Research Article

APPLIED FOOD BIOTECHNOLOGY, 2021, 8 (3): 201-212
Journal homepage: www.journals.sbmu.ac.ir/afb

pISSN: 2345-5357
eISSN: 2423-4214

Characterization of Effective Native Lactic Acid Bacteria as Potential Oral Probiotics on Growth Inhibition of *Streptococcus mutans*

Maryam Golshahi[1], Mohammad Mehdi Pirnia[2], Parvaneh Jafari[3], Elahe Ebrahimi[4], Farzaneh Tafvizi[5], Mahsa Dameshghian[6], Maryam Tajabadi Ebrahimi[6*]

1. Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran
2. Department of Biophysics, Institute of Biochemistry and Biophysics Researches, University of Tehran, Tehran, Iran
3. Department of Microbiology, Faculty of Science, Arak Branch, Islamic Azad University, Arak, Iran
4. Microbiology Department, Islamic Azad University of Pharmaceutical sciences Branch, Tehran, Iran
5. Department of Biology, Parand Branch, Islamic Azad University, Parand, Iran
6. Department of Biology, Central Tehran Branch, Islamic Azad University, Tehran, Iran

Abstract

Background and Objective: Probiotics' effects on harmful oral bacteria have been verifed. As antibiotic resistance becomes a major problem, searching for novel potential species is important. The objective of this study was to select novel safe strains of lactic acid bacteria with potentials as oral probiotics. Furthermore, ability of these strains to suppress growth and attachment of *Streptococcus mutans* as the most important cariogenic bacteria in tooth decay was investigated.

Material and Methods: Initial identification tests, including Gram staining and catalase and oxidase tests, were carried out on 22 strains of lactic acid bacteria isolated from Iranian traditional dairy products. Safety of the strains was assessed using hemolysis test and antibiotic resistance assessment. Strains were then assessed for probiotic characteristics such inhibition of *Streptococcus mutans* growth, tolerance to lysozyme enzymes and ability of adhesion as well as ability of decreasing *Streptococcus mutans* adhesion. Selected strains were identified using16S rRNA molecular method.

Results and Conclusion: Of all strains, four strains with the optimal probiotic characteristics were selected. These included one *Lactobacillus brevis*, one *Lactobacillus casei* and two *Lactobacillus paraceasei*. These four strains showed strong antimicrobial characteristics against *Streptococcus mutans*, were resistant to oral lysozyme enzymes and included high adhesion abilities to polystyrene wells. Furthermore, they decreased *Streptococcus mutans* attachment; thus, biofilm formation by this bacterium was prevented. These strains were recognized as safe strains since they were approved in assessments of antibiotic susceptibility and hemolytic activity. Therefore, these four strains are suggested as oral probiotics.

Conflict of interest: The authors declare no conflict of interest.

Article Information

Article history:
Received 12 Jan 2021
Revised 5 Feb 2021
Accepted 14 April 2021

Keywords:
- lactic acid bacteria
- oral probiotics
- safety assessment
- *Streptococcus mutans*
- tooth decay

***Corresponding author:**
Maryam Tajabadi Ebrahimi,
Department of Biology, Central Tehran Branch, Islamic Azad University, Tehran, Iran

Tel: +98-21-44600196
E-mail:
m.tajabadi@iauctb.ac.ir

How to cite this article
Golshahi M, Pirnia MM, Jafari P, Ebrahimi E, Tafvizi F, Dameshghian M, Tajabadi Ebrahimi M. Characterization of Effective Native Lactic Acid Bacteria as Potential oral Probiotics on Growth Inhibition of Streptococcus mutans. Appl Food Biotechnol. 2021; 8(3): 201-212.
http://dx.doi.org/10.22037/afb.v8i3.33704

1. Introduction

Tooth decay is one of the most common oral infectious diseases; in which, acid produced by the bacteria in fermentation of carbohydrates destructs tooth enamel and structure [1]. When food and sweet drinks are consumed regularly, acidogenic bacteria increase in the oral cavity. Over time, these changes interfere with the balance of degradation and restoration of enamel, resulting in dental caries [2]. To control risk factors of dental caries, changes in diets such as decreases in consumption of sweets and increases in host resistance are recommended; however, complete elimination of caries associated microorganisms is difficult and almost impossible [2]. In the mouth, a diverse

population of nearly 1000 bacterial species exists on the tongue, teeth, gums and inner cheeks. Streptococci consist nearly 20% of this population. Naturally, oral health is affected by the oral bacteria as well as age, health, nutrition and lifestyle of the individuals [3]. Several studies have reported the beneficial effects of probiotics, including increased immune responses and treatment or prevention of infectious diseases of the genital and respiratory system. Furthermore, allergies and atopic diseases in children, who use probiotic productions are apparently diminished [4]. It has been shown that probiotic bacteria such as *Lactobacillus* spp. and *Bifidobacterium* spp. are capable of good colonization in digestive system of humans. Recent studies on the bacteria of oral and dental infections indicate their roles in increasing systemic diseases such as diabetes, respiratory diseases and cardiovascular diseases (CVD). This illustrates importance of oral and especially teeth health [4]. *Lactobacillus* spp. isolated from healthy teeth can decrease growth of *Streptococcus* (*S.*) *mutans in vitro*. Furthermore, combination of microbiota varies between the people with periodontitis and those with healthy conditions [4]. Probiotic microorganisms should be able to withstand oral and peripheral environmental conditions and grow in saliva-coated surfaces [5]. Major probiotic mechanisms for controlling oral and dental diseases include enhancing adhesion to target mucosa and inhibiting pathogenic adhesion, eliminating competitiveness of pathogens, producing antimicrobial agents and balancing immune system [6]. Every strain includes various effects on people because of especial microflora and antibiotic resistance among bacteria is growing; therefore, this the most logical way to investigate novel safe species with probiotic characteristics. Regarding problems and failures to produce effective vaccines, probiotic bacteria that can attach to enamels seem appropriate for the prevention of dental caries. In general, efficacy of probiotic strains for oral use is characterized by the ability to produce antimicrobial metabolites against cariogenic bacteria and tolerance against oral lysozyme enzymes. A probiotic strain that is effective against decay should be able to adhere to tooth surfaces, where decay bacterial agents are located. The probiotic strain then must be able to occupy binding sites and form biofilms in competition with the pathogens to decrease their implant-tation. In this study, oral probiotic characteristics of novel probiotic strains were investigated. These characteristics included safety (non-hemolysis and susceptibility to antibiotics), production of antimicrobial substances, resistance to lysozyme enzyme effective adherence and decrease of *S. mutans* implantation.

2. Materials and Methods

2-1- Bacterial strains

In this study, 22 strains of lactic acid bacteria (LAB) have been used. All strains were previously isolated by Tajabadi Ebrahimi from Iranian traditional dairy products, including various yogurts, cheeses, fermented milks, Dooghs, Kashks and Tarkhinehs (Iranian traditional foods) [7,8]. Bacterial strain of *S. mutans* PTCC1683 was provided by the Scientific and Industrial Research Center, Tehran, Iran.

2-2- Biochemical Tests

Bacteria were inoculated into 50 ml of MRS broth (Merck, Germany) and incubated at 37 °C for 48 h under 5% atmospheric CO_2. To ensure purity of the bacteria and their appearance investigation, bacteria were cultured in MRS agar (Merck, Germany) plates and incubated for 48 h at similar conditions. Colonies were investigated and then Gram staining and oxidase and catalase tests were carried out [9,10]. Lyophilized cultures of these bacteria were inoculated into nutrient broth (QueLab-393506, QueLab, Canada) and incubated at 37 °C for 24 h.

2-2- Safety assessment test

Safety of the probiotic strains was assessed based on the World health Organization (WHO) guidelines as follows [11].

2-2-1- Hemolysis test

To investigate ability of hemolysin production, strains were cultured in nutrient agar (QueLab-393506, QueLab, Canada) containing 5% of fresh defibrinated sheep blood. Plates were incubated at 37 °C for 24-48 h. Then, plates were studied for hemolytic ability. *Staphylococcus aureus* PTCC 1431 was used as positive control with the ability of producing β-hemolysins [12].

2-2-2- Antibiotic resistance assessment

Antibiotic resistance patterns of the strains were assessed based on the Clinical and Laboratory Standards Institutes (CLSI) guidelines [13-15]. Antibiotics included the most commonly used ones in clinical treatments of oral cavity (16-18). Briefly, antibiotic disks of erythromycin, gentamicin, vancomycin, azithromycin, tetracycline, clindamycin, ampicillin, chloramphenicol and ciprofloxacin (Mast, UK) were used via disk diffusion method [19]. Overnight culture suspensions of bacteria with 0.5 McFarland concentrations were prepared and 100 µl of the suspensions were spread on MRS agar plates uniformly. Generally, MRS agar is a better medium for the growth of LAB, compared to Mueller-Hinton media. Studies have shown poor irregular growth of LAB on Mueller-Hinton media [20]. Then, antibiotic disks were placed on plates and incubated at 37 °C for 24 h. Inhibitory zones were measured using caliper and strains were classified based on the zone diameters [15] as sensitive (≥ 21 mm),intermediate (≥ 16 mm to ≤ 20 mm) and resistant (≤ 15 mm).

2-3- Assessment of the bacterial efficacy

2-3-1- Resistance to lysozyme

Probiotic strains in mouth should be resistant to oral lysozyme enzymes. Based on a method by Shukla et al., 5% of the overnight bacterial cultures with 0.5 McFarland concentrations were inoculated into MRS broth completed with 22 IU ml^{-1} lysozyme (EWL, EC 3.2.1.17; Sigma-Aldrich, Germany). A sample was inoculated into MRS broth as control with no lysozymes. The bacterial count was carried out after incubating at 30 °C for 0 and 2 h. A serial dilution of the bacterial suspension was prepared in PBS buffer and cultured in MRS media as cross-culture. After incubating, number of the live bacteria was counted. *Micrococcus luteus* ATCC 11454 was used as positive control [21].

2-3-2- Assessment of the bacterial ability to produce bacteriocins

Ability of the selected strains from the previous stages to produce bacteriocins against *S. mutans* was investigated using two methods of agar-well diffusion [22] and disk diffusion [23]. For the two methods, fresh cultures of *S. mutans* with 1% concentration were prepared and 100 µl of these cultures with 0.5 McFarland concentrations were spread on BHI agar (Merck, Germany) plates. In well diffusion method, 50 µl of each extract of lactobacilli were poured into 5-mm deep wells. In Disk diffusion method, 100 µl of each strain extract were added to blank disks in three steps. After 24 h incubation at 37 °C, diameters of the clear zones around each well/disk were measured. The third method, turbidimetry, was carried out to ensure correct selection of the strains [24]. Purpose of these three methods included non-repeatability of the well diffusion and disk diffusion methods. In turbidimetry method, 5% concentrations of the neutral extracts were used. The probiotic ability of decreasing growth of the indicator bacteria in BHI broth was assessed after 24 h of incubation. Spectrophotometer (Analytik Jena, model SPECORD 250, Analytik Jena, Germany) was used at 600 nm. Results were compared to results of a specimen with *S. mutans* and the media. Four strains of 6, 7, 21 and 22 were selected as superior strains.

2-3-3- Minimum inhibitory concentration and minimum bactericidal concentration assessments

Ten various concentrations of the selected *Lactobacillus* extracts from 1 to 10% with 1% of the indicator bacteria were prepared. After 24 h of incubation, the minimum inhibitory concentration (MIC) was reported. To indicate the minimum bactericidal concentration (MBC), various concentrations of lactobacilli extracts from MIC concentration to 11% were cultured on nutrient agar and incubated for 24 h. The minimum concentration of extracts; at which, no colonies grew was selected as the MBC [25].

2-3-4- Biofilm formation capacity of the acid lactic bacteria

Biofilm formation capacity of the selected strains was assessed based on a method by Tahmoures Pour et al. [21]. Standard suspensions of 0.5 McFarland with turbidity of 1.5×10^8 CFU ml^{-1} bacteria were prepared in trypticase soy broth (TSB)(Merck, Germany) supplemented with 1% of sucrose. Then, 250 µl of this suspension were transferred to the wells of a 96-well polystyrene plate. Culture media with no bacterial suspensions were used as controls. After 24 h, contents of the wells were removed and each well was washed three times with 300 µl of sterile physiology saline. After stabilizing with 250 µl of ethanol for 5 min, attached cells were stained using 200 µl of 2% crystalline violet dye. Then, quantitative biofilm formation assay was carried out by adding 200 µl of 33% acetic acid to each well. Crystal violet stain was dissolved in acid and assessed at 492 nm using ELISA reader (BioTek-ELX800, BioTek, USA). Classification of the isolates was carried out based on their optical absorption (OD) as follows: average optical absorption of the strain, OD; the mean optical absorption of the well, ODc; no binding, OD < ODc; poor binding, ODc < OD < 2OD; medium binding, 2ODc < OD < 4ODc; and strong binding, 4ODc < OD.

2-3-5- Prevention of Streptococcus mutans biofilm formation

In this assay, effects of *Lactobacillus* extracts on the binding of *S. mutans* were assessed using method by Tahmoures Pour et al. [21]. In this method, a mixture of similar volumes of *Lactobacillus* spp. and *S. mutans* was used. After 24 h of incubation, differences between the optical absorption of control wells (containing *S. mutans*) and the target wells (containing *Lactobacillus* spp. and *S. mutans*) was were assessed to calculate the probiotic prevention effects on the pathogen binding. Briefly, suspensions of 0.5 McFarland of *S. mutans* and probiotic strains were cultured in TSB culture media supplemented with 1% of sucrose. Then, 200 µl of a similar volume of the *Streptococcus* and probiotic suspension were transferred to microtiter plate wells. Suspensions of *S. mutans* or probiotic strain were separately pipetted to two control wells. After incubation for 24 h, a quantitative biofilm test was carried out using crystal violet colorimetric device.

2-4- Molecular identification of the lactic acid bacteria

The four selected strains were identified using 16s rRNA sequencing molecular identification method. First, DNA was extracted using extraction kit (MBST, Iran) Gram-positive bacteria. Quantitative and purity of the extracted DNA were assessed using NanoDrop (Thermo-Fisher Scientific, USA). The PCR reaction was optimized in a final volume of 25 µl, containing 10 µl of master mix buffer (Ampliqon, Denmark), 0.5 µl (0.4 mM) of specific primers

(F: 5´-GAGAGTTTGATCCTGGCTCAG-3´, R: 5´-CTACGGCTACCTTGTTACGA-3´) (Takapoozist, Iran) and 40 ng of the DNA. The PCR was carried out using Bio-Rad PCR System (Bio-Rad, USA) with the following conditions of initial denaturation at 94 °C for 2 min; then, 30 cycles of 94 °C for 60 s, 56 °C for 60 s and 72 °C for 60 s, and final extension at 72 °C for 5 min. To investigate genome amplification, 5 µl of the PCR products were electrophoresed on 2% agarose gels at 100 V (Bio-Rad, USA). Gels were visualized using gel documentation device (UVITECH, UK). Sequencing of the PCR products was carried out using Sanger method (Bioneer, South Korea). Complete sequences of the 16S rRNA genes were achieved using Vector NTI Software v.11 (Thermo Fisher Scientific, USA). To identify 16S rRNA gene sequences of the isolates, Blastn online tool of the NCBI BLAST database was used (www.ncbi.nlm.nih.gov/BLAST). Matching of the 16S rRNA gene sequences was carried out using ClustralW method (www.genome.jp/tools/clustalw/). The phylogenic tree was prepared using neighbor-joining (NJ) method and the bootstrap number was calculated with a replication of 1000 using MEGA Software v.4 (www.megasoftware.net) [26].

2-5- Statistical analysis

Graph Pad Prism Software v.6 (GraphPad, USA) was used for t-test and one-way ANOVA-test at the significance of 0.05 for the analysis of safety and efficacy test results.

3. Results and Discussion

3-1- Biochemical tests

Results of the biochemical tests showed that all 22 strains of Gram-positive, catalase-negative and oxidase-negative bacteria belonged to *Lactobacillus* spp.

3-2- Safety assessment of the strains

3-2-1- Hemolysis test

To investigate the safety of bacteria, hemolytic activity of the lactobacilli was assessed. Based on the test, no hemolytic activity was observed for α and β-hemolysis and all strains included negative hemolysis; therefore, strains did not destroy red blood cells (RBC) and were approved for the test.

3-2-2- Resistance to antibiotics

Table 1 presents the bacterial resistance patterns to various antibiotics based on diameters of the inhibition zones. Table 2 shows antibiotic resistance patterns of all the strains based on the inhibition zone diameters. Results of antibiotic resistance assessment showed that eight strains were resistant to antibiotics. Therefore, these strains were eliminated and the other strains were transferred to strain efficacy assessment. Antibiotics were categorized in various groups of effective on cell wall, protein synthesis and nucleic acid based on the CLSI standards for lactobacilli. Diameters of the non-growth zones were measured and classified into sensitive, moderate and resistant groups according to declared standard lactobacilli. In general, one strain from 22 strains was resistant to ampicillin (4%), one strain to azithromycin (4%), three strains to erythromycin (13%), four strains to vancomycin (18%) and two strains (9%) to tetracycline. Therefore, the most resistant pattern was seen to vancomycin. All strains were sensitive to ciprofloxacin, clindamycin and gentamicin. Resistance of *Lactobacillus* spp. to glycopeptide antibiotics such as vancomycin is often described as an inherent resistance and in most reports, *Lactobacillus* spp. have been resistant to vancomycin [24].

Table 1. The bacterial antibiotic resistance patterns (mm) based on the CSLI guidelines

Antibiotics	Resistant (R)	Intermediate (I)	Sensitive (S)
Ciprofloxacin (5 µg)	≤15	16-20	≥21
Erythromycin (15 µg)	≤15	16-20	≥21
Gentamicin (10 µg)	≤12	13-14	≥15
Vancomycin (30 µg)	≤9	10-11	≥12
Azithromycin (15 µg)	≤13	14-17	≥18
Tetracycline (30 µg)	≤18	19-22	≥23
Clindamycin (2 µg)	≤15	16-18	≥19
Ampicillin (10 µg)	≤12	13-15	≥16

In literatures, no suggestions have been seen that the bacterial resistance of vancomycin is transmitted. Resistance to vancomycin is due to the production of cell wall peptidoglycan precursors that end in D-alanine-D-lactate, amino acid residues that vancomycin is unable to bind [27]. Antibiotic susceptibility is highly various, depending on the strain characteristics. Use of gentamicin in livestock has caused resistance of enterococci, which depends on the presence of genes such as *aph* (2") or *aph* [2]-*aph* [6] transmitted by plasmids and transposons [27]. According to European Food Safety Authority (EFSA), antibiotic resistance of *L. paracaei* strain has not been documented and the strain is considered safe for livestock food production [21].

3-3- Assessment of the bacterial efficacy

3-3-1- Resistance to lysozyme

Investigating, *Lactobacillus* spp. were exposed to lysozyme enzyme.

Table 2. The bacterial antibiotic resistance patterns based on the diameter (mm) of the inhibition zones. (S) Sensitive, (I) intermediate and (R) resistant. Eight strains were eliminated due to the antibiotic resistance genes

Ciprofloxacin	Erythromycin	Gentamycin	Vancomycin	Azithromycin	Tetracycline	Clindamycin	Ampicillin	Chloramphenicol	Antibiotic Strain
22(S)	26(S)	15(S)	12(S)	19(S)	20(I)	19(S)	12(R)	21(S)	L1
26(S)	20(I)	15(S)	12(S)	18(S)	20(I)	20(S)	22(S)	21(S)	L2
21(S)	27(S)	15(S)	11(I)	18(S)	23(S)	20(S)	21(S)	23(S)	L3
25(S)	35(S)	16(S)	8(R)	18(S)	19(I)	20(S)	24(S)	21(S)	L4
25(S)	26(S)	16(S)	13(S)	19(S)	23(S)	20(S)	20(S)	22(S)	L5
23(S)	26(S)	17(S)	12(S)	20(S)	26(S)	21(S)	22(S)	21(S)	L6
21(S)	31(S)	15(S)	13(S)	19(S)	26(S)	19(S)	25(S)	21(S)	L7
23(S)	17(R)	16(S)	13(S)	9(R)	23(S)	20(S)	24(S)	14(R)	L8
21(S)	17(R)	15(S)	13(S)	18(S)	18(R)	20(S)	26(S)	23(S)	L9
21(S)	26(S)	15(S)	11(I)	18(S)	25(S)	21(S)	27(S)	15(R)	L10
25(S)	27(S)	16(S)	13(S)	19(S)	23(S)	20(S)	20(S)	21(S)	L11
23(S)	30(S)	15(S)	13(S)	15(I)	19(I)	20(S)	21(S)	23(S)	L12
21(S)	31(S)	15(S)	8(R)	16(I)	18(I)	19(S)	26(S)	24(S)	L13
21(S)	31(S)	17(S)	11(I)	19(S)	22(I)	20(S)	22(S)	23(S)	L14
25(S)	20(I)	16(S)	12(S)	20(S)	19(I)	21(S)	25(S)	21(S)	L15
23(S)	26(S)	15(S)	12(S)	20(S)	26(S)	21(S)	22(S)	21(S)	L16
21(S)	26(S)	16(S)	9(R)	18(S)	24(S)	21(S)	23(S)	14(R)	L17
21(S)	25(S)	17(S)	13(S)	18(S)	22(I)	20(S)	24(S)	21(S)	L18
23(S)	13(R)	17(S)	9(R)	19(S)	17(R)	20(S)	25(S)	22(S)	L19
21(S)	27(S)	16(S)	13(S)	20(S)	25(S)	19(S)	25(S)	23(S)	L20
25(S)	27(S)	17(S)	13(S)	20(S)	26(S)	21(S)	27(S)	23(S)	L21
23(S)	28(S)	16(S)	13(S)	19(S)	26(S)	21(S)	22(S)	21(S)	L22

After incubation, colony counts and optical absorption of the strains were compared with those of non-exposed strains. In general, number of colonies decreased significantly ($p < 0.05$), indicating the bacterial sensitivity to this enzyme. Result of Fig. 1 showed that strains nos. 3, 11, 12 and 20 were susceptible to lysozyme. Therefore, 12 strains were removed from the safety assessment and ten strains were used in the next assessment.

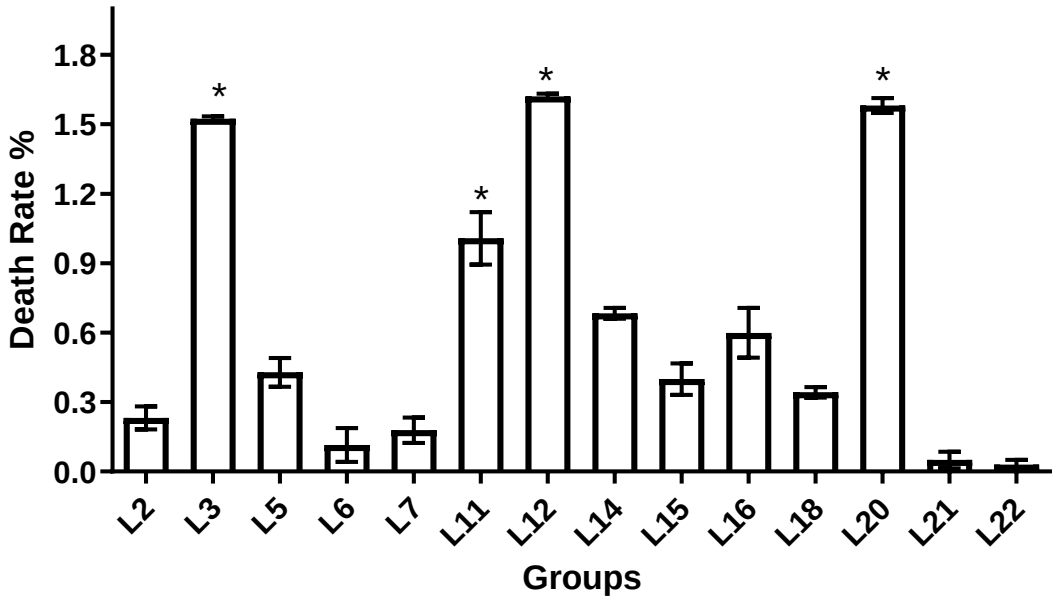

Figure 1. Resistance to lysozyme enzyme. Graphs comparing the percentages of decreases in colony numbers of the strains. Strain nos. 3, 11, 12 and 20 were susceptible to the lysozyme enzyme ($p < 0.05$)

No significant differences were seen between the numbers of decreased colonies in other strains, compared to non-exposed strains. Therefore, these strains were considered resistant. Koll et al. investigated characteristics of oral probiotics of *Lactobacillus* spp. Safety assessments, including antibiotic resistance and resistance to lysozyme enzymes, were carried out [18]. In general, results from the current study were similar to those results. In a study by Shukla et al. in 2010, oral lactobacilli were assessed for resistance to lysozyme [28]. Result supported results from the present study. In several studies, the major cause of sensitivity of *Lactobacillus* spp. to lysozyme was due to the pantothenic acid deficiency in the bacterial cell walls, which decreased synthesis of lipids, prevented normal absorption and maintenance of the extracellular amino acids and greatly increased sensitivity of the cells to lysozyme [29]. Bacteria are naturally susceptible to lysozyme in exponential phase, while they may be resistant to the enzyme in stationary phase [30].

3-3-2- Assessment of the bacterial ability to produce bacteriocins Disk diffusion, well diffusion and turbidimetry assays

Diameters of inhibition zones by ten *Lactobacillus* strains in disk diffusion and well diffusion methods are reported in Table 3. As shown in the table, four strains of 6, 7, 21 and 22 included the maximum inhibitory effects on growth of *S. mutans*. Data from turbidimetry of the ten strains are presented in Table 3. Results of this method supported results from the former methods that strains of 6, 7, 21 and 22 included the maximum effects on growth inhibition of *S. mutans*. Therefore, bacteriocins produced by these four bacteria are likely to affect this oral pathogen. Figure 2 shows inhibition zones for the selective strains in disk diffusion method.

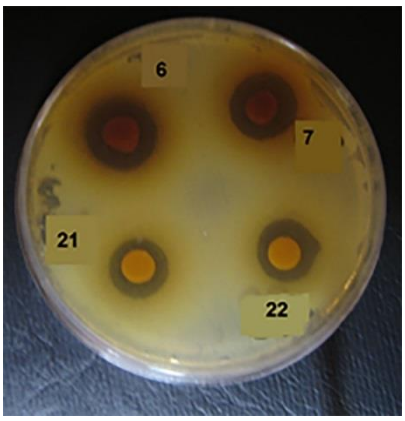

Figure 2. Inhibition zones for the four selective strains in disk diffusion method. Strains nos. 6, 7, 21 and 22 included the most inhibitory effects on growth of *S. mutans*

3-3-3- Minimum inhibitory concentration and minimum bactericidal concentration assessments

To investigate the minimum effective doses of lactobacilli, MIC and MBC were assessed. Results of MIC for strains of 6, 7, 21 and 22 included 70, 70, 80 and 60 µl/ml respectively and the MBC results included 100 µl/ml for all the strains. The MBC for strain no. 22 is shown in Figure 3.

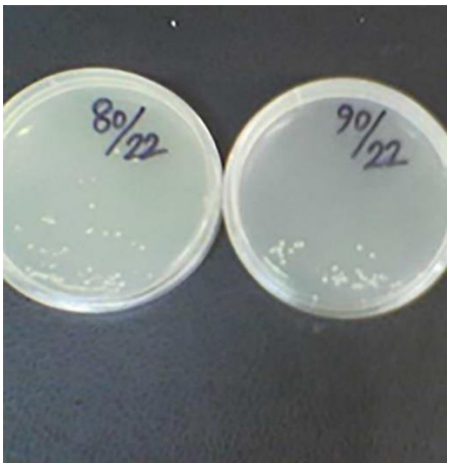

Figure 3. Concentration of 100 µl ml^{-1} was reported as MBC for strain no. 22

Effects of antimicrobial activity on *S. mutans* were assessed in Koll et al. study and most strains could inhibit growth of this cariogenic bacterium [18]. Teanpaisan et al. (2011) studied effects of 357 *Lactobacillus* strains on prevention of *S. mutans* growth using disk diffusion method [31]. Their results supported results from the current study. Biochemical identification and safety assessment were carried out for 22 strains. One of the characteristics of probiotics is the good adhesion [21].

Strains of 2, 5, 6, 7, 14, 15, 16, 18, 21 and 22, which were verified for safety and resistance to lysozyme, were assessed for the ability to adhere to the polystyrene plate and strains that were stronger for adherence were selected to assess their ability to decrease adherence of tooth decay bacteria.

3-3-4- Biofilm formation capacity of the acid lactic bacteria

The adherence ability of *Lactobacillus* spp. was assessed through a colorimetric method using optical absorption and ELISA reader. At this stage, bacterial ability to adhere was investigated using the highlighted equations [21]. Based on the optical absorption, results were analyzed using PRISM Software and t-test. Comparison of the strain adherence with the control sample adherence sample showed significant differences ($p < 0.05$) (Figure 4).

Table 3. Inhibition zone diameters (mm) from disk diffusion and well diffusion methods for ten *Lactobacillus* strains and results of turbidimetry method. Standard deviation for the three methods is shown in the last column

Strain No.	2	5	6	7	14	15	16	18	21	22	SD	Blank	Control
Disk diffusion	12	13	14	14	12	10	12	12	13	14	1.26		
Well diffusion	25	21	26	30	21	20	25	27	28	27	3.33		
Turbidimetry	.83	0.695	0.093	0.208	0.327	0.707	0.310	0.312	0.131	0.282	0.26	0	0.66

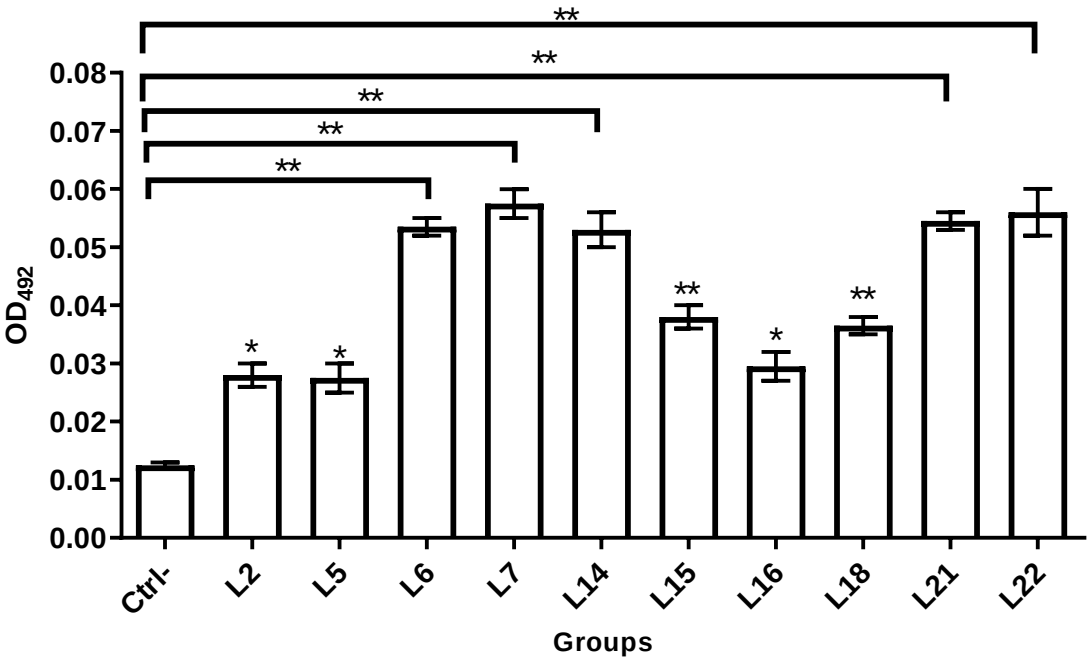

Figure 4. Comparison of strain adherence with the control adherence using optical absorption. Strains nos. 2, 5, 15, 16 and 18 included medium and strains nos. 6, 7, 14, 21 and 22 included strong adherences to polystyrene wells ($p < 0.05$)

Strains of 2, 5, 15, 16 and 18 included medium adherence and strains of 6, 7, 14, 21 and 22 included strong adherence to polystyrene wells. Therefore, strong-adherence strains were selected to assess their decrease ability of binding (forming biofilm) in pathogenic bacteria.

3-3-5- Prevention of Streptococcus mutans biofilm formation

In this experiment, effects of lactobacilli on the adherence of *S. mutans* were assessed. Results revealed decreases of binding in presence of *Lactobacillus* spp. Statistical analysis of the results of strain adherence comparison with control sample showed significant differences ($p < 0.05$). Figure 5 shows decreases in adherence of *S. mutans* by each strain of lactobacilli. Based on the results, strains of 7, 21, 22 and 6 included the most decreasing rate respectively and could inhibit the biofilm formation of *S. mutans*. In a study by Haukioja et al. in 2010, colonization potentials of various probiotics and dairy *Lactobacillus* spp. from feces were investigated. The researchers used microtiter wells covered with human saliva and hydroxyapatite. Results showed that adherence of *Lactobacillus* spp. to hydroxyapatite was much stronger than that of *Bifidobacterium* spp. [4]. Tahmores Pour et al. (2008) and Widyarman et al. (2019) used lactobacilli to decrease adherence of *S. mutance* and showed that these strains included antibiofilm activities against *S. mutans* [21,32]. Lactobacilli from the present study could inhibit biofilm formation of *S. mutans* by 20-40%. Therefore, results were similar to results by the highlighted researchers. Nowadays, Researchers search for bacteria that prevent spread of oral biofilms in sufficient competence with cariogenic bacteria. Furthermore, experts try to link specific bacteria to oral diseases. When appropriate probiotics with accurate clinical and epidemiological assessments are characterized, they can be used as a complementary method for the substitution of pathogenic bacteria with beneficial bacteria to create a healthy oral cavity and prevent dental and gum diseases [33].

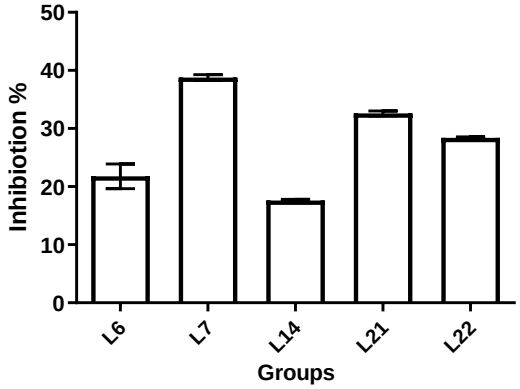

Figure 5. Results of decreases in adherence of *Streptococcus mutans* by *Lactobacillus* spp. Strains nos. 7, 21, 22 and 6 respectively included the most effects on growth decreases and could inhibit the biofilm formation of *S. mutans* ($p < 0.05$)

Dental decay induced from *S. mutans* is majorly attributed to three various factors. The *S. mutans* creates enduring colonies strengthened with polymeric structure of the extracellular matrix and boosted with significant quantities of glucan that are synthesized from environmental sucrose. This strain turns a vast variety of carbohydrates into organic acids through biochemical pathways, lowering pH of the environment (acidogenicity). High viability in stressful situations such as acidic environment (aciduricity) [34] is the last major characteristic of *S. mutans*, which makes the bacteria cariogenic. Establishing environments reach in polysaccharides with low pH in comparison with the natural oral cavity pH, *S. mutans* modifies the environment in benefit of other aciduric species to grow [35]. Since forming biofilms is the most important factor in development of oral caries and diseases, decreases of adherence can be effective in decreasing risks of dental caries [36]. It has been reported that *Lactobacillus* spp. of probiotic products are only colonized in the oral cavity if they are in contact with the oral media. However, most of these strains cannot maintain prolonged colonization of the teeth and oral cavity. This is the reason for the absence of subsequent tooth decay by these *Lactobacillus* strains that produce acid lactic [37].

3-4- Molecular identification

3-4-1- DNA extraction

Electrophoresis of the isolate PCR products (1500-bp amplicons) on 2% agarose gel is shown in Figure 6.

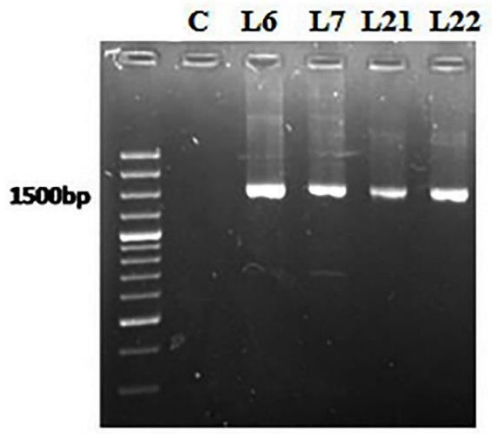

Figure 6. PCR products (~1500-bp amplicons) on 2% agarose gel resulted from the amplification of 16S rRNA genes of the isolates. (M) Molecular marker and (N) negative control

3-4-2- Sequencing results of 16S rRNA multiplied fragments

Four strains of L6, L7, L21 and L22 respectively recorded as TD3, T2, T16 and TD10 were recognized as safe strains with oral probiotic potentials. In this study, these strains were selected for molecular identification and phylogenetic analysis using sequencing method. Based on the phylogeny analysis, it is suggested that bacteria with 97% similarity in sequences of 16S rRNA genes belong to similar species [38]. After the sequencing, comparative comparison was carried out using NCBI Blastn and 100% similarity to *L. brevis* for the isolate of TD10, 99% similarity to *L. paracasei* for the isolates of TD3 and T16 and 99% similarity to *L. casei* for the isolate of T2 were reported. Thus, isolates belonged to *L. brevis*, *L. paracasei* and *L. casei* groups. Sequences of these strains were annotated in GenBank database with the accession numbers listed in Table 4. Figure 7 illustrates phylogenic relationships between the isolates and the reference bacteria from GenBank. In the phylogenic tree, two major clades of A and B were observed. Clade A included smaller clusters. The T2, T16 and TD3 isolates with 100% bootstraps were grouped in Clade A with other *L. casei* and *L. paracasei*. The TD10 isolate with 100% similarity was grouped in Clade B with other *L. brevis* isolates.

Table 4. The selected bacterial strains with their accession numbers in GenBank database

Strain	Accession no.
Lactobacillus brevis TD10	KP165838
Lactobacillus casei T2	KP165840
Lactobacillus paracasei TD3	KP165841
Lactobacillus paracasei T16	KP165842

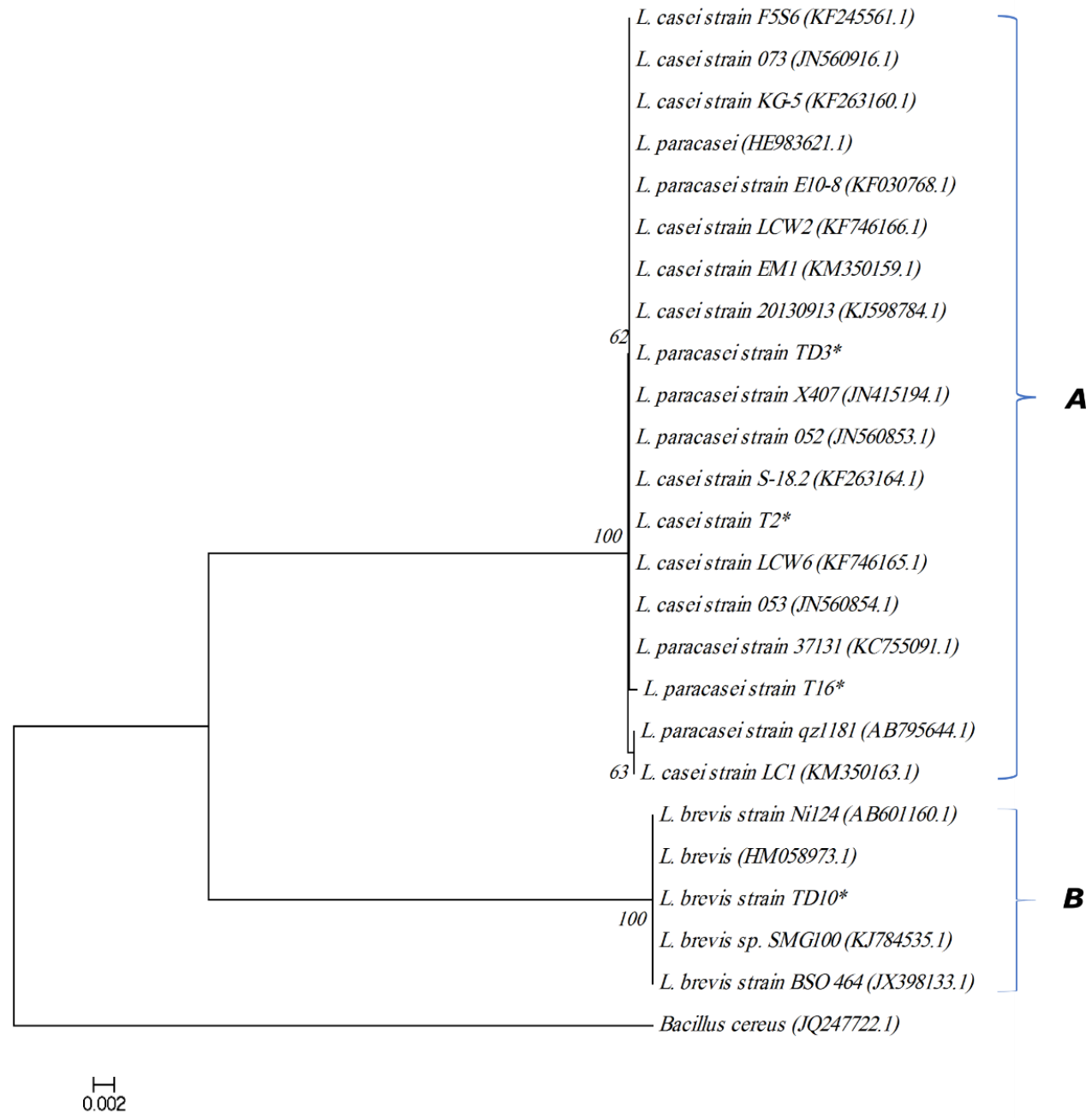

Figure 7. The phylogenic tree representing relationships between the 16S rRNA sequences of the bacterial isolates (marked by asterisks) and reference sequences in GenBank. Numbers in class nodes represent the bootstrap values (%)

4. Conclusion

The four selected *Lactobacillus* strains were assessed for antibiotic resistance and hemolytic activity. All strains were non-hemolytic and sensitive to the antibiotics. Effects of bacteriocins of these strains on *S. mutans* were stronger than those of other strains. They were investigated in terms of probiotic adherence profile. These lactobacilli had strong adherence and could inhibit residence and colonization of the *S. mutans*. Additionally, strains were resistant to oral lysozyme enzyme, that suggests they are suitable for oral cavity. These results suggest the present four Lactobacillus strains as potential probiotics. These bacteria can decrease risks of dental caries and other oral cavity diseases by affecting the binding process of *S. mutans* to the teeth, the most important factor in teeth diseases. The four strains identified as *L. casei*, *L. paracasei* and *L. brevis* using 16s rRNA sequencing molecular method.

5. Acknowledgements

The authors appreciate Miss. Maryam Fallahi for language edition.

6. Conflict of Interest

The authors report no conflicts of interest.

References

1. Yadav K, Prakash S. Dental caries: A review. Asian J Biomed Pharm Sci. 2016;6(53):01.

2. Amara A, Shibl A. Role of probiotics in health improvement, infection control and disease treatment and management. Saudi pharm J. 2015; 23(2): 107-14. doi: 10.1016/j.jsps.2013.07.001

3. Allaker RP, Stephen AS. Use of probiotics and oral Health. curr oral health rep. 2017; 4(4): 309-318. doi: 10.1007/s40496-017-0159-6

4. Haukioja A. Probiotics and oral health. Europ J dent. 2010;4(3):348. doi: 10.1055/s-0039-1697851

5. Rastogi P, Saini H, Dixit J, Singhal R. Probiotics and oral health. Nati J Maxillofac Surg. 2011; 2(1): 6-9 doi: 10.4103/0975-5950.85845

6. Bermudez-Brito M, Plaza-Diaz J, Munoz-Quezada S, Gomez-Llorente C, Gil A. Probiotic mechanisms of action. Ann Nutr Metab. 2012;61(2):160-174. doi: 10.1159/000342079

7. Tajaabady EM, Bahrami H, Ziyary Z. Tarkhineh source of probiotic lactic acid bacteria. J Anim Physiol Dev . 2011;4:1-8

8. Ebrahimi MT, Ouweh AC, Hejazi MA, Jafari P. Traditional Iranian dairy products: A source of potential probiotic lactobacilli. Afr J Microbiol Res. 2011; 5(1):20-7.

9. Garrity GM, Bell JA, Lilburn T. The Revised Road Map to the Manual. Bergey's Manual® of Systematic Bacteriology: Springer; 2005: 159-187.

10. Muyanja C, Narvhus JA, Treimo J, Langsrud T. Isolation, characterisation and identification of lactic acid bacteria from bushera: a Ugandan traditional fermented beverage. Int J Food Microbiol. 2003; 80(3): 201-210. doi: 10.1016/S0168-1605(02)00148-4

11. Ganguly NK, Bhattacharya SK, Sesikeran B, Nair GB, Ramakrishna BS, Sachdev HPS, Batish VK, Kanagasabapathy AS, Muthuswamy V, Kathuria SC, Katoch VM. ICMR-DBT guidelines for evaluation of probiotics in food. Indian J Med Res. 2011;134(1):22.

12. Pisano MB, Viale S, Conti S, Fadda ME, Deplano M, Melis MP, Deiana M, Cosentino S. Preliminary evaluation of probiotic properties of *Lactobacillus* strains isolated from Sardinian dairy products. BioMed Res Int. 2014; 2014:2-9. doi: 10.1155/2014/286390

13. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. CLSI supplement M100. 2017.

14. Vlkova E, Rada V, Popelarova P, Trojanova I, Killer J. Antimicrobial susceptibility of bifidobacteria isolated from gastrointestinal tract of calves. Livest Sci. 2006;105(1-3):253-9. doi: 10.1016/j.livsci.2006.04.011

15. Kaktcham PM, Zambou NF, Tchouanguep FM, El-Soda M, Choudhary MI. Antimicrobial and safety properties of lactobacilli isolated from two Cameroonian traditional fermented foods. Sci Pharm. 2012;80(1):189-204. doi: 10.3797/scipharm.1107-12

16. Angulo FJ, Collignon P, Powers JH, Chiller TM, Aidara-Kane A, Aarestrup FM. World Health Organization ranking of antimicrobials according to their importance in human medicine: A critical step for developing risk management strategies for the use of antimicrobials in food production animals. Clin Infec Dis. 2009;49(1):132-141. doi: 10.1086/599374

17. Korhonen JM, van Hoek AHAM, Saarela M, Huys G, Tosi L, Mayrhofer S, Von Wright A. Antimicrobial susceptibility of *Lactobacillus rhamnosus*. Beneficial Microbes. 2010; 1: 75-80 doi: 10.3920/BM2009.0002

18. Koll P, Mandar R, Marcotte H, Leibur E, Mikelsaar M, Hammarstrom L. Characterization of oral lactobacilli as potential probiotics for oral health. Oral Microbiol Immunol. 2008; 23(2): 139-147. doi: 10.1111/j.1399-302X.2007.00402.x

19. Charteris WP, Kelly PM, Morelli L, Collins JK. Antibiotic susceptibility of potentially probiotic *Lactobacillus* species. J Food Proc. 1998;61(12):1636-1643. doi: 10.4315/0362-028X-61.12.1636

20. Sharma C, Gulati S, Thakur N, Singh BP, Gupta S, Kaur S, Mishra SK, Puniya AK, Gill JP, Panwar H. Antibiotic sensitivity pattern of indigenous lactobacilli isolated from curd and human milk samples. 3 Biotech. 2017 ;1:7(1):53. doi: 10.1007/s13205-017-0682-0

21. Tahmourespour A, Kermanshahi RK. The effect of a probiotic strain (*Lactobacillus acidophilus*) on the plaque formation of oral Streptococci. Bosnian J Basic Med Sci. 2011;11(1):37. doi: 10.17305/bjbms.2011.2621

22. Rammelsberg M, Radler F. Antibacterial polypeptides of *Lactobacillus* species. J Appl Bacteriol. 1990; 69(2): 177-184. doi: 10.1111/j.1365-2672.1990.tb01507.x

23. Ming L, Zhang Q, Yang L, Huang J-A. Comparison of antibacterial effects between antimicrobial peptide and bacteriocins isolated from *Lactobacillus plantarum* on three common pathogenic bacteria. Int J Clin Exp Med. 2015; 8(4): 5806-5811.

24. Batdorj B, Dalgalarrondo M, Choiset Y, Pedroche J, Metro F, Prevost H, Chobert JM, Haertle T. Purification and characterization of two bacteriocins produced by lactic acid bacteria isolated from Mongolian airag. J Appl Microbiol. 2006;101(4):837-848. doi: 10.1111/j.1365-2672.2006.02966.x

25. Ben Slama R, Kouidhi B, Zmantar T, Chaieb K, Bakhrouf A. Anti-listerial and anti-biofilm activities of potential Probiotic *L actobacillus* strains isolated from T unisian traditional fermented food. J Food Safe. 2013; 33(1): 8-16. doi: 10.1111/jfs.12017

26. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol. 2007;24(8):1596-1599. doi: 10.1093/molbev/msm092

27. Khanafari A, Porgham SH, Ebrahimi MT. Investigation of probiotic chocolate effect on Streptococcus mutans growth inhibition. Jundishapur J Microbiol. 2012; 5(4): 590. doi: 10.5812/jjm.3861

28. Shukla G, Sharma G, Goyal N. Probiotic characterization of lactobacilli and yeast strains isolated from whey beverage and

therapeutic potential of Lactobacillus yoghurt in murine giardiasis. Am J Biomed Sci. 2010; 2: 248-261.
doi: 10.5099/aj100300248

29. Zago M, Fornasari ME, Carminati D, Burns P, Suarez V, Vinderola G, Reinheimer J, Giraffa G. Characterization and probiotic potential of *Lactobacillus plantarum* strains isolated from cheeses. Food Microbiol. 2011; 28(5): 1033-1040.
doi: 10.1016/j.fm.2011.02.009

30. Neujahr HY, Borstad B, Logardt I-M. Factors affecting the resistance of *Lactobacillus fermenti* to lysozyme. J Bacteriol. 1973;116(2):694-698.

31. Teanpaisan R, Piwat S, Dahlen G. Inhibitory effect of oral Lactobacillus against oral pathogens. Letters in Applied Microbiology. 2011 Oct;53(4):452-9.
doi: 10.1111/j.1472-765X.2011.03132.x

32. Widyarman AS, Bachtiar EW, Bachtiar BM, Seneviratne CJ. Inhibitory effect of probiotic lactobacilli against *Streptococcus mutans* and Porphyromonas gingivalis biofilms. Sci Dent J. 2019; 3(2): 50-55
doi: 10.4103/SDJ.SDJ_8_19

33. Cannon ML. A review of probiotic therapy in preventive dental practice. Probiotics Antimicrob. 2011; 3(2): 63-67.
doi: 10.1007/s12602-011-9072-9

34. Lemos JA, Burne RA. A model of efficiency: stress tolerance by *Streptococcus mutans*. Microbiol. 2008;154(11):3247-3255.
doi: 10.1099/mic.0.2008/023770-0

35. Lemos JA, Palmer SR, Zeng L, Wen ZT, Kajfasz JK, Freires IA, Abranches J, Brady LJ. The Biology of *Streptococcus mutans*. Gram-Positive Pathogens. 2019: 435-48.
doi: 10.1128/9781683670131.ch27

36. Pitts NB, Zero DT, Marsh PD, Ekstrand K, Weintraub JA, Ramos-Gomez F, Tagami J, Twetman S, Tsakos G, Ismail A. Dental caries. Nat Rev Dis Primers. 2017 ; 25: 3(1):1-6.
doi: 10.1038/nrdp.2017.30

37. Twetman S, Keller MK. Probiotics for caries prevention and control. Adv Dent Res. 2012; 24(2): 98-102.
doi: 10.1177/0022034512449465

38. Vetrovsky T, Baldrian P. The variability of the 16S rRNA gene in bacterial genomes and Its consequences for bacterial community analyses. Plos. 2013.
doi:10.1371/journal.pone.0057923

Research Article

APPLIED FOOD BIOTECHNOLOGY, 2021, 8 (3): 201-212
Journal homepage: www.journals.sbmu.ac.ir/afb

pISSN: 2345-5357
eISSN: 2423-4214

بررسی تاثیر باکتری‌های لاکتیک اسید به‌عنوان زیست‌یارهای بالقوه دهانی بر مهار رشد *استرپتوکوکوس موتانس*

مریم گلشاهی[۱]، محمد مهدی پیرنیا[۲]، پروانه جعفری[۳]، الهه ابراهیمی[۴]، فرزانه تفویضی[۵]، مهسا دمشقیان[۶]، مریم تاج آبادی ابراهیمی[۶*]

۱- گروه زیست شناسی، واحد علوم و تحقیقات، دانشگاه آزاد اسلامی، تهران، ایران
۲- گروه بیوفیزیک، انستیتو تحقیقات بیوشیمی و بیوفیزیک، دانشگاه تهران، تهران، ایران
۳-گروه میکروبیولوژی، دانشکده علوم پایه، واحد اراک، دانشگاه آزاد اسلامی، اراک، ایران
۴-گروه میکروبیولوژی، دانشگاه آزاد اسلامی واحد علوم دارویی، تهران، ایران
۵-گروه زیست شناسی، واحد پرند، دانشگاه آزاد اسلامی، پرند، ایران
۶- گروه زیست شناسی، واحد تهران مرکز، دانشگاه آزاد اسلامی، تهران، ایران

چکیده

سابقه و هدف: یکی از ویژگی های تایید شده زیست‌یارها[۱]، اثر آن‌ها بر باکتری‌های مضر دهانی است. از آنجا که مقاومت آنتی بیوتیکی مشکل مهمی است، یافتن سویه‌های مستعد جدید، اهمیت دارد. هدف این مطالعه، انتخاب سویه‌های جدید و ایمن باکتزی‌های لاکتیک اسید دارای خصوصیات زیست‌یاری دهانی است. علاوه براین، توانایی این سویه‌ها در جلوگیری از رشد و اتصال *استرپتوکوکوس موتانس* به‌عنوان مهمترین باکتری عامل پوسیدگی دندان بررسی شده است.

مواد و روش ها: آزمون‌های شناسایی اولیه، شامل رنگ‌آمیزی گرم، آزمون‌های کاتالاز و اکسیداز، روی ۲۲ سویه باکتری های اسید لاکتیک جدا شده از محصولات لبنی سنتی ایران انجام شد. ایمنی سویه‌ها با آزمون فعالیت همولیتیکی و مقاومت آنتی‌بیوتیکی بررسی شد. سپس ویژگی‌های زیست‌یاری سویه‌ها از جمله مهار رشد *استرپتوکوکوس موتانس*، مقاومت به آنزیم لیزوزیم، توانایی اتصال سویه ها، توانایی سویه‌ها در کاهش اتصال *استرپتوکوکوس موتانس* مورد ارزیابی قرار گرفتند. سویه‌های انتخاب شده با استفاده ازروش مولکولی 16S rRNA شناسایی شدند.

یافته‌ها و نتیجه‌گیری: از بین تمام سویه‌ها، ۴ سویه با بهترین ویژگی زیست‌یار دهانی انتخاب شدند. این سویه‌ها شامل یک سویه *لاکتوباسیلوس برویس*، یک سویه *لاکتوباسیلوس کازئی* و دو سویه *لاکتوباسیلوس پاراکازئی* بودند. این چهار سویه دارای ویژگی‌های ضدمیکروبی قوی در برابر *استرپتوکوکوس موتانس*، مقاوم به آنزیم لیزوریم دهانی و دارای توانایی اتصال قوی به چاهکهای پلی استرنی بودند. همچنین، این سویه‌ها اتصال *استرپتوکوکوس موتانس* را کاهش دادند، از این رو، از تشکیل زی‌لایه[۲] توسط این باکتری جلوگیری شد. از آنجا که در این ارزیابی ثابت شد، این سویه ها فعالیت همولیتیکی و مقاومت آنتی بیوتیکی ندارند، به‌عنوان سویه‌های ایمن شناسایی شدند. بنابراین، این چهار سویه ها به‌عنوان زیست‌یارهای دهانی پیشنهاد می‌گردند.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

تاریخچه مقاله

دریافت ۱۲ ژانویه ۲۰۲۱
داوری ۵ فوریه ۲۰۲۱
پذیرش۱۴ آوریل ۲۰۲۱

واژگان کلیدی

- *استرپتوکوکوس موتانس*
- پوسیدگی دندان
- باکتری‌های اسید لاکتیک
- ارزیابی ایمنی
- زیست‌یارهای دهانی

***نویسنده مسئول**

مریم تاج آبادی ابراهیمی
گروه زیست شناسی، واحد تهران مرکز، دانشگاه آزاد اسلامی، تهران، ایران.
تلفن: ۴۴۶۰۰۱۹۶ ۹۸+

پست الکترونیک:
m.tajabadi@iauctb.ac.ir

[۱] probiotics
[۲] biofilm