowsi University of Mashhad, Iran. The pathogens were employed for the auto-aggregation and co-aggregation tests.

The *Lactobacillus* species were cultured on De Man Rogosa Sharpe (MRS) agar (Merck Co., Germany) at 37 °C under microaerophilic conditions. The pathogenic strains were grown on Mueller–Hinton agar (MHA), Mueller–Hinton broth (MHB), and Brain heart infusion (BHI) agar and broth (Merck Co., Germany) and propagated at 37 °C under aerobic conditions.

Assays of probiotic activity

Resistance to acidic conditions (low pH)

The strains were incubated at 30 °C for 48 h before being centrifuged at 6000 g for 15 min. The supernatant was discarded, and the cells were washed with sterile phosphate-buffered saline (PBS; 8.01 gr/L NaCl, 0.2 gr/L KCL, 1.78 gr/L $Na_2HPO_4{\cdot}2H_2O$, 0.27 gr/L KH_2PO_4) with a pH value of 2.5 before being incubated at 37 °C for 4 h. Before and after incubation, these samples were serially diluted in sterile saline solution (0.85% NaCl), and the viable cell population was determined by the spread plate method using MRS Agar. Finally, the plates were incubated at 37 °C for 48 h [15].

The percentage survival of the bacteria was calculated as follows:

$$Survival\ (\%) = \frac{Log\ cfu\ of\ viable\ cells\ survived}{Log\ cfu\ of\ initial\ viable\ cells\ \text{inoculated}} \times 100 \quad (1)$$

Resistance to bile salts and simulated gastric and intestinal juices

The MRS broth containing 0.3% (w/v) of bile salts (bovine bile, Sigma-Aldrich) was inoculated with 100 µl of the grown bacteria (incubated at 30 °C for 48 h) in a test tube to determine the strain tolerance to bile salts. The mixture was incubated at 37 °C for 4 h. The viable cell count was determined at the beginning of and 4 h after incubation through the spread plate method. The percentage survival of the bacteria was determined using Eq. 1 [16].

To test the percentage survival of the strains in the presence of pepsin (Sigma-Aldrich, Milan, Italy), the simulated gastric juice which was prepared by suspending 3 mg/mL pepsin in sterile saline solution (0.85% NaCl, w/v) and adjusted to pH 2.5 was inoculated with the active LAB cultures (incubated at 30 °C for 48 h) at an inoculum size of 1% (v/v) and incubated at 37 °C for 4 h. The simulated intestinal juice which was prepared by dissolving 1 mg/mL pancreatin (Sigma-Aldrich) in sterile saline solution and adjusted to pH 8.0 was used in the pancreatin resistance test. This juice was inoculated with the active LAB cultures at an inoculum size of 1% (v/v) and incubated at 37 °C for 6 h. The viable cell population was determined by the spread plate method before and after the incubation of the MRS agar plates. The percentage survival of the bacteria was calculated using Eq. (1) [15].

Surface hydrophobicity

The LAB cells were harvested by centrifugation at 6000 g for 10 min, washed twice in 50 mM K_2HPO_4 at pH 6.5, and resuspended in the same buffer to obtain an A_{560} nm value of approximately 0.8 to 1. 3 ml of the solution along with 0.6 ml of n-hexadecane which was transferred to a test tube, and the mixture was stirred for 120 s. After that, the tubes were left standing at 37 °C for 30 min to separate into two phases. The aqueous phase was carefully removed, and the A_{560} value was measured [17].

$$H\% = A0 - \frac{A}{A0} \times 100 \quad (2)$$

A_0 = Absorbance value before extraction with n-hexadecane.

A = Absorbance value after extraction with n-hexadecane.

Auto-aggregation and co-aggregation assays

The LAB strains were grown at 37 °C for 24 h in MRS broth under microaerophilic conditions. Then, the pellets were resuspended in 10 ml of PBS to approximately 10^8 cfu/ml after centrifugation at 1372 × g for 10 min (A_{550} nm = 0.2–0.3). Each suspension was vortexed for 10 s and incubated at room temperature for 6 h. One milliliter of the upper part of the suspension was collected every hour, and its absorbance value was measured with a spectrophotometer (Lightwave S2000UV-Vis) at 600 nm. The following formula was used to quantify the percentage of auto-aggregation [18].

$$Auto - aggregation = 1 - \left(\frac{At}{A0}\right) \times 100 \quad (3)$$

A_t = absorbance at different times, A_0 = absorbance at time zero.

Three milliliters of the tested bacterial suspensions and pathogens *E. coli* O157 H7 NCTC 12,900, *S. enterica* subsp. *enterica* ATCC 13,076, and *L. monocytogenes* ATTC 7644 with a concentration of 10^8 cfu/ml and A_{600} nm value of 0.25–0.05 was vortexed for 10 s and incubated at room temperature for 6 h without shaking for the determination of the co-aggregation percentage. As the control, the samples containing 6 ml of the bacterial suspension were used. During 6 h of incubation at room temperature, 1 ml of the upper part of each suspension was withdrawn every hour, and the absorbance value of each pair of the bacterial suspensions (probiotic and pathogen) was measured. Absorbance value was measured for both the mixtures and the bacterial suspensions separately.

The percentage of co-aggregation was determined using Eq. (4), where Ax and Ay are the individual aggregation properties of the lactobacilli and the pathogen, and $A\ (X+Y)$ is the combined aggregation of the lactobacilli and the pathogen. All of the experiments were performed in duplicate [18].

$$Co-aggregation\% = \frac{\left(\frac{(Ax+Ay)}{2} - A(X+Y)\right)}{(Ax+Ay)/2} \times 100 \quad (4)$$

Antibiotic susceptibility

The antibiotic resistance for all the LAB strains used in this study was checked by the broth micro-dilution method. First, 95 µl of MRS broth was poured into all the wells of the 96-well microtiter plate. Next, 100 µl of each of the solutions of ampicillin, kanamycin, erythromycin, chloramphenicol, and tetracycline (Sigma-Aldrich, USA) was added to the first row of the plate, except the positive control row, at an initial concentration of 1 mg/l. The serial dilutions were prepared, and subsequently 5 µl of each strain solution was added to each well, except the negative control row, at 0.5 McFarland. Eventually, the plates were incubated at 37 °C for 24 h, and the turbidity of the wells was measured using an ELISA reader, ELX808, Biotek). Minimal inhibitory concentration (MIC) was defined as the lowest concentration of a given antibiotic at which the tested bacteria could not grow [19, 20].

Animals and ethics statement

Female BALB/C mice were purchased from Pasteur Institute of Iran and raised in the Animal House unit (Bu Ali Research Institute, Mashhad University of Medical Sciences, Iran).

Five groups of 5 mice (6–8 weeks old, weight: 16–19 g) were used in each experiment. The animals were housed in polycarbonate cages at room temperature and provided with commercial food and tap water ad libitum.

The mice pellet diets contained 10% moisture, 0.5% salt, 1% calcium, 0.65% phosphorus, 0.25% tryptophan, 0.33% methionine, 1.15% lysine, 0.7% threonine, minimum raw fat of 4%, and maximum raw fiber of 4%. All the ethical criteria of the biomedical committee of Ferdowsi University of Mashhad regarding practical work with mice used with the ethical code of IR.UM.REC.1400.004 were observed.

Animal studies are reported in compliance with ARRIVE guidelines. All such items included of study desine, sample size, and statistical method from Arrival guidelines were followed [21–24].

Feeding procedure

In this study, the mice were fed with the native strains of *L. gasseri* (52b), *L. plantarum* (M11), *L. acidophilus* (AC2), and *L. fermentum* (19SH).

The test strains were inoculated in 10 ml of MRS broth and cultivated overnight at 37 °C under anaerobic conditions. Then, they were collected by centrifugation at 4000 g for 30 min at 4 °C, washed three times with sterile PBS and resuspended in PBS. Afterwards, the suspensions were prepared using spectrophotometry (WPA, S2000 UV–Vis) for feeding at 0.5 McFarland (1.5×10^8 cfu/ml) until the 24th day and at 4 McFarland (1.2×10^9 cfu/ml) from the 24th to the 38th day. The mice were fed with 0.5 ml of the suspensions daily until the 11th day, every other day from the 17th to 24th day and daily from the 24th to 38th day by gavage.

The feeding paused for 6 days (11th–17th) to quantify the adhesion of the bacteria tested. The fecal samples were collected on the 0th, 3rd, 7th, 14th, 17th, 27th and 38th days (Fig. 1) [25, 26].

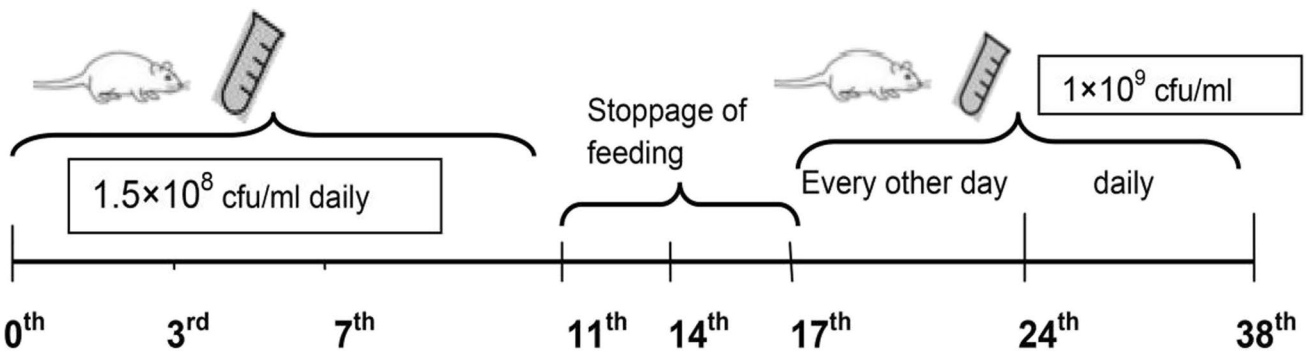

Fig. 1 Feeding procedure.The mice were administered with 0.5 ml of the suspensions every day until the 11th day, every other day from the 17th to 24th day and daily from the 24th to 38th day by gavage. The administration paused for 6 days (11th–17th) to measure the adhesion of the tested bacteria

Fecal sampling

Miles and Misra methods were used to count the number of intestinal microbiota colonies in mice. Briefly, 1 g of the feces of each mouse was transferred to a test tube containing 1 ml of PBS. After complete mixing, serial dilutions were prepared, and the specific agar culture media were used to detect each colony. The concentration of the bacteria was measured in colony forming units (cfu) per 1 g of the feces solution. MRS agar, Trypticase soy agar, MacConkey agar, *Salmonella Shigella* agar, and plate count agar, all supplied from Merck Co., Germany, were used to detect the feces LAB, Gram-negative bacteria, coliforms, *Salmonella*, *Shigella*, and total bacteria count, respectively [9, 27].

Statistical analysis

The findings were presented as the mean standard deviation (SD) of two replications (in vitro). Minitab version 19 (Minitab ®19.2, Minitab Inc., State College, PA, USA) was used for statistical analysis. Statistical significance was determined by *P* values less than 0.05. Furthermore, Shapiro–Wilk was applied to test the normality distribution, and the equality of variance test was performed by Leven's test for each groups of mice (in vivo). There was non-normal distribution among groups of PCA, SSA, and TSA from 5 experimental groups. However, the variances were equal in all groups. Therefore, all groups of mice were assessed by a non-parametric test. The comparison of fed groups at fecal sampling days was made by Kruskal–Wallis test, and to compare the effect of before and after the bacteria fed in each group at specific days was used by Wilcoxon-signed ranks test. Continuous variables are expressed as the median ± SEM (standard error of the median). The Bonferroni correction was obtained 0.008 for the groups of MRS culture and 0.01 for other culture media. The *P* values less than these ones were reported significant. The statistical software (IBM SPSS, Armonk, NY, version 23.0) was used to evaluate the data, and the significance threshold was set at $p < 0.05$. Graph pad version 8 was used to create the graphs.

Results

Probiotic properties

Resistance to low pH and bile salts

Exposure of bacteria to the acidic conditions of stomach is the first step in the diagnosis and evaluation of their probiotic properties, because it enables them to survive when passing through the GI tract [21]. In this research, the survival ability and acid (pH = 2.5) tolerance of 8 strains were examined in vitro. All the strains were highly viable under the acidic conditions (Table 1), except *L. gasseri* ATCC 33,323, *L. plantarum* subsp. *plantarum* ATCC 14,917, and 19SH which could not tolerate such conditions. 52b and M11 were the most viable ones ($p < 0.0001$).

Resistance to bile salts is one the most significant properties of probiotics. Since a relatively high content of bile salts exists in both the small intestine and colon, which is toxic and lethal to live cells, the duration of the presence of such bacteria under these conditions is of great importance [15].

All the tested strains were remarkably resistant to bile salts, except for *L. gasseri* ATCC 33,323 which formed no colony at time zero and 4 h after incubation (Table 2).

Table 1 Acid tolerance of LAB strains in PBS (pH = 2.5)

N	Species	Strain number	Source	Initial counts Time (0 h) cfu/ml	Log (cfu/ml)	Survival after Time (4 h) cfu/ml	Log (cfu/ml)	Survival%
1	*L. gasseri*	52b	Vaginal	8.1×10^7	7.91 ± 0.00	1.9×10^7	7.28 ± 0.02	92.04[a]
2	*L. gasseri*	ATCC 33,323	Human	0	0	0	0	0[e]
3	*L. plantarum*	M11	Milk	4.3×10^7	7.63 ± 0.01	1.6×10^7	7.20 ± 0.01	94.38[a]
4	*L. plantarum*	ATCC 14,917	Fermented products	0	0	0	0	0[e]
5	*L. fermentum*	19SH	Horre	8.2×10^7	7.91 ± 1	0	0	0[e]
6	*L. fermentum*	ATCC 9338	Human	3.4×10^7	7.53 ± 0.1	0.81×10^6	5.91 ± 0.00	78.46[c]
7	*L. acidophilus*	AC2	Sourdough Fermented products	4.1×10^8	8.61 ± 0.1	1.9×10^6	6.27 ± 0.2	72.82[d]
8	*L. acidophilus*	ATCC 4356	Human	7.8×10^7	7.89 ± 0.00	6.5×10^6	6.81 ± 0.01	86.33[b]

Results are reported as the mean of two replications

Data are presented as mean ± standard deviation and analyzed by ANOVA one-way test with Tukey test

Different lowercase letters in the percentage survival of LAB strains column show significant differences ($P < 0.0001$)

Table 2 Bile salts tolerance of LAB strains

N	Species	Strain number	Source	Initial counts Time (0 h) cfu/ml	Logcfu/ml))	Survival after Time (4 h) cfu/ml	Log (cfu/ml)	Survival%
1	*L. gasseri*	52b	Vaginal	0.95×10^6	5.98 ± 0.01	1.3×10^7	7.11 ± 0.00	100^a
2	*L. gasseri*	ATCC 33,323	Human	0	0	0	0	0^c
3	*L. plantarum*	M11	Milk	2.2×10^6	6.34 ± 0.00	2.5×10^6	6.40 ± 0.01	100^a
4	*L. plantarum*	ATCC 14,917	Fermented products	1.05×10^8	8.02 ± 0.02	9×10^7	7.95 ± 0.1	99^a
5	*L. fermentum*	19SH	Horre	2.5×10^8	8.4 ± 0.3	1.4×10^8	8.16 ± 0.2	97^a
6	*L. fermentum*	ATCC 9338	Human	1.3×10^7	7.11 ± 0.11	1.8×10^7	7.26 ± 0.02	100^a
7	*L. acidophilus*	AC2	Sourdough Fermented products	4×10^8	8.60 ± 0.2	3.6×10^6	6.56 ± 0.01	76^b
8	*L. acidophilus*	ATCC 4356	Human	0.22×10^6	5.34 ± 0.14	4.8×10^6	6.68 ± 0.01	100^a

Results are reported as the mean of two replications

Data are presented as mean ± standard deviation and analyzed by ANOVA one-way test with Tukey test

Different lowercase letters in the percentage survival of LAB strains column show significant differences ($P<0.0001$)

Survival under conditions simulating the human GIT

In the present study, pepsin solution (pH = 2.5) and pancreatin solution (pH = 8.0) were used as simulated gastric and intestinal juices, respectively. In the pepsin test, only *L. gasseri* ATCC 33,323 and 19SH could survive, and the other strains could not tolerate this condition. In the pancreatin test, all the strains had a high percentage survival, except M11 and *L. acidophilus* ATCC 4356 which were not tolerant towards this condition at all (Tables 3 and 4).

Resistance to antibiotics

The antibiotic susceptibility of the tested bacteria was evaluated using MIC. According to Table 5, 52b had a high resistance to ampicillin and kanamycin as their MICs were equal to 0.5 mg/l. This strain was rather resistant to erythromycin and susceptible to tetracycline. Furthermore, no MIC was observed in the case of chloramphenicol. *L. gasseri* ATCC 33,323 was tolerant of kanamycin and tetracycline, while being susceptible to the other antibiotics. M11 was resistant to erythromycin and ampicillin whose MICs were equal to 0.5 and 0.25 mg/l, respectively. However, it was sensitive to the other tested antibiotics. The standard strain of *L. plantarum* subsp. *plantarum* ATCC 14,917 was susceptible to all the antibiotics and showed no tolerance to them. 19SH was only tolerant of kanamycin and tetracycline with MICs of 0.5 mg/l. *L. fermentum* ATCC 9338 was resistant to kanamycin and tetracycline. AC2 was sensitive to all the antibiotics tested, and the standard strain of *L. acidophilus*

Table 3 Percentage survival of LAB strains in simulated gastric conditions (pepsin, (PH = 2.5) solution)

N	Species	Strain number	Source	Initial counts Time (0 h) cfu/ml	Log (cfu/ml)	Survival after Time (4 h) cfu/ml	Lo(cfu/ml)	Survival%
1	*L. gasseri*	52b	Vaginal	0.1×10^5	4.00 ± 0.1	0.25×10^5	4.40 ± 0.01	$<10^{6a}$
2	*L. gasseri*	ATCC 33,323	Human	3.5×10^8	8.5 ± 0.04	1.5×10^8	8.17 ± 0.02	96^b
3	*L. plantarum*	M11	Milk	> 2000	-	0	-	-
4	*L. plantarum*	ATCC 14,917	Fermented products	1.5×10^7	7.17 ± 0.01	0	0	-
5	*L. fermentum*	19SH	Horre	1.1×10^8	8.04 ± 0.00	3.5×10^7	7.5 ± 0.2	93^b
6	*L. fermentum*	ATCC 9338	Human	1.3×10^4	3.82 ± 0.04	0	-	-
7	*L. acidophilus*	AC2	Sourdough Fermented products	6.8×10^7	7.83 ± 0.00	0	-	-
8	*L. acidophilus*	ATCC 4356	Human	1×10^7	7.00 ± 0.05	0	-	-

no significant

Results are reported as the mean of two replications

Data are presented as mean ± standard deviation and analyzed by ANOVA one-way test with Tukey test

Different lowercase letters in the percentage survival of LAB strains column show significant differences ($P<0.0001$)

Table 4 Percentage survival of LAB strains in simulated intestinal conditions (pancreatin, (PH = 8)

N	Species	Strain number	Source	Initial counts Time (0 h) cfu/ml	Log (cfu/ml)	Survival after Time (4 h) cfu/ml	Log)cfu/ml)	Survival%
1	*L. gasseri*	52b	Vaginal	0.1×10^7	6.00 ± 0.11	0.5×10^6	5.70 ± 0.00	94.98^{b}
2	*L. gasseri*	ATCC 33,323	Human	7×10^8	8.84 ± 0.1	3×10^8	8.47 ± 0.01	95.8^{a}
3	*L. plantarum*	M11	Milk	0.84×10^4	3.92 ± 0.00	0	-	-
4	*L. plantarum*	ATCC 14,917	Fermented products	7×10^7	7.84 ± 0.25	6×10^7	7.77 ± 0.2	99.1^{a}
5	*L. fermentum*	19SH	Horre	7.5×10^7	7.87 ± 0.1	7×10^7	7.84 ± 0.01	99.6^{a}
6	*L. fermentum*	ATCC 9338	Human	0.4×10^7	6.60 ± 0.00	0.63×10^7	6.80 ± 0.00	100.00^{a}
7	*L. acidophilus*	AC2	Sourdough Fermented products	4.3×10^7	7.63 ± 0.1	1.6×10^7	7.2 ± 0.1	94.3^{b}
8	*L. acidophilus*	ATCC 4356	Human	0.94×10^7	6.97 ± 0.00	0.00	-	-

no significant

Results are reported as the mean of two replications

Data are presented as mean ± standard deviation and analyzed by ANOVA one-way test with Tukey test

Different lowercase letters in the percentage survival of LAB strains column show significant differences ($P < 0.0001$)

ATCC 4356 was only tolerant towards erythromycin with a MIC of 0.25 mg/l (Table 5).

Cell surface characteristics

Some researchers have declared a correlation between the hydrophobicity and adhesion ability of probiotic strains. At the same time, some other scientists claimed that there was no correlation between the two features [28].

In this study, the most surface hydrophobicity belonged to the human strains of *L. gasseri*, as 52b (56.15%) and the standard strain of *L. gasseri* ATCC 33,323 (51%) had the highest surface hydrophobicity. M11 (25.83%), *L. plantarum* subsp. *plantarum* ATCC 14,917 (33.4%) and *L. acidophilus* ATCC 4356 (30.38%) had medium surface hydrophobicity, and the other strains were weakly surface hydrophobic. The three groups were significantly different ($p < 0.0001$) (Fig. 2).

Auto- and co-aggregation are two vital properties of probiotic bacteria, because it seems that the co-aggregation ability is associated with the adhesion ability to epithelial cells [29]. In the present research, the highest percentage of auto-aggregation was, respectively, related to 19SH (62%), *L. plantarum* subsp. *plantarum* ATCC 14,917 (50%), M11 (45.36%), *L. fermentum* ATCC 9338 (44.88%), and *L. acidophilus* AC2 (43.24%) (Fig. 3). In addition, the co-aggregation test was conducted for examining the interference with three human intestinal pathogens, namely *E. coli* O157:H7 NCTC 12,900, *S. enterica* subsp. *enterica* ATCC 13,076, and *L. monocytogenes* ATCC 7644. According to Fig. 4, the largest amount of co-aggregation pertained to AC2 (*L. monocytogenes* (85%), *S. enteritidis* (100%), and *E.*

Table 5 Minimum inhibitory concentration (MIC, mg/l) of 5 antibiotics on LAB strains

N	Species	Strain number	Source	Erythromycin	Ampicillin	Chloramphenicol	Kanamycin	Tetracycline
1	*L. gasseri*	52b	Vaginal	0.125 ± 0.00^{b}	0.5 ± 0.00^{a}	ND	0.5 ± 0.00^{a}	0.062 ± 0.00^{c}
2	*L. gasseri*	ATCC 33,323	Human	0.015 ± 0.00^{b}	ND	0.005 ± 1.8^{b}	0.5 ± 0.00^{a}	0.5 ± 0.00^{a}
3	*L. plantarum*	M11	Milk	0.5 ± 0.00^{a}	0.25 ± 0.00^{a}	ND	0.14 ± 1.1^{b}	0.031 ± 0.00^{bc}
4	*L. plantarum*	ATCC 14,917	Fermented products	0.015 ± 0.00^{b}	ND	ND	ND	0.375 ± 1.3^{ab}
5	*L. fermentum*	19SH	Horre	0.008 ± 0.12^{b}	0.001 ± 0.00^{b}	0.006 ± 1.3^{b}	0.5 ± 0.00^{a}	0.5 ± 0.00^{a}
6	*L. fermentum*	ATCC 9338	Human	0.009 ± 0.94^{b}	0.004 ± 0.9^{b}	ND	0.25 ± 0.00^{a}	0.125 ± 0.00^{abc}
7	*L. acidophilus*	AC2	Sourdough Fermented products	0.0025 ± 0.4^{b}	ND	ND	ND	0.002 ± 0.00^{c}
8	*L. acidophilus*	ATCC 4356	Human	0.25 ± 0.00^{a}	0.19 ± 2.1^{b}	ND	0.03 ± 0.00^{b}	0.1405 ± 1.00^{bc}

ND: No detriment

Data are presented as mean ± standard deviation and analyzed by ANOVA one-way test with Tukey test. Different lowercase letters in the same column show significant differences (*P* values of kanamycin, erythromycin, ampicillin, chloramphenicol, and tetracycline are, respectively < 0.0001, 0.003, 0.011, 0.001, 0.002)

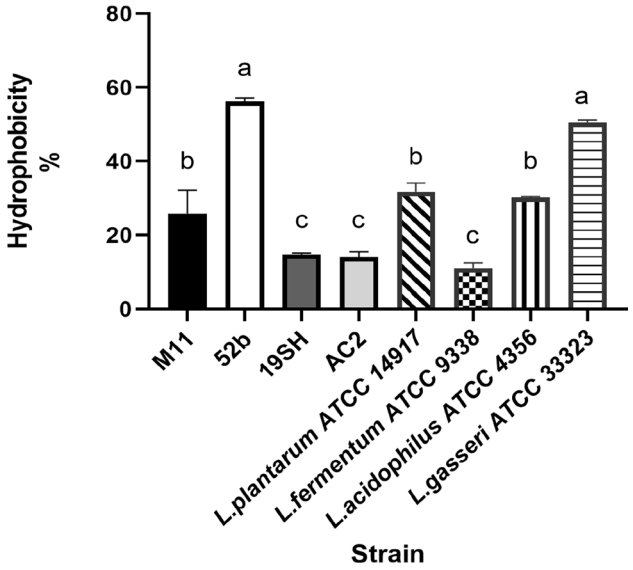

Fig. 2 Surface hydrophobicity of LAB strains. Data are presented as mean ± standard deviation and analyzed by ANOVA one-way test with Tukey test. Different lowercase letters in the same column show significant differences ($P < 0.0001$). The graphs were drawn using graph pad version 8

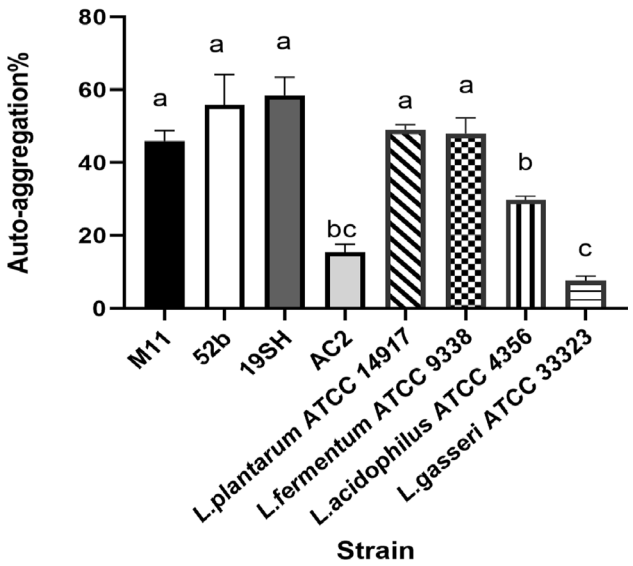

Fig. 3 Percentage of auto-aggregation ability of different LAB strains. Data are presented as mean ± standard deviation and analyzed by ANOVA one-way test with Tukey test. Different lowercase letters in the same column show significant differences ($P < 0.0001$). The graphs were drawn using graph pad version 8

coli (38.73%)) followed by 19SH (*L. monocytogenes* (93%), *S. enteritidis* (51%), and *E. coli* (28%)) and *L. plantarum* subsp. *plantarum* ATCC 14,917 (*L. monocytogenes* (92%), *S. enteritidis* (42%), and *E. coli* (31%)) which had a significant difference with the other strains ($p < 0.0001$).

Establishment and surface adhesion of native strains administrated in vivo

Subsequently, the native strains of 52b, AC2, 19SH, and M11, as well as their mixtures, were utilized for oral administration to female BALB/C mice. The mice fecal samples were taken on 0^{th}, 3^{rd}, 7^{th}, 14^{th}, 17^{th}, 28^{th}, and 38^{th}.

The LAB counts of the fecal samples of the mouse groups were approximately equal on 0th day ($P = 0.079$) which was not significant among the groups. The LAB count increased on the third day after treatment at 0.5 McFarland; at the same time, the rise was significant for the administered strains ($P = 0.005$). As feeding proceeded from the 3^{rd} to the 11^{th} day, the number of LAB lowered in the fecal samples. With the 6-day stoppage of feeding on the 11^{th} to the 17^{th} day, the LAB count decreased in the mice intestine. As feeding was continued at 10^9 cfu/ml, the population of the administered strains significantly ($P = 0.005$) increased on the 28th day (compared with the 17^{th} day) in the feces of the mice fed with them (Fig. 5).

Based on the statistical analysis performed on the data of the 38-day period, all strains were established in the mice intestines on the 3^{rd} day. At the same time, according to the 6-day stoppage of feeding which was done from the 11^{th} to the 17^{th} day to confirm the degree of the strains adhesion, in addition to the medians comparison of the log LAB counts of the fecal samples from the 3^{rd} to the 17^{th} day, a significant decrease occurred in the whole strains, in terms of the lowest bacterial reduction; the highest adhesion was related to *L. gasseri* (52b) strain ($3^{th} = 7.75 \pm 1.12$, $17^{th} = 6.75 \pm 1.5$, $P = 0.005$) (Fig. 5).

Effect of oral administration of probiotic bacteria on intestinal microbiota of BALB/C mice

PCA was used to determine the total count of the intestinal bacteria. The total count of the intestinal microbiota of the mice fed with these strains followed a decreasing trend from the 0th to the 14th day, which was significant on the 14^{th} day (compared with the 0th day) for all the bacteria ($P = 0.005$). As feeding proceeded, the total count of the mice gastrointestinal microbiota was elevated from the 14th to the 38th day ($P = 0.005$). There was no significant difference among the fed groups at the same days; P value of the groups was observed, respectively ($0^{th} = 0.068$, $3^{rd} = 0.079$, $7^{th} = 0.079$, $14^{th} = 0.068$, $28^{th} = 0.068$, $38^{th} = 0.072$), (Fig. 6A).

TSA culture medium was applied to examine the effect of the administered strains on the Gram-negative bacteria. The population of the Gram-negative bacteria was significantly ($P = 0.005$) reduced from the 0^{th} to the 14^{th} day in the intestine of the mice fed with M11, MIX, and 52b. In the case of the mice fed with AC2 and 19SH, the number of the Gram-negative bacteria followed a decreasing trend

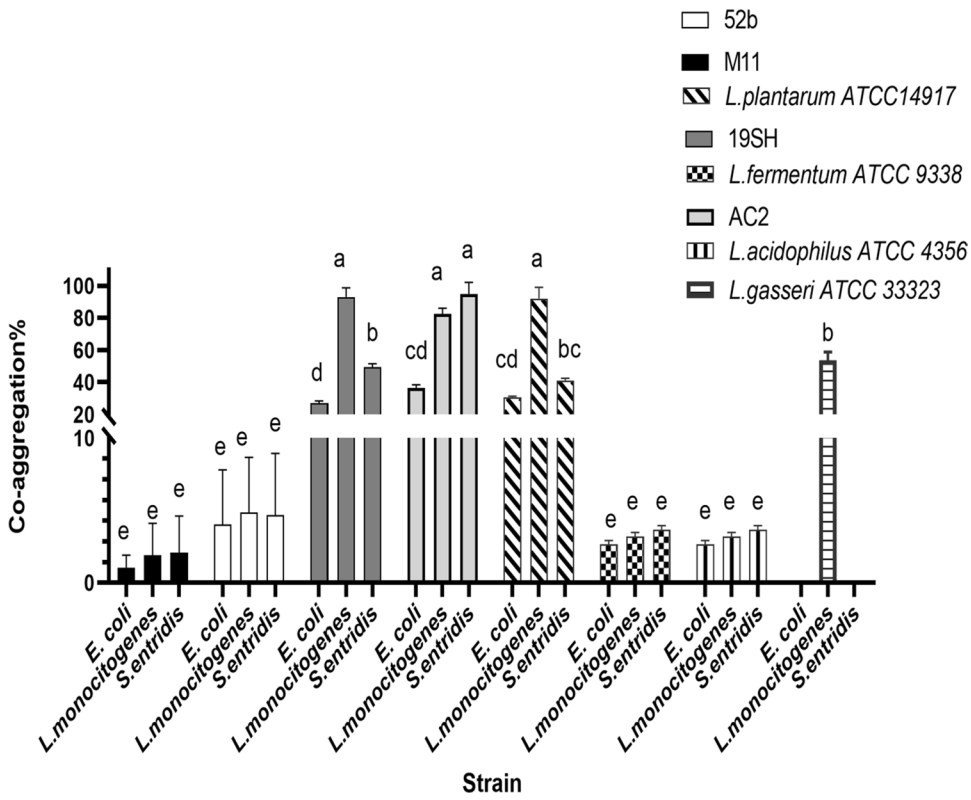

Fig. 4 Comparison between co-aggregation abilities of different LAB strains with intestinal pathogens. Data are presented as mean ± standard deviation and analyzed by ANOVA one-way test with Tukey test. Different lowercase letters in the same column show significant differences ($P < 0.0001$). The graphs were drawn using graph pad version 8

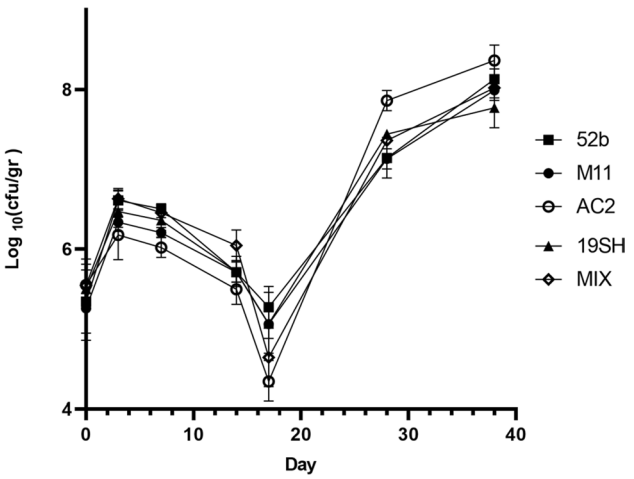

Fig. 5 Establishment of probiotic strains at feeding intervals. There was no significant difference among the fed groups at the same days; *P* value of groups was observed, respectively ($0^{th} = 0.079$, $3^{rd} = 0.072$, $7^{th} = 0.068$, $14^{th} = 0.122$, $17^{th} = 0.081$, $28^{th} = 0.076$, $38^{th} = 0.068$), but there was significant difference in each fed groups at specific days ($P = 0.005$), All groups of mice were assessed by non-parametric test. The comparison of fed groups at Fecal sampling days was made by Kruskal–Wallis test, and to compare the effect before and after the bacteria fed at specific days in each group was used by Wilcoxon-signed ranks test. The median ± SEM is used to represent continuous variables (standard error of the median). The statistical software (IBM SPSS, Armonk, NY, version 23.0) was used to evaluate the data, and the significance threshold was set at *P* 0.05. Graph pad version 8 was used to create the graphs

($P = 0.005$) from the 3rd to the 7^{th} and from the 3^{rd} to the 14^{th} day, respectively. Although this reduction continued until the 38th day, it was not significant at 95% confidence level. There was no significant difference among the fed groups at the same days, *P* values of the groups were, respectively ($0^{th} = 0.068$, $3^{rd} = 0.072$, $7^{th} = 0.072$, $14^{th} = 0.067$, $28^{th} = 0.068$, $38^{th} = 0.068$) (Fig. 6B).

Coliforms are a group of Gram-negative bacteria, which generally belong to four genera of *Enterobacteriaceae*, namely *Citrobacter freundii*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *E. coli*, and *Klebsiella pneumonia*. Among these, *E. coli* is the most famous one on which the effects of different probiotic bacteria differ [30].

Given the statistical analysis, the coliform counts of the fecal samples of the mice fed with 19SH and 52b, significantly decreased from the 3rd to the 14th day and for AC2, the response significantly ($P = 0.005$) decreased from the 3^{rd} to the 7^{th} day. They also lowered until the 38^{th} day although the reduction was significant ($P = 0.005$). Moreover, the population of the fecal coliforms of the mice supplemented with M11 and MIX significantly ($P = 0.005$) decreased from the 0th to the 14th day. This decrease was continued at a constant rate until the 38th day. There was no significant difference among the fed groups at the same days. *P* values of the groups were, respectively ($0^{th} = 0.068$, $3^{rd} = 0.061$, $7^{th} = 0.068$, $14^{th} = 0.067$, $28^{th} = 0.068$, $38^{th} = 0.067$) (Fig. 6C).

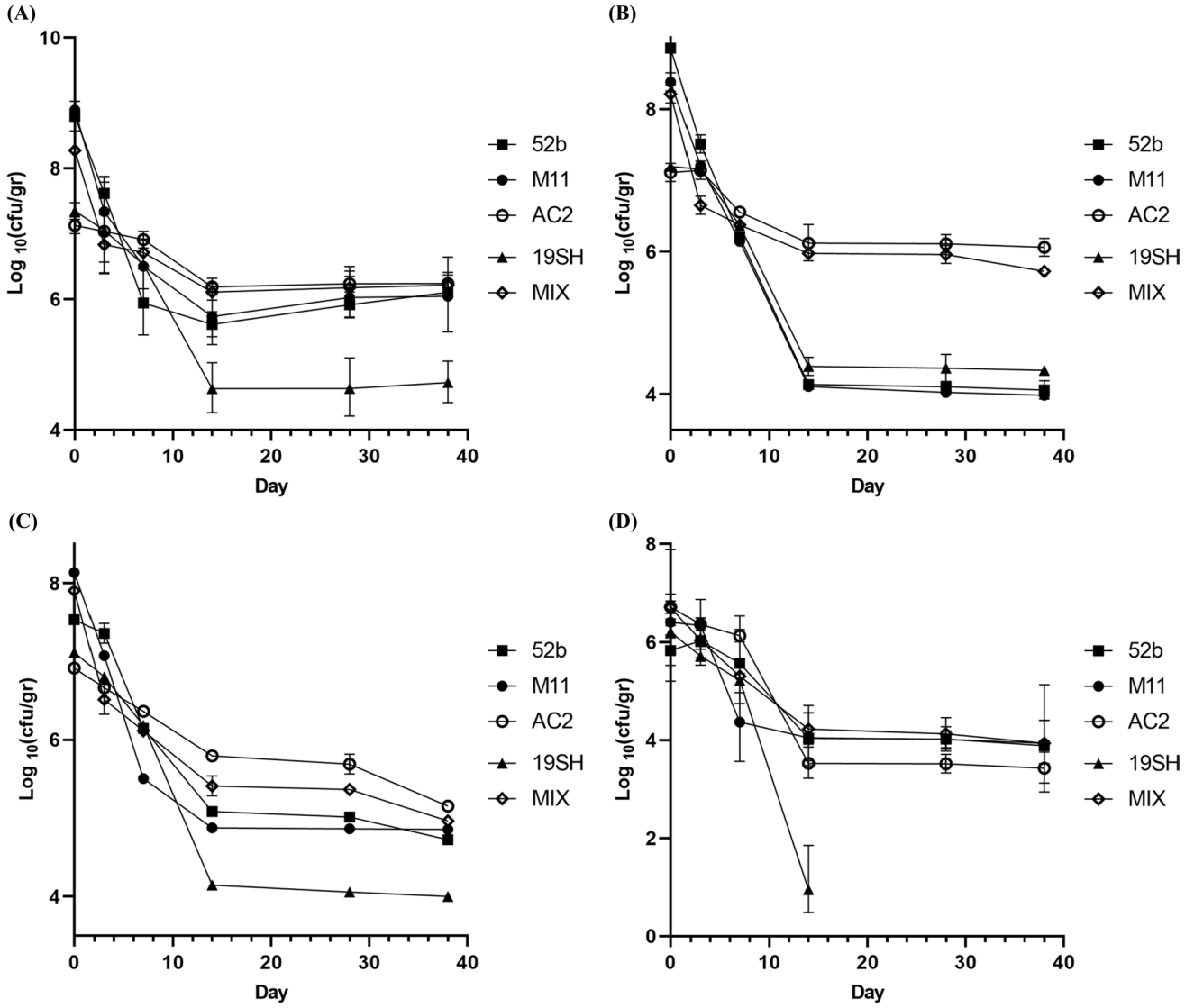

Fig. 6 Effect of administered probiotic strains on intestinal microbiota of BALB/C mice model using. (PCA) (**A**)**.** There was no significant difference among the fed groups at the same days; *P* value of the groups was observed, respectively ($0^{th}=0.068$, $3^{rd}=0.079$, $7^{th}=0.079$, $14^{th}=0.068$, $28^{th}=0.068$, $38^{th}=0.072$), but there was significant difference in each fed groups at specific days ($P=0.005$). (TSA, Gram-negative bacteria) (**B**)**.** There was no significant difference among the fed groups at the same days; *P* values of the groups were, respectively ($0^{th}=0.068$, $3^{rd}=0.072$, $7^{th}=0.072$, $14^{th}=0.067$, $28^{th}=0.068$, $38^{th}=0.068$), but there was significant difference in each fed groups at specific days ($P=0.005$). (MCA, coliforms) (**C**). There was no significant difference among the fed groups at the same days. *P* values of the groups were, respectively ($0^{th}=0.068$, $3^{rd}=0.061$, $7^{th}=0.068$, $14^{th}=0.067$, $28^{th}=0.068$, $38^{th}=0.067$), but there was significant difference in each fed groups at specific days ($P=0.005$). (*SS*A, *Salmonella* and *Shigella* bacteria (**D**). There was no significant difference among the fed groups at the same days; *P* values of the groups were, respectively ($0^{th}=0.079$, $3^{rd}=0.094$, $7^{th}=0.068$, $14^{th}=0.079$, $28^{th}=0.077$, $38^{th}=0.119$), but, there was significant difference in each fed groups at specific days ($P=0.005$). All groups of mice were assessed by non-parametric test. The comparison of the fed groups at fecal sampling days was made by Kruskal–Wallis test, and to compare the effect before and after the bacteria fed at specific days in each group was used by Wilcoxon-signed ranks test. The median ± SEM is used to represent continuous variables (standard error of the median). The statistical software (IBM SPSS, Armonk, NY, version 23.0) was used to evaluate the data, and the significance threshold was set at $P<0.05$. Graph pad version 8 was used to create the graphs

Similar to *Salmonella* and *Yersinia*, *Shigella* is generally categorized as a lactose-negative pathogen. On the other hand, considering DNA homology, *Shigella* and *E. coli* closely resemble each other, and some researchers believe that *Shigella* is subspecies of *E. coli* [31]. Based on the statistical analysis, 52b brought about the populations of *Salmonella* and *Shigella* to decrease significantly ($P=0.005$) in the mice intestine on the 14^{th} day. After the oral administration of M11 to the mice, it was revealed that this strain could reduce the *Salmonella* and *Shigella* counts

from the 3rd to the 38th day significantly ($P=0.005$). Supplementation with AC2 and MIX had the populations of *Salmonella* and *Shigella* significantly ($P=0.005$) decrease in the mice intestine from the 7th to the 14th day, which proceeded at a constant rate until the 38th day. 19SH could also lower the number of *Salmonella* and *Shigella* in the mice intestine from the 7th to the 14th day and reduce it to zero on the 28th day. There was no significant difference among the fed groups at the same days; *P* values of the groups were, respectively ($0^{th}=0.079$, $3^{rd}=0.094$, $7^{th}=0.068$, $14^{th}=0.079$, $28^{th}=0.077$, $38^{th}=0.119$) (Fig. 6D). Overall, 19SH produced the greatest effect on the populations of *Salmonella* and *Shigella* in the mice intestine.

Discussion

According to the obtained results, most of the tested strains could tolerate the acidic conditions (pH = 2.5) in vitro, except *L. plantarum* subsp. *plantarum* ATCC 14,917, *L. gasseri* ATCC 33,323 and 19SH. M11 (*L. plantarum* isolated from milk), and 52b (*L. gasseri* isolated form healthy woman vagina) were the most resistant strains to low pH. The high tolerance of M11, as the most compatible *Lactobacillus* species, could be due to its large genome, capability of metabolizing different carbon sources and growth ability. Similarly, *L. gasseri* is usually found in the human digestive system and often in breast milk, which can easily tolerate the stomach acidic conditions [32]. In addition, it can be stated that commercial stains are so weak that cannot tolerate acid and bile stresses, while native strains can easily do [33].

All the strains showed a high resistance to bile salts. Resistance to low pH and bile salts ensures the successful passage of probiotics through the stomach and their establishment in the intestine. Cell wall integrity and biochemical structure play key roles in protecting a strain against stresses. Therefore, the difference in the tolerance of strains to acidic and bile stresses originates from the difference between their cell walls [34].

Of the 8 tested strains, only 19SH and *L. gasseri* ATCC 33,323 could successfully survive the simulated gastric juice (pepsin, pH = 2.5), and the other strains did not grow at all after 4 h of incubation. Rushdy and Gomaa [35] declared that the resistance of lactobacilli to gastric juice was associated with the activity of hydrogen ATPase pump, the cell membrane compounds, type of bacteria, type of the culture medium, and incubation conditions.

All the strains, except M11 and *L. acidophilus* ATCC 4356, were highly resistant to the simulated intestinal juice (pancreatin, pH = 8). The contents of the digestive juices (gastric and intestinal) vary depending on the time they stay in the GI tract, the GI tract length, and the host. At the same time, the conditions simulated in this research could not probably represent these variations perfectly. Sometimes, some foods can also help the bacteria survive in human body. Such variations were not taken into account in our simulated digestive system in vitro.

52b and its corresponding standard strain had high surface hydrophobicity, whereas that of M11 and its corresponding standard strain was at the medium level. Surface properties, including auto-aggregation and hydrophobicity, are also regarded as a measure of adhesion to cell monolayers. Some researchers have discovered a correlation between the surface hydrophobicity and adhesion ability of probiotic bacteria [15].

Barinov et al. [36] examined the genomes of 12 *Lactobacillus* species and found out that the cell surface proteins, including the membrane ones, accounted for a considerable fraction (19.9–29.3%) of the total protein content. In other words, the genomes of the members of the *L. acidophilus* group (*L. acidophilus*, *Lactobacillus Johnsonii*, and *L. gasseri*), which are often utilized as probiotics, encoded a large number of proteins containing LPXTG-motif (Leu-Pro-any-Thr-Gly). The results demonstrated that half of these proteins were capable of adhering to mucus. Cell wall-anchored proteins are attached to the cell wall either covalently by sortases (LPXTG proteins) or non-covalently (through a LysM motif or cell wall-binding domains). In addition, other researchers claimed that *L. plantarum* WCFS-1 could adhere to mucus through LPXTG proteins [37].

The largest amount of auto-aggregation respectively belonged to 19SH, *L. plantarum* subsp. *plantarum* ATCC 14,917, M11, AC2, and *L. fermentum* ATCC 9338. Moreover, the highest percentage of co-aggregation was related to AC2 (*L. monocytogenes* (85%), *S. enteritidis* (100%) and *E. coli* (38.73%)) followed by 19SH (*L. monocytogenes* (93%), *S. enteritidis* (51%), and *E. coli* (28%)) and *L. plantarum* subsp. *plantarum* ATCC 14,917 (*L. monocytogenes* (92%), *S. enteritidis* (42%), and *E. coli* (31%)) which were significantly different from the other strains at $P<0.05$ (Figs. 3 and 4).

Scientists believe that there is a correlation between auto- and co-aggregation abilities. Campana et al. [13] indicated that the co-aggregation ability of probiotic LAB with pathogens was associated with the competition for adhering to the surface of the intestinal epithelial cells. This relationship could be ascribed to the presence of some specific molecules on the surface of the membrane of probiotic LAB, which act either as ligands and bind with pathogens or as an adhesive and cause the strains to adhere to the intestinal epithelial cells. In the present study, the strains 19SH and AC2 which had high auto-aggregation ability also had the highest co-aggregation ability in vitro and in vivo. However, in the case of the strains 52b and M11 which had high auto-aggregation ability in vitro, it was proved that they had high

co-aggregation ability with Gram-negative pathogens and coliforms in vivo.

In the antibiotic susceptibility test, each strain was resistant to a specific antibiotic, except for AC2 which was susceptible to all the antibiotics. It cannot be declared that antibiotic tolerance is a remarkable characteristic of probiotics, because, according to the literature, the probiotics which resist different classes of antibiotics including glycopeptides (vancomycin), aminoglycosides (streptomycin and gentamycin), monoketates (aztreonam), and fluoroquinolones (ciprofloxacin) raise concerns as it has been proven that such strains transfer antibiotic-resistant genes to the commensal microbiota [13, 38].

All the above-mentioned results, pertaining to the evaluation of probiotic properties, were obtained in vitro, and it was concluded that the native strains tested were all probiotic. It is difficult to find a strain possessing the whole probiotic properties. Consequently, the criteria for selecting potential probiotic candidates depend on the product purpose. In this research, the native strains were selected for mice feeding to investigate their effects on the intestinal microbiota.

PCA is bacteriological culture medium which is used to determine the total count of live aerobic bacteria in a sample. The total bacteria count of the fecal samples of all the mouse groups decreased after the 3rd day of administration. This revealed that the population of the other tested bacteria was reduced on the 3rd day. In fact, the intestinal Gram-negative bacteria, coliforms, and *Salmonella* and *Shigella* counts decreased on the 3rd day. These reductions in the population of the intestinal bacteria caused the total bacteria count to decrease significantly ($P = 0.005$) in PCA on the 3rd day. However, the strains administered at certain intervals were the main reason behind this decrease. By counting the colonies of the fecal samples on the 3rd day of administration, it was understood that the number of all the LAB present in the mice feces increased and concluded that the fed strains were established on the 3rd day (Figs. 5 and 6A). Colonization on the intestine wall is a desirable feature of probiotic bacteria. As a result, the ability to adhere to the intestinal epithelial cells (known as the prerequisite for colonization) is considered an important criterion for choosing the probiotic bacteria [39].

Teichoic acid and peptidoglycan are also very vital molecules, as the host immune cells detect them. The surface of Gram-positive bacterial cells is composed of a thick peptidoglycan layer, several polysaccharides (such as teichoic acid and lipoteichoic acid) and numerous cell-surface proteins (including the S layer proteins). These compounds are actually the major constituents of the cell-surface structures of bacteria. These surface structures are in direct contact with the cell environment, and the cell-surface proteins participate in a variety of physiological processes. Moreover, adhesion factors, antigens, enzyme receptors, and transporters are the other proteins present on the surface of bacterial cells. Lipoteichoic acid also acts as an adhesive molecule and adheres to the intestinal epithelial cells. Consequently, the difference in the adhesion of probiotics to these cells originates from the difference in the structure of their lipoteichoic acid molecules [40, 41].

Considering Fig. 5, after the 6-day stoppage of administration, only *L. gasseri* (52b) had the highest adhesion compared to other fed groups on the 17th day. Moreover, based on the results of surface hydrophobicity which were positively correlated with those of the adhesion ability, 52b had the highest percentage of adhesion.

Additionally, based on the results of surface hydrophobicity which were positively correlated with those of the adhesion ability, 52b had the highest percentage of adhesion. Shiraishi et al. [42] investigated the cell wall of *L. gasseri* JCM 1131 and understood that Hex4DAG and acyl-Hex4DAG present in the hook-like part of the glycolipid moiety of lipoteichoic acid were involved in a different type of interaction between the host and the bacterial cell. They also deduced that this difference was due to the modulation of the host immune system. This confirms the high adhesion ability of 52b in the present study. As the population of probiotic bacteria increases in the intestine, they produce antimicrobial compounds including short-chain fatty acids such as propionic acid, acetic acid, valeric acid, diacetyl, and bacteriocins which are antimicrobial proteins and lethal to the bacteria that are in the same family as the generating strain. Bacteriocins can easily be decomposed by proteolytic enzymes, especially the proteases of mammals` digestive system [43].

As can be seen in Fig. 5, the LAB count was lowered from the 3rd to the 11th day, which was probably owing to the presence of antimicrobial agents, in particular bacteriocins, in the intestine. As the administration proceeded at 10^9 cfu/ml, the number of the LAB strains was significantly elevated in the mice feces on the 28th day ($P = 0.005$). Therefore, the total bacteria count was raised from the 14th to the 28th day (Fig. 6A).

In parallel with the reduction in the LAB count from the 3rd to the 14th day of feeding, the pathogens also experienced a considerable decrease. In the case of the effects of the administered bacteria on the Gram-negative ones and coliforms, which were examined using TSA and MCA, it can be said that all the fed strains produced decreasing effects on the intestinal pathogens, with M11 and MIX having the most substantial impacts such that these reductions became significant from the 0th to the 14th day ($P = 0.005$) (Fig. 6B, C). Additionally, in the case of the intestinal *Salmonella* and *Shigella*, it can be cited that all the administered bacteria caused them to be reduced until the 14th day (Fig. 6D), with 19SH having the greatest effect. Overall, 19SH, 52b, and

M11 had the most profound effects on reducing the Gram-negative bacteria count, while 19SH produced the greatest effect on decreasing the intestinal coliform, *Salmonella* and *Shigella* counts.

The high co-aggregation ability of 19SH probably enabled it to reduce the population of *Salmonella Shigella*, coliforms, and Gram-negative bacteria until the 14th day. It should be noted that co-aggregation is an effective mechanism through which probiotics prevent pathogens from adhering to the intestinal epithelial cells. The co-aggregation ability of probiotic LAB with pathogens is related to their capability of adhering to the intestinal epithelial cells, and this relationship depends on the presence of some specific molecules (LysM proteins, CWBD proteins, LPXTG proteins, etc.) on the surface of the membrane of the probiotic LAB. Such molecules act either as ligands and bind with pathogens or as an adhesive and bring about the strains to adhere to the intestinal epithelial cells [37].

In addition to the co-aggregation ability with pathogens, production of bacteriocins, short-chain fatty acids, diacetyl, etc. by the probiotic bacteria plays a role in destructing the pathogens.

The antimicrobial nature of bacteriocins has been exploited to control the pathogenic microbiota in poultry. Ogunbanwo et al. [44] proposed that plantaricin (isolated from *Lactobacillus plantarum* F1) could be used as a viable alternative to antibiotics against colibacillosis in broiler Chickens.

Likewise, the production of plantaricin may be the reason why M11 could faster reduce the number of the intestinal Gram-negative bacteria and coliforms, relative to the other administered strains. Furthermore, it can be declared that the presence of M11 in MIX resulted in the production of the same effect on the pathogens.

Plantaricin CLP29 and enterocin CLE34 have dramatic antimicrobial effects on *Salmonella* pullorum and *E. coli* [45]. Kahouli et al. [46] and Wang et al. [47] investigated the fatty acids produced by *L. fermentum* NCIMB 5221 and *L. acidophilus* RD758 isolated from fermented foods. They showed that *L. fermentum* NCIMB 5221 was able to produce ferulic acid as an antioxidant compound which inhibited the growth of cancer cells in colon and brought about the natural growth of the intestinal epithelial cells by generating short-chain fatty acids.

Acetic acid is the major short-chain fatty acid in the large intestine and accounts for more than half of the total short-chain fatty acid content in feces. pH reduction is one the major roles of short-chain fatty acids, which in turn restrains the growth of pathogens and raises the absorption of some nutrients. Moreover, compounds like butyrate can alter the intestinal epithelial cells and increase the mucus production, leading to the improved adhesion of bacteria [48]. In this study, 19SH and AC2 had the intestinal epithelial cells grow and destroyed the pathogens, probably by producing short-chain fatty acids [46, 47].

In conclusion, the results of this research showed that the native strains of *L. gasseri* (52b), *L. plantarum* (M11), *L. fermentum* (19SH), and *L. acidophilus* (AC2) had probiotic properties and were much stronger than their corresponding standard strains in this respect. The probiotic properties of the native strains, including adhesion, auto-, and co-aggregation in vitro, were in agreement with the corresponding ones in vivo. 52b had the highest adhesion ability both in vitro and in vivo, and 19SH had the most co-aggregation with *L. monocytogenes*, *S. enteritidis* and *E. coli* in vitro. This strain also produced the most profound decreasing effect on the mice intestinal microbiota and pathogens in vivo. In general, the membrane proteins and lipoteichoic acid of LAB act as adhesive molecules and adhere to the intestinal epithelial cells. The difference in the adhesion ability of various LAB can be attributed to the amount of D-alanine, the replacement ratio of the repeating glycero-phosphate (GroP) unit, and the percentage of the hexoses present in the glycerolipid anchor of their cell walls. As a result, adhesion to the mucus surface is a prerequisite for the colonization of bacteria in the GI tract and considered a competitive advantage for microorganisms in that ecosystem. Therefore, the host immune cells identify them, resulting in the reinforcement of the host immune system and health promotion in humans and animals. In conclusion, 52b, M11, 19SH, and AC2 are the best candidates for the investigation of the cell wall and its effect on the host immune system.

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Declarations

Ethics approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This study involves animal testing (mice). The ethical criteria with the code of IR.UM.REC.1400.004 (Ferdowsi University of Mashhad, Iran) were observed.

Consent for publication All authors consent for publication.

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