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Molecular study and probiotic potency of lactic acid bacteria isolated from dairy products in Mazandaran Province of Iran and it antagonistic effect on *Pseudomonas aeruginosa*

Monavar-ul-Sadat Mousavizadeh[1], Sedigheh Mehrabian[1]*, Fatemeh Ashrafi[1], Maryam Tajabadi Ebrahimi[2]

1. Microbiology Group, Biological Sciences Faculty, North Tehran Branch of Islamic Azad University, Tehran, Iran,
2. Microbiology Group, Biological Sciences Faculty, Center Tehran Branch of Islamic Azad University, Tehran, Iran

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ABSTRACT

Probiotics are living dietary supplements that exert their health benefits by improving the intestinal microbial balance in the host. lactic acid bacteria are the most common type of bacteria that have been identified as probiotics. This study isolated lactic acid bacteria from the traditional yogurt, dooghd and, curd of Mazandaran Province in Iran and investigated their probiotic potency and antimicrobial effect on *Pseudomonas aeruginosa*. lactic acid bacteria were isolated by phenotypic and biochemical tests of acid resistance and bile salt tolerance tests, and their primary probiotic index was evaluated. The inhibitory effect of the isolated bacteria on *P. aeruginosa* was investigated by the disk-diffusion agar method. The non-growth halo diameter was measured around the disk. In order to reduce errors, each test was repeated at least three times, and the mean non-growth halo diameter was measured on Mueller-Hinton agar and its antagonistic effect was investigated. The findings showed that adding a lactobacillus dose increases its inhibitory effects. The *Lactobacillus plantarum* were identified using specific primer pairs of the 16S rRNA gene, the established specific band, and PCR product sequencing. Rec A gene PCR was performed for differentiating the members of this group using specific primers and five *L. plantarum* strains were identified. According to the results of this study, lactobacilli were well able to inhibit the growth of pathogenetic strains. In this study, *L. plantarum* had an inhibitory effect on *P. aeruginosa*.

1. Introduction

Dairy products play an important role in human nutrition. They provide a high-quality protein source and are good sources of vitamins B, D, and A, as well as calcium, phosphorus, magnesium, and zinc. Dairy products have a long history of human nutrition. They contain essential nutrients including protein, fat and fatty acids, vitamins, and minerals for humans. traditional yogurt, dooghd and, curd are produced in Mazandaran Province of Iran and its summer resorts. These products are made from cow's milk and no starter culture is used in their process of production. Recent studies have shown that dairy products have a diverse and specific microbial population associated with their traditional production technology and the

*Corresponding author: Prof. Sedigheh Mehrabian
Tel: +982122950955
E-mail address: mehrabians20212@gmail.com

geographical area in which they are produced. Lactic acid bacteria are the most important group of microorganisms in dairy products and there is a long history of their consumption by humans to produce and store food. These bacteria are commonly found in nutrient-rich environments such as milk, yogurt, cheese, meat, beverages, and vegetables. They are also isolated from the soil, lakes, and intestines of animals and humans. Lactic acid bacteria have long played an important role in food technology. These bacteria are of several genera and have a significant number of species (Tohidpour and Mehrabian, 2011). The common characteristics of these bacteria include being gram-positive and catalase-negative, growth in diverse conditions, from microaerophilic to completely anaerobic, and producing lactic acid. Carbohydrate fermentation testing is one of the most important tests for determining the species of bacterial genera. There are different fermentation ways for identifying the genus or species of bacteria. Bacterial fermentation is performed by two methods, namely Homofermentation and Heterofermentation. In homofermentation, glucose is broken down by bacteria into lactic acid and small amounts of other substances, such as acetate, formate, and ethanol. In heterofermentation, bacteria break down glucose into lactic acid and other substances such as acetic acid, formic acid, ethyl alcohol and CO_2 and sometimes glycerol. Arabinose, inositol, sucrose, raffinose, rhamnose, cellobiose, ribose, glucose, fructose, galactose, trehalose, lactose, mannose, mannitol, melezitose, and melibiose are used to perform this test. The products of the fermentation of these sugars by bacteria depend on factors such as type of bacteria, the substrate nature, and sometimes environmental factors such as temperature and pH. In general, the compounds that may be produced include lactic acid, acetic acid, formic acid, ethanol, acetoin, and CO_2, succinic acid (decomposed into propionic acid and CO_2), and butyric acid (Pourahmad, 2004). Non-Starter Lactic Acid Bacteria (NSLAB) are made up of different microbial populations, and certain strains and biotypes of them are present in the production process of dairy products based on the dominating selected conditions and other ecosystem conditions. These bacteria are widely used as starter culture in the production of fermented products such as yogurt and cheese, meat products, breads, and vegetables. These bacteria produce several antibacterial substances such as bacteriocins and metabolites such as hydrogen peroxide, lactic acid, and other organic acids and ethanol, which inhibit the growth of harmful microorganisms (Guy et al., 2014). Lactic acid bacteria are among the most important bacteria that have probiotic properties. These bacteria have a positive effect on people's health by improving the intestinal microflora balance and mucosal defense against pathogens. The functional properties of probiotics include immunomodulation and reduced serum cholesterol, gastrointestinal infections, chronic travelers' diarrhea, and cancer rates (Birri et al., 2013). Pseudomonas aeruginosa is a gram-negative, motile bacillus that is widespread in nature and can be colonized in normal individuals and act opportunistically. This bacterium causes disease in people who have weak immunity and disorders of the immune system (Jawetz,2010).

One of the benefits of lactic acid bacteria is the production and secretion of inhibitory substances that have an antagonistic effect on a wide range of microorganisms. These inhibitory substances include formic acid, free fatty acids, ammonia, ethanol, bacteriocins, antibiotics, hydrogen peroxide, etc. (Mohammadi et al., 2017).

2. Methods and Materials

2.1. samples

Dairy products, including traditional yogurt, dooghd and, curd, were collected in different parts of Mazandaran Province in 2018.

2.2. Isolation of lactic acid bacteria

The samples were transferred to MRS broth and incubated in a jar at 37 °C for 48-72 hours in microaerophilic conditions. They were then transferred to MRS Agar and incubated in a jar at 37 °C for 48-72 hours in microaerophilic conditions (Microbiology Anaerocult C merk) for isolation. For this purpose, after identification of gram-positive bacilli colonies without oxidase and catalase, pure culture was prepared in MRS agar medium. Unfortunately, it was not isolated from lactobacilli whey samples.

2.3. Determination of probiotic potential

acid resistance and bile salt tolerance tests and gram staining tests were then performed on the grown colonies.

After identifying lactobacilli, the following tests were performed to evaluate their probiotic potency:

The study of gastric acidic conditions is as follows: in vitro, the acidic environment of the stomach is simulated, and the tolerance of the bacteria obtained from dairy products (traditional yogurt and, dooghd) was examined.

NaCl (125 mmol/l), KCL (7 mmol/l), NaHCO3 (45 mmol/l), and pepsin (3 g/l) (uFlka, Sigma-Aldrich) were used to simulate gastric conditions, and hydrochloric acid (HCl) was used to reach a pH of 2 and 3. The desired bacterium with a turbidity of 0.5 McFarland was then added to the acidic medium for 24 hours in microaerophilic conditions, it was incubated at 37 °C. After 24 hours, it was cultured on solid culture medium and examined for the growth of bacteria with isolated probiotic potential. (Charernjiratragul et al., 2010).

The next comparison was between the effect of bile salts on bacteria with probiotic potency isolated from dairy products (traditional yogurt and, dooghd). The bile was prepared with dilution rates of 0.4, 0.5, and 0.6%, and incubated in an anaerobic jar for 2.0 hours. Control (no bile salts) and test cultures were evaluated at 2 and 24 hours for the presence or absence of growth by streaking samples onto MRS agar. (Menconi et al., 2014).

Then, in order to identify and determine the isolated lactobacilli, PCR test was performed randomly on five samples to identify and determine definitively.

2.4. 16S rRNA gene sequencing

The following steps were taken for 16S rRNA gene sequencing, Bacterial culture from a single colony, Genomic DNA extraction from bacterial culture using FavorPrep™ Tissue Genomic DNA Extraction Mini Kit (Favorgen Co.) and Polymerase Chain Reaction was performed. The following primers were used to amplify the 16S rRNA gene.

4F: 5'- TATCGGAGAGTTTGATCCTGG -3'
1541r: 5'- AAGGAGGTGATCCAGCCGCA -3'

The PCR was implemented in 35 cycles according to the following table:

Table 1. PCR Protocol

PCR Protocol		
Innitial denaturation	95 °C	3 '
Denaturation	93 °C	45"
Annealing	55 °C	60"
Extention	72 °C	90"
Final Extention	72 °C	'10

Electrophoresis was performed to evaluate the PCR product. The electrophoresis confirmed 1500 nucleotide pairs (Figure 1).

Table 2. DNA concentration after purification at 260 nm

	Strain code	DNA Concentration (ng/µl)
1	ID -980717-2037-05/01 (M15)	145.6
2	ID -980717-2037-05/02 (M18)	156.8
3	ID -980717-2037-05/03 (M19)	153.3
4	ID -980717-2037-05/04 (M25)	133
5	ID -980717-2037-05/05 (M27)	143.5

After observing the appropriate band, the PCR product was purified using FavorPrep purification Mini kit (Favorgen Co.).

The DNA concentration was measured after purification at 260 nm.

The results were as follows:

The pure PCR products were sent to Bioneer Co. in Korea for sequencing. Sequencing was performed using the following primers.

27f : 5'- GAGTTTGATCCTGGCTCAG -3'
16r339: 5'-ACTGCTGCCTCCCGTAGGAG -3'
16f358: 5'- CTCCTACGGGAGGCAGCAG -3'
704f: 5'- GTAGCGGTGAAATGCGTAGA -3'

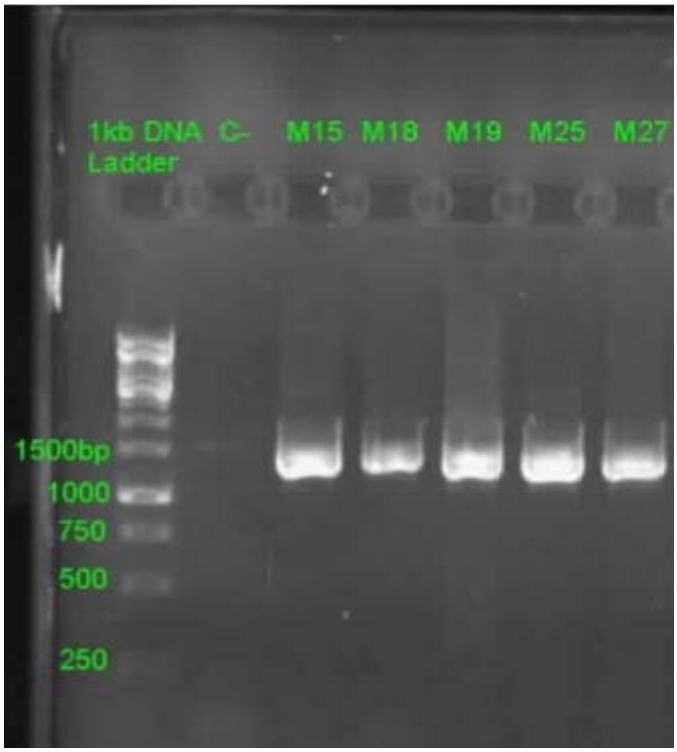

Figure 1. PCR product electrophoresis of five strains of *Lactobacillus plantarum*

2.5. Antibiogram testing of P. aeruginosa on lactic acid bacteria isolated from dairy products

To investigate antagonism activity, lactic acid bacteria isolated by disk diffusion were tested on *P. aeruginosa* (ATCC: 27853) in vitro. Tube dilution and turbidimetry were used to determine the minimum inhibitory concentration (MIC) of antimicrobial substances that prevent bacterial growth after 24 or 72 hours. The lactic acid strains were stored for 24 hours in MRS broth. The supernatant was prepared from each strain of lactic acid bacteria by centrifugation at 5000 rpm for 10 min. The supernatant was then filtered with a 0.22-µm filter. *P. aeruginosa* were introduced into Mueller Hinton broth and stored at 37 °C for 24 hours to create a turbidity similar to 0.5 McFarland. 10 µl of *P. aeruginosa* suspension turbidity equivalent to 0.5 McFarland tube. was measured in a spectrophotometer, on a plate poured with sterile swabs, a table was cultured in Muller Hinton agar. Then, to make different disk dilutions, a 10-µl sampler and a sterile sampler head were used to add 10, 20, 30, 40 µl to the blank disks based on the standard 0.5-McFarland sample.

For values above 30 µl, due to the lack of capacity of the blank disk, it was necessary to wait for the disk to dry and re-pour the metabolite on it. The drying process was performed in a hot air oven at 60 °C for 15 min. Then, by numbering and dividing different areas on the plate, the disks were placed on the medium with different dilutions. The plates were then transferred to a jar containing gas pack to be incubated under microaerophilic conditions for 24-48 hours. Finally, the results were determined based on whether growth inhibition halos were or were not observed around the disk and their diameter was measured (Allahdori, 2013).

After purification of gram-positive, oxidase and catalase-negative bacteria, PCR test was performed randomly on five samples to identify and determine definitively.

3. Results

In Phenotypic results for the lactic acid bacteria isolated from traditional yogurt and, dooghd in MRS medium After gram staining, the isolated gram-positive bacilli proved negative in the catalase, oxidase and motolity

tests. These bacteria were then examined for probiotic potency (resistance to acid and bile).

In a study of the results of acid resistance, 10 to 60 percent of lactic acid bacteria survived at pH = 2,3 for one hour. Also, these bacteria were able to tolerate 4.5%, 5.5% and 6.5% of bile salts for one hour.

By comparing the sequence obtained with the nucleotide sequences in databanks including NCBI, Ribosomal Database Project, and Eztaxon, the following results were obtained:

3.1. 16S rRNA gene analysis results

A 1315-nucleotide sequence was obtained from the 16S rRNA gene, strain M15.

ID-980717-2037-05/01 (Strain M15)

A 1438-nucleotide sequence was obtained from the 16S rRNA gene, strain M18.

ID-980717-2037-05/02 (Strain M18)

A 1506-nucleotide sequence was obtained from the 16S rRNA gene, strain M19.

ID-980717-2037-05/03 (Strain M19)

A 1513-nucleotide sequence was obtained from the 16S rRNA gene, strain M25.

ID-980717-2037-05/04 (Strain M25)

A 1506-nucleotide sequence was obtained from the 16S rRNA gene, strain M27.

ID-980717-2037-05/05 (Strain M27)

The PCR product was examined by sequencing and comparing its sequence with those available in the gene bank.

The sequences obtained for M15, M18, M19 and M27 were 100% similar to the same gene from *L. pentosus* DSM 20314 (T) strain, whose entire genome is registered as AZCU01000047 in NCBI, and it is 99.93% for M25(difference in one nucleotide).

Also, the sequences obtained for M25 were 99.93% similar to the same gene from the *L. plantarum* subsp. *plantarum* ATCC 14917(T) strain, whose entire genome is registered as ACGZ01000098 in NCBI (difference in one nucleotide), and it is 100% for M15, M18, M19 and M27.

According to the results, the strains belong to the *L. plantarum* group.

In order to differentiate members of this group, rec A gene PCR was performed using specific primers.

3.2. The rec A gene PCR results

Due to the high similarity of the 16S rRNA gene of the *L. plantarum* species, rec A gene sequencing or the comparison of the PCR products obtained from specific primers can be used to differentiate the species (Bringel et al., 2005; Toriani et al., 2001).

Specific forward primers including planF and pentF and the common reverse primer pREV were used to separate the plantarum species from the pentosus. planF (59-CCG TTT ATG CGG AAC ACC TA-39). pentF (59-CAG TGG CGC GGT TGA TAT C-39). pREV (59-TCG GGA TTA CCA AAC ATC AC-39)

PCR with the above primers in the case of strains belonging to the *L. plantarum* species led to the amplification of a fragment of about 300 nucleotides, while in the case of *L. pentosus* strains, the amplified fragment was about 200 nucleotides. Figure 3 shows the PCR product electrophoresis with the above primers and the DNA of the M15, M18, M19, M25, and M27 strains.

As shown in Figure 2, no 200-nucleotide fragment is observed in the PCR product of any of the strains with the pentF specific primer, while an approximately 300-nucleotide fragment is observed for all the strains in the PCR with the PlanF primer. PTCC 1745 (*L. plantarum*) and PTCC 1872 (*L. pentosus*) were used as controls.

Due to the fact that the PCR product bands with the planF primer related to the M25 and M27 strains seemed a little ahead of the PTCC 1745 control band, the PCR was repeated for the rec A gene of these two strains as a multiplex using a ladder on both sides of the gel. The results showed that the product band was completely parallel to the PTCC 1745 control band.

According to these findings, all five strains belonged to the *L. plantarum* species.

In the study of antagonistic activity of different doses of lactic acid bacteria isolated from traditional yogurt and, dooghd by disc release on *P. aeruginosa* (antibiogram), the diameter of the aura did not grow at a dose of 20, 0.6 - at a dose of 30, 0.6 and at a dose of 40, 1.2 mm.

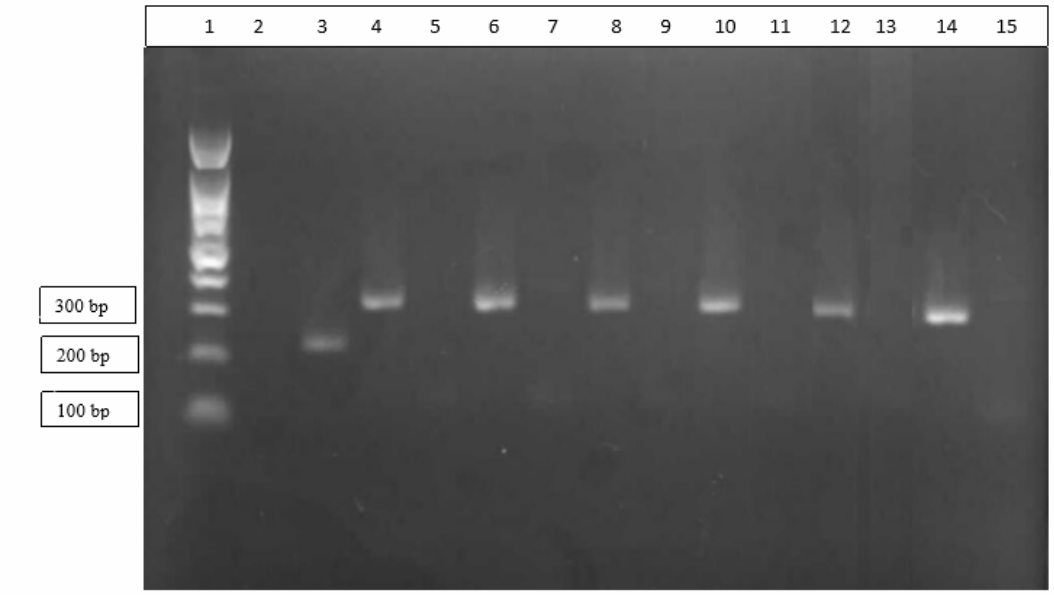

Figure 2: Gel electrophoresis: 100 bp ladder, 2: L. pentosus PTCC1872 with planF primer, 3: L. pentosus PTCC1872 with pentF primer, 4: L. plantarum PTCC1745 with planF primer, 5: L. plantarum PTCC1745 with pentF primer, 6: M15 with planF primer, 7: M15 with pentF primer, 8: M18 with planF primer, 9: M18 with pentF primer, 10: M19 with planF primer, 11: M19 with pentF primer, 12: M25 with planF primer, 13: M25 with pentF primer, 14: M27 with planF primer, 15: M27 with pentF.

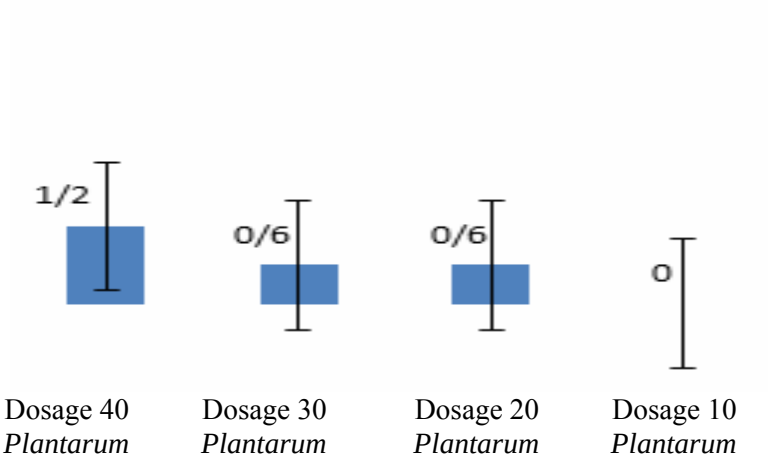

Figure 3: The mean *Lactobacillus* halo diameter on *Pseudomonas aeruginosa*

Table 3. A description of the Lactobacillus halo diameter on Pseudomonas aeruginosa

	N	*Minimum*	*Maximum*	*Mean*	
	Statistic	*Statistic*	*Statistic*	*Statistic*	*Std. Error*
Plantarum dosage 10	*3*	*.00*	*.00*	*.0000*	*.00000*
Plantarum dosage 20	*3*	*.40*	*.80*	*.6000*	*.11547*
Plantarum dosage 30	*3*	*.50*	*.70*	*.6000*	*.05774*
Plantarum dosage 40	*3*	*1.00*	*1.40*	*1.2000*	*.11547*
Valid N (listwise)	*3*				

4. Discussion

Lactobacilli can be used as natural preservatives because they produce antimicrobial compounds such as bacteriosis (Zhou et al., 2008). The production of these compounds is important because lactic acid bacteria are the predominant microflora of many fermented products that play an important role in the processing and control of pathogens. Moreover, the consumption of probiotic products can modulate the intestinal microflora and inhibit the growth of pathogenic bacteria in the intestine (Rawa et al., 2013; Stoyanova et al., 2012).

Saranya and Hemashenpagam (2011) examined the antimicrobial activity of lactic acid bacteria against standard strains of *E. coli*, *Klebsiella pneumoniae*, *P. aeruginosa*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Proteus*. The most potent inhibitor pertained to *L. plantarum*, with a non-growth halo diameter against *P. aeruginosa*, as consistent with the present study.

P. aeruginosa is a gram-negative and opportunistic bacillus that is abused in immunocompromised individuals.

Izadi et al. (2010) studied yogurt in Shahr-e-Babak in Kerman, Iran, and isolated *Lactobacillus acidophilus* from yogurt samples, which is consistent with the isolation of Lactobacillus in this study. Qobadi Dana et al. (2012) examined dairy products such as yogurt, kefir and raw milk from the pristine areas of Kermanshah, Kurdistan, Lorestan, Ilam and Markazi provinces in Iran and confirmed new species of Lactobacillus. Farahbakhsh et al. (2012) studied the isolation of lactobacilli from traditional yogurt in rural areas in Rafsanjan, Iran, and isolated *casei*, *rhamnosus*, *Plantarum*, *acidophilus*, *bulgaricus*, *delbrueckii*, *fermentum*, and brevis species of *Lactobacillus*. They also examined their antibacterial effects and showed that probiotic bacteria have antibacterial activity against some pathogenic bacteria, which is consistent with the results of the present study. Sultan Dalal et al. (2015) investigated the isolation and identification of lactic acid bacteria with probiotic potential in traditional yogurt in Yazd Province and showed the presence of a high diversity of lactic acid bacteria. They isolated *L. plantarum*, *Lactobacillus brevis*, and *fermentum fermentum* species, as consistent with the present study. Aghajani et al. (2018) In a study of the antagonistic effect of lactobacilli isolated from the soil of local yogurt processing site against pathogenic bacteria identified three species of *Lactobacillus casei*, *L. plantarum* and *Lactobacillus delbrocci* on the bacteria *S. aureus* and *Pseudococcus schizophrenia*, *Escherichia coli*. which is consistent with the present study.

Intestinal gram-negative bacteria, especially *Salmonella*, *Shigella*, and *E. coli*, are among the most important causes of foodborne diseases and diarrhea in developing countries. Moreover, as drug resistance is increasing every day in these bacteria, the competitive inhibition of pathogenic bacteria and probiotics, including lactic acid bacteria, especially lactobacilli, is currently used to prevent the growth of pathogenic bacteria (Adesokan et al., 2008; Tajabadi et al., 2009). Obadina et al. (2006) identified the antagonistic effect of certain lactobacilli species on *Pseudomonas*, *E. coli* and *S. aureus* and found results that were consistent with the present findings. which is consistent with the present study.

Datta et al. (2013) Lactobacillus isolated fermentom, plantarum, kazai, and bravis from dairy products to test for antimicrobial activity, and examined the growth spurt on several pathogenic bacteria, which had the greatest inhibitory effect. *Plantarum* was observed on *P. aeruginosa*, which is consistent with the present study.

Puttalingamma et al. (2006) also investigated the antagonistic effect of *L. plantarum* on *E. coli*, *Salmonella*, *S. aureus*, and *Bacillus subtilis* and found similar results as consistent with the present study in terms of the *L. plantarum* isolation.

Coconnier et al. (1998) reported that the consumption of the supernatant of *Bacillus fermentum*, *casei*, and *acidophilus* culture has an antibacterial effect on a wide range of gram-positive and gram-negative pathogenic bacteria. Elvin et al. (2017) studied the antibacterial properties of lactic acid bacteria on food pathogenic bacteria and found that *L. acidophilus* has an inhibitory effect on *E. coli*. This study is consistent with the isolation of *Lactobacillus* and its inhibitory effect on pathogenic bacteria.

Martin et al. (2009) examined the presence of lactic acid bacteria with probiotic potency in pig's milk, which protects piglets against

infectious diseases through a variety of mechanisms. They identified different types of lactobacilli, especially *L. plantarum* and *L. brevis*, as consistent with the present study.

Lim and Im (2009) screened and identified bacteria with probiotic potential from fermented food products in Korea, and isolated *L. plantarum* in their dairy-based foods, which is consistent with the present study.

Noraphat et al. (2010) isolated lactic acid bacteria from the dairy-based food Kung Som in Thailand, which, in their case, was *L. plantarum*, and had inhibitory effects on *E. coli*, as consistent with the present study in terms of the *L. plantarum* isolation.

Abdullahand and Osman (2010) found that lactic acid bacteria can be widely used as starter culture in the production of fermented products such as yogurt, cheese, meat products, and breads and vegetables. They also isolated *L. brevis* and *L. fermentum* and unidentified lactobacillus spp. from these sources, as consistent with the present study in terms of the lactobacillus isolation.

Conclusion

The results showed that lactobacilli were well able to inhibit the growth of pathogenetic strains. In this study, *L. plantarum* had an inhibitory effect on *E. coli*. It should be noted that increasing the dose of lactobacilli and their continuous consumption in meals and replacing lactobacilli as the dominant flora can provide immunity to these bacteria in living organisms and play an effective role in nutrition. Also, the increasing incidence of congenital anomalies, chronic diseases, ineffectiveness of antibiotics, microbial resistance, and other complications known as current health problems in human societies have been attributed to the lack of probiotic consumption. Also, the use of traditional dairy products that have long been used in history plays an important role in people's health. Therefore, the use of beneficial bacteria that do not have harmful health and environmental consequences while maintaining the desired characteristics is of interest to researchers throughout the world. All this evidence suggests that common lactobacilli species are available in different communities. Given that there is ample evidence about lactobacilli's vital role in promoting the health of organisms by preventing various diseases and increasing the efficiency of their survival, these beneficial bacteria can be easily used in other dairy products and traditional dairy products can be used instead of imported starters.

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