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Lactobacillus spp. Inhibit the Biofilm Formation of *Pseudomonas aeruginosa*

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Abstract: Probiotic bacteria are microbial food supplements with beneficial properties on human health. This study aimed to isolate *Lactobacillus* spp. from the traditional homemade dairy and evaluate their inhibitory effects on the biofilm formation of *P. aeruginosa*. In this study, 10 samples of traditional homemade yogurt and milk samples were collected from five separated rural areas of Miyana, Azerbaijan, Iran, from May-June 2016. *Lactobacillus* spp. was isolated from traditional homemade dairy using microbiological methods. Finally, *Lactobacillus* spp. effects on *P. aeruginosa* biofilm were determined by the modified micro-plate test. Fifty-eight acid-tolerance bacteria were isolated from milk and yogurt samples. *L. plantarum* and *L. casei* had antibiofilm activity. The percentage of biofilm inhibition by *L. plantarum* and *L. casei* isolated from yogurt and milk were obtained zero to 52.1% and 5.7% to 48.8%; and zero to 25.8% and 5.2% to 16.1%, respectively. *L. plantarum* and *L. casei* isolated from traditional milk and yogurt have an inhibition effect on the biofilm formation of *P. aeruginosa*. It suggests the traditional homemade dairy is one of the best probiotics against biofilm-producing *P. aeruginosa*.

Keywords: lactic acid bacteria; probiotic bacteria; biofilm; antibiofilm activity; traditional dairy; *Pseudomonas aeruginosa.*

1. Introduction

Traditional dairy products are homemade and natural products, which could be a valuable source of lactic acid bacteria [1, 2]. These products are part of the daily diet of most people around the world. Lactic acid bacteria have fermentation activities that can result in the inhibition of pathogenic and spoilage bacteria. The antimicrobial effects of *Lactobacillus* spp. May result in the production of antimicrobial substances such as bacteriocins, lactic acid, diacetyl, and hydrogen peroxide [3-5]. The microbiological characteristic of several traditional dairy products have been studied in Iran [6-9] and other countries [10, 11]. Lactobacillus species are Gram-positive, non spore-forming, and catalase-negative bacilli, producing lactic acid as the main product of carbohydrates fermentation [12]. Lactobacillus spp. are presented in milk and dairy products such as yogurt, cheese, and fermented milk [13]. Lactobacillus spp. are used as a starter bacterium for different fermented foods [2]. *P. aeruginosa* is a Gram-negative and oxidase-positive bacillus isolated from various infections and is one of the most

antibacterial resistant bacteria. The mortality rate of *P. aeruginosa* infections is 50% in cancer patients, patients with acquired immune deficiency syndrome, cystic fibrosis disease, and severe skin burns [14, 15]. Biofilm formation causes the reduction of antibiotic susceptibility compared to planktonic cells in *P. aeruginosa*, which could play an important role in the pathogenesis of *P. aeruginosa* [16-18]. In Azerbaijan, traditional milk and yogurt are widely produced and consumed. At the same time, these products constitute a natural reservoir of Lactobacillus spp., which have not yet been studied their activity against *P. aeruginosa*. Several studies demonstrated the antibiotic and anti-biofilm effects of different genera of bacteria [19], foods [20, 21], herbal extracts [22-24], and agents [25-28] on the biofilm formation and growth of *P. aeruginosa*. In the present study, *Lactobacillus* spp. isolated from the traditional homemade milk and yogurt were evaluated for biofilm inhibition activity against *P. aeruginosa.*

2. Materials and Methods

2.1. Sampling.

Five samples of traditional homemade yogurt and five samples of traditional homemade milk were collected from the rural area; in Miyana, Azerbaijan, Iran, from May-June 2018. The yogurt and milk samples were collected in sterilized bottles and transferred to the laboratory at 4ºC and were stored at 4ºC. They were used in experiments [29].

2.2. Isolation of bacteria and selection of acid-resistant isolates.

One milliliter of each yogurt and milk sample was shaken and were suspended in 10 mL of sterilized peptone (Sigma, The USA) (8.5 gr/L NaCl (Sigma, The USA) and 1 gr/L peptone). One mL of each prepared suspensions was added to 10 mL of De Man, Rogosa, and Sharpe (MRS) broth medium (Biolife, Milano, Italy) and was incubated at 37ºC for 24h in microaerophilic and anaerobic condition (Mart microbiology B.V., The Netherland). Afterward, 1 mL of suspensions was added to phosphate buffer saline (PBS) under pH 2.5-3 and was incubated at 37ºC for 2h. For detection of acid-resistant lactobacilli and to removing unwanted bacteria, PBS was centrifuged; the sedimentation was transferred to the MRS broth and was incubated at 37ºC for 24h. The MRS broth was streaked on the MRS agar (Merck, German) at 37ºC for 48-72h in anaerobic condition. Finally, Lactobacillus spp. were identified by colony morphology, biochemical tests, Gram's staining, catalase reaction, growth at acidic medium, growth in MRS broth at 15ºC and 37ºC, and carbohydrates fermentation patterns [30]. We also identified Lactobacilli by the API 50 CHL system (Biomerioux, France). All of the isolated strains were maintained at − 80 °C in the presence of 20% glycerol (Sigma, France).

2.3. Determination of biofilm production by P. aeruginosa.

The fresh overnight culture of *P. aeruginosa* (equal to 0.5 McFarland) was prepared. Fifty µL of the suspension was inoculated to 150 µL of Trypticase soy broth (Merck, Germany) contains 3 % glucose (Sigma, the USA) prepared in a sterile 96-well flat-bottomed plastic tissue culture plate with a lid. A well was considered as a negative control that contained TSB with 3 % glucose. The plate was incubated at 37 ºC for 48h. The wells were washed 3 times with 200 µL of PBS (pH 7.3). The biofilm produced was stained with 200 µL crystal violet (Sigma, The USA) 0.5 % at 37^{ºC} for 15 min. The excess stain was decanted and washed 3 times with sterile

deionized water and left to air dry. Two hundred µL ethanol 95% was added to the wells and shacked gently. The optic density (OD) of each well was measured at 492 nm by an ELISA reader (DANA, Iran) [28, 31].

2.4. Determination of Lactobacilli inhibitory effect on P. aeruginosa biofilm.

To determine the inhibitory effect *of Lactobacillus* spp. against *P. aeruginosa* biofilm, the modified micro-plate test was used as described by Stepanovic *et al*. [31]. Briefly, Overnight fresh culture of *P. aeruginosa* and *Lactobacillus* spp. (equal to 0.5 McFarland) was prepared; 50 µL of the suspension of *P. aeruginosa* was inoculated to 100 µL of TSB contains 3 % glucose and 50 µL of *Lactobacillus* spp. Were prepared in a sterile 96-well flat-bottomed plastic tissue culture plate with a lid. The plate was incubated at 37ºC for 48h. The wells were washed 3 times with 200 µL of PBS (pH 7.3). The biofilm produced was stained with 200 µL crystal violet 0.5 % at 37ºC for 15 min. The excess stain was decanted and washed 3 times with sterile deionized water and left to air dry. Two hundred µL ethanol 95% were added to the wells and shacked gently. The OD of each well was measured at 492 nm by ELISA counter. Biofilm inhibition percent by Lactobacillus spp. was evaluated by biofilm inhibition percent = (OD control – OD treat) * 100 / OD control. All experiments were done three times, and the answers were averaged.

3. Results and Discussion

Fifty-eight acid-tolerance bacteria were isolated from 10 samples. *L. casei* and *L. saniviri* were isolated from all milk and yogurt samples, follow as *L. parabuchneri* were the most isolated bacteria (n=9, 15.51%) (Table 1). *L. plantarum* was isolated from milk samples of two villages as well as were isolated from yogurt samples of four villages. On other hands, *L. casei* and *L. plantarum* were isolated from milk samples of five villages and two villages, respectively. Besides, *L. casei* and *L. plantarum* were isolated from yogurt samples of 5 regions and 4 villages, respectively. *L. plantarum* and *L. casei* had antibiofilm activity.

The range of OD means for biofilm formation inhibition by *L. plantarum* and *L. casei* isolated from milk were recorded from 0.1 to 0.681 and 0.77, respectively. The mean percentage for inhibition of biofilm formation by *L. plantarum* and *L. casei* isolated from milk were obtained 12.9% and 10.65%, respectively. In the milk samples, *L. plantarum* had the most inhibition of biofilm formation (25.8 %), and *L. casei* had 16.1%.

In addition, the range of means of OD for inhibition of biofilm formation by *L. plantarum* and *L. casei* isolated from yogurt were recorded from zero to 0.43933 and 0.46967 to 0.86967, respectively. The mean percentage for inhibition of biofilm formation by *L. plantarum* and *L. casei* isolated from yogurt were 26.05% and 27.25%, respectively. The results demonstrated that *L. plantarum* isolated from yogurt also had the most inhibition of biofilm formation (52.1 %) and followed by *L. casei* (48.8 %).

Probiotic bacteria carry out various activities in hosts, such as normal flora, provide necessary vitamins and ingredients for hosts, and have broad-spectrum activity against pathogenic bacteria [32]. Lactic acid bacteria are associated with fermented foods and are used as starter cultures or probiotics in these foods [33]. Lactic acid bacteria play a main role in various dairy products [1, 34].

In this study, 58 bacterial isolates with probiotic potential from traditional milk and yogurt were isolated from five rural areas in Azerbaijan, Iran. These results are consistent with

Abdelbasset *et al*. study that 52 lactic acid bacteria from 13 traditional fermented samples were isolated [35].

Table 1. The isolated bacteria from dairy products.

Bacteria	Milk N (%)	Yogurt N (%)	Total N (%)
L. acidifarinae	1 (4.34)	2 (5.71)	3 (5.17)
L. brevis	1 (4.34)	0	1 (1.72)
L. casei	5 (21.73)	5 (14.28)	10 (17.24)
L. collinoides	2 (8.69)	2 (5.71)	4 (6.89)
L. duranis	0	1 (2.85)	1 (1.72)
L. frintoshensis	2 (8.69)	2 (5.71)	4 (6.89)
L. mali	0	2 (5.71)	2 (3.44)
L. manihotivorans	0	3 (8.57)	3 (5.17)
L. parabuchneri	5 (21.73)	4 (11.42)	9 (15.51)
L. paracasei	0	3 (8.57)	3 (5.17)
L. plantarum	2 (8.69)	4 (11.42)	6 (10.34)
L. rapi	0	1 (2.85)	1 (1.72)
L. rosoiae	0	1 (2.85)	1 (1.72)
L. saniviri	5 (21.73)	5 (14.28)	10 (17.24)
Total	23 (100)	35 (100)	58 (100)

Acid tolerance bacteria is the main indicator for probiotic bacteria [36], which created a difference between probiotic strains, was assessed by determining this aspect. The pH range is so important for the survival of Lactic acid bacteria [36, 37]. Since low pH is considered one of the main features of probiotic bacteria [37], a high acidic tolerance method was used to detect probiotic bacteria in the present study. Dairy products include high microbial diversity. Moreover, isolation of each of these strains is so time-consuming. Therefore, by acidifying an area, the non-probiotic strains were mostly removed. So, in our study, a selective screening method in acidic conditions was used. Since, from 10 cases of traditional homemade milk and yogurt, 14 species were isolated as an acid-resistant bacterium.

Lactobacillus spp. is the normal flora of the intestine. It plays an important role in human health by preventing intestinal infection, reducing cholesterol, stimulating the immune system, and reducing the risk of clone cancer [38]. Boris *et al*. identified that *Lactobacillus* spp. Isolated from dairy inhibit growing of *P. aeruginosa*, *S. aureus*, *E. coli* and *S. typhimurium*. The most inhibition effect was for *S. aureus* and *E. coli* [39]. Coconnier *et al*.'s study demonstrated that *L. acidophilus* possesses an inhibition effect on growing steps of various pathogenic bacteria, which are active in the urinary tract and vagina like *P. aeruginosa*. *L. acidophilus* reduces these organisms' attachment to epithelial cells and decreases the colony count of these bacteria [40].

In this study, *L. casei* and *L. plantarum* isolated from traditional milk and yogurt could inhibit the biofilm formation of *P. aeruginosa*. Among them, the most inhibition effect of biofilm formation of *P. aeruginosa* was for *L. plantarum*. Abdelbasset *et al*. demonstrated that bacteriocins of lactic acid bacteria show broad antibacterial activity against Gram-negative bacteria [35]. Acid, lactic bacteria are most common bacteria that consider as a probiotic. These bacteria produce various low molecular mass components such as acids, carbon dioxide, alcohols, hydrogen peroxide, diacetyl, and other metabolites. Many of these components have a broad activity spectrum against other bacteria, and their production is largely affected by the food matrix itself [32]. Frostier *et al*. provided that the growth of some isolates isolated from clinical specimens such as *P. aeruginosa* was significantly reduced by staying near Lactobacillus spp. The colony count had decreased [41]. Valdez assessed the inhibition effect of *L. plantarum* on the pathogenicity of *P. aeruginosa*, which was isolated from a wound. The

results illustrated that *L. plantarum* had an inhibition effect on the production of haemosiderin and lactoferrin elastase enzyme and biofilm formation. Also, the mice with burn infection, which infected by *P. aeruginosa*, treatment of these mice with *Lactobacillus* spp. recovered faster than control mice [42]. The interaction between pathogens and probiotics is strain-related; therefore, extended study and other probiotics are required. It remains to be distinguished whether the inhibition effect in our study is species-specific or would have an effect on other pathogens.

Increasing consumption of industrial dairy products may reduce probiotics bacteria. However, the usage of probiotic bacteria may be useful for the prevention and treatment of some bacterial infections.

4. Conclusions

Our data demonstrate *L. plantarum* and *L. casei* isolated from the traditional dairy have the most biofilm inhibition effect and suggested the traditional dairy is one of the best probiotic foods against *P. aeruginosa*. Therefore, bacterial-mediated therapy could be considered a novel and effective treatment.

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Conflicts of Interest

The authors declare no conflict of interest.

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