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ORIGINAL ARTICLE

Anti-biofilm Effect of Lactic Acid Bacteria Isolated from Abali Local Buttermilk against Urinary *Enterococcus faecalis*

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KEYWORDS

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ABSTRACT: *Enterococcus faecalis* is a biofilm-producing bacterium and the cause of urinary tract infection. This bacterium is resistant to many antibiotics today and new methods such as use of lactic acid bacteria are needed. The present study aimed to isolate lactic acid bacteria with antagonistic and anti-biofilm effects against *E. faecalis* as a candidate for new treatment. In order to study of the anti -biofilm effect of lactic acid bacteria against *E. faecalis*, 30 samples of urinary *E. faecalis* were collected and their biofilm formation was investigated by a 96-well microtiter plate method. 10 samples of Abali traditional buttermilk (Dough) were collected and lactic acid bacteria were isolated and purified. Biochemical tests were done for the identification of lactic acid bacteria. The antagonistic effect of lactic acid bacteria supernatant against *E. faecalis* was investigated by determining the minimum growth inhibitory and lethal concentration in the 96-well microtiter plate. The anti-biofilm effect of the isolates was investigated by the 96-well microtiter plate method. The best isolates were molecularly identified in terms of anti-biofilm properties by the ribotyping method. Out of 30 *E. faecalis* bacteria samples, 12 samples (40%), 10 samples (33.33%), and 7 samples (23.33%) produced strong, moderate, and weak biofilm, respectively. 1 sample (3.33) was unable to form biofilm. From traditional buttermilk samples, 9 lactic isolates were isolated, all of which had antagonistic and anti-biofilm effects. According to molecular identification, D4 (*Lactobacillus fermentum*) was identified with the highest anti-biofilm effect against *E. faecalis*. Based on the results of this research, the lactic isolate (*L. fermentum*) with antagonistic and anti-biofilm properties can be a candidate for the treatment of urinary infections caused by *E. faecalis* in the future.

INTRODUCTION

Urinary tract infections (UTIs) are a common problem affecting millions of people worldwide. One of the most common bacteria causing UTIs is *Enterococcus faecalis*. This bacterium is a gram-positive, facultative anaerobe that is commonly found in the gastrointestinal tract of humans and animals. While *E. faecalis* is typically harmless in the gut, it can cause serious infections when it enters other parts of the body, including the urinary tract [1]. Urinary *E. faecalis* infections are a significant health concern, particularly in hospitalized patients and those with compromised immune systems. These infections can be difficult to treat, as *E. faecalis* is often resistant to multiple antibiotics [2]. The pathogenesis of *E. faecalis* UTIs is complex and not yet fully understood. However, several factors have been identified that contribute to the ability of *E. faecalis* to cause UTIs. These include the ability of the bacteria to adhere to and invade the urinary tract epithelium, the production of virulence factors, and biofilm formation [3]. One of the important features of *E. faecalis* in pathogenicity is ESP

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protein, cytolysin, and aggregates that play a vital role in the biofilm formation and pathogenesis of this bacterium. In hospital environments, microbial biofilm on various surfaces is considered a reservoir of infection transmission, and *Enterococcus*, especially *E. faecalis*, is the cause of 65% of hospital infections caused by biofilm, which causes the bacteria to become resistant to antibiotic treatments [4]. In recent years, bacterial resistance mechanisms (e.g., uropathogenic bacteria) have resulted in serious efforts to find out new therapeutic strategies such as anti-adhesive strategies, microbiota transplantation, nanomaterials, phage therapy, probiotics consumption and vaccination [5].

Buttermilk (Dough) is an acidic dairy drink and one of the ancient dairy products of Iran, which can be considered a low-viscosity drinkable yogurt pamphlet. Fermented dairy products are important because they are rich in lactic acid bacteria (LAB) [6]. Lactic acid bacteria are a large family of bacterial genera in the Firmicutes, Gram-positive, rod or cocci, and chemoautotrophs that require vitamins, amino acids, growth factors, and many nutrients for growth. They are a diverse group of bacteria that are commonly found in fermented foods and dairy products. These bacteria are known for their ability to produce lactic acid, which gives fermented foods their characteristic sour taste and helps to preserve them. In addition to their role in food fermentation, LAB has also been studied for its potential health benefits, particularly as probiotics [7]. The lactic acid bacteria are considered potential probiotic candidates. The most important criteria for the LAB strains to be used as probiotics include the following: (1) being safe, (2) resistant to low pH and high bile concentration and surviving in gastrointestinal fluids, (3) adhesion characteristics, (4) antagonistic activity, and (5) surviving during storage. Lactic acid bacteria with probiotic potential can antagonize pathogenic bacteria and compete with them to obtain food, and binding site on epithelial cells. They inhibit the production of toxins by pathogenic groups or other protective activities through the regulatory effect of the immune system [8].

This research aims to molecular identification of the most effective lactic acid bacterium from locally sourced buttermilk in the Abali region and evaluate its potential as an anti-biofilm agent against *E. faecalis*, a common cause of urinary tract infections.

MATERIALS AND METHODS

Bacterial strain and Sample preparation

30 samples of *Enterococcus faecalis* were prepared from Islamic Azad University, Damghan branch, and cultured on Muller Hinton Broth (MHB). The culture media used in this research was obtained from Merck Company, Germany. To isolate lactic acid bacteria, 10 samples of buttermilk (Dough) were collected from the AbAli region and transported to the laboratory by Cool Box according to the standard principles of microbiology (transfer under sterile conditions and temperature of 4°C).

Biofilm Formation of E. faecalis

To assess the biofilm formation of *E.faecalis* semi-quantitative adhesion test on a microtiter plate was done. Briefly, an overnight culture of each *E.faecalis* in Trypticase-Soy Broth (TSB) was diluted 1:100 in new TSB containing 2% glucose. 200 microliters of this medium were added to the wells and incubated at 37 °C for 24 h. The well containing TSB culture medium alone was used as a negative control. After emptying and washing wells with phosphate buffer saline, wells were filled with 200 microliters of 96% ethanol for 15 minutes to fix the samples. The contents of the wells were emptied and the microtitre plate was dried at laboratory temperature. The wells were stained with 2% crystal violet for 5 minutes and washed with city water. Acetic acid solvent 33% (200 microliters was added to the wells and incubation was done for 15 minutes at 37°C. The optical absorbance of the wells was read at 492 nm by a Microtiterplate reader (Biot Trend, Cat# NB-12-0035). The formation of biofilm was calculated based on the following formula:

Biofilm Formation (BF) = Stained attached Bacteria (AB) - Stained Control Wells (CW)

The OD values <0.100 and 0.100 – 0.199 were regarded as negative and weak biofilm-producing *E. faecalis*, respectively. The OD 0.200 – 0.299 were considered moderate biofilm producers (MP). An OD value ≥ 0.300 was indicative of strong biofilm-producing *E. faecalis* [9].

Isolation and purification of lactic acid bacteria

First, the enrichment of lactic acid bacteria was done by adding 2 ml of each Dough sample in an MRS broth culture medium containing 0.1 g of nystatin at 37 ℃ for 48 hours in an anaerobic Jar (two Times). Isolation of lactic isolates was done based on dilution (pour plate method) with dilutions of 10^{-1} to 10^{-10}) in MRS broth. One milliliter of each dilution was poured into empty plates and the sample was slowly mixed with the melted MRS. The plates were incubated at 37 ℃ for 48 hours. Purification of lactic acid bacteria was done by strict method from single colonies with different appearances (shape, color, and consistency) on MRS agar, and purification was repeated three times. The glycerol stock of each purified isolate was prepared and stored at -20°C [10].

Identification of lactic acid bacteria

In order to observe microscopically, gram staining was done and the composition and shape of the isolates were examined with a light microscope. To confirm the basic biochemistry of the isolated bacteria, catalase tests were performed using 3% hydrogen peroxide and oxidase tests were performed using a disc containing reagent. The isolates that were negative for the presence of catalase and oxidase enzymes were selected for additional biochemical tests as follows:

Briefly, the motility ability test was done in Sulfide Indole Motility medium (SIM) [11], Sugars fermentation, (Glucose, lactose, galactose, dextrin, arabinose, trehalose, sucrose, maltose, sorbitol, mannitol, and xylose), by isolated bacteria, was investigated in phenol red broth [12]. The tolerance of isolates to acidic conditions was investigated in pH (2.5, 4.5 and 6.5) in the MRS broth medium and the bacterial population was counted by serial dilution method. The hemolytic power of the isolates was investigated on a blood agar culture medium, and temperature tolerance of 15 to 45°C was performed. In all test incubation condition was at 37°C for 48 hours [13].

Supernatant preparation

In order to prepare the supernatant of isolates, 50 ml of 24-hour cultivation of each isolate in MRS broth culture medium was poured into sterile falcons. This process was repeated once again by transferring the supernatant to a new sterile falcon until a single-handed and clear liquid was obtained. Sterilization of the clear liquid was done using a milliliter filter 0.22-micron filter and stored at - 20°C. This supernatant was used for the antagonistic and anti-biofilm effects of isolates [14].

Minimum growth inhibitory concentration (MIC) and lethal concentration (MBC) of isolates against E. faecalis

The investigation of (MIC) and (MBC) of the supernatant of isolates against *E. faecalis* was carried out by the 96-well microtiter plate method. First, the wells were filled with 50 microliters of MHB medium, 50 microliters of the supernatant of each isolate at concentrations of 1000-16.25 µg ml $^{-1,}$ and 50 microliters of *E. faecalis*. The negative control well contained only the culture medium and the positive control well contained the culture medium and pathogen. The first well in which turbidity was not observed was considered as MIC. To determine MBC, 1µl of each well (without growth) was cultured on the MHA medium and incubated at 37°C for 24 hours. After incubation, the lowest concentration of lactic isolate supernatant that stopped the bacterial growth was considered as MBC. Ciprofloxacin was used as a control [15].

Anti-biofilm effect of isolates against E. faecalis by 96-well microtiter plate method

In order to investigate the anti- biofilm effect of the isolates, 80 µl of *E. faecalis* (McFarland half) samples producing strong biofilm, and 80 µl of the supernatant of each lactic acid isolate were transferred to the wells of a 96-well microtiter plate. The control wells contained *E. faecalis* and MHB medium. The incubation was done at 37°C for 24 hours. The wells were empty and washed with phosphate buffer twice. The wells were fixed with 96% ethanol for 15 minutes and stained with 2% crystal violet for 10 minutes. The wells were washed with city water and after the wells dried, 100 µl of 33% acetic acid was added to them. The amount of light absorption was measured at a wavelength of 490 nm, and the anti-

biofilm percentage was calculated from the formula below [16].

Antibiofilm effect % = [(absorbance of control well – absorbance of test well) / absorbance of control well] × 100

Molecular identification of the best lactic acid bacterium with anti-biofilm effect

DNA Extraction

First, genomic DNA extraction of the best isolate with the highest anti-biofilm effect against *E. faecalis* producing a strong biofilm was performed using the Cinnagen extraction kit EX6081. The quality of the extracted DNA was evaluated using electrophoresis on agarose gel. 3 microliters of the extracted DNA were mixed with 1 microliter of sampling buffer and loaded into the wells by a sampler and connected to an electrical source with a voltage of 95 volts for 40 minutes. From this time, the gel was removed from the tank and after staining with ethidium bromide, the quality of the bands was examined using a transilluminator device (Biorad, UAS).

PCR Reaction

To perform the PCR reaction, the 16S rRNA gene primer (F-5′AGAGGTTCCTGAGCTCAG3′, R-5′ ACAGCTTCCTTGTTACGATT3′) sequence was used, which was designed using the oligo7 software and the NCBI website. The PCR reaction was performed using the Taq DNA Polymerase Master Mix kit (Amplicon). The PCR reaction (30 cycles) temperature program included the primary denaturation step (180 seconds at 95°C,), secondary denaturation (60 seconds at 95°C), annealing (30 seconds at 52°C) extension (30 seconds at 72°C), and final extension (180 seconds at 72°C). The PCR product (1 microliter) along with 3 microliters of loading dye was electrophoresed on a 2% agarose gel with a current of 95 volts for 45 minutes, and the identity of the resulting bands was determined by comparison with a molecular weight marker of 10 bp .

Phylogeny tree

The sequence related to the isolated strain and other lactic acid bacteria sequences held in the Gene bank with a high percentage of similarity with the desired sequence as well as a non-similar sequence were entered into the MEGA7 software and the phylogenetic tree was drawn using the Neighbor Joining method.

Statistical analyses

Experiments were done with three repetitions and data analysis was done using SPSS version 23 software and graphs were drawn by calculating standard deviation using Excel software.

RESULTS

Biofilm formation of E. faecalis

Based on the results obtained, 12 samples (40%), 10 samples (33.33%), and 7 samples (23.33%) were able to produce strong, moderate, and weak biofilm, and one sample (3.3%) lacked the ability to form biofilm. Biofilm formation by 30 samples of *E. faecalis* is shown in Figure 1.

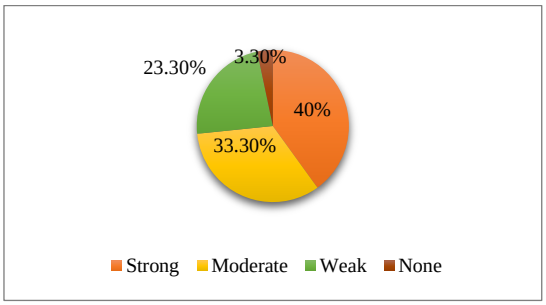

Figure 1. Biofilm formation of *E. faecalis.*

Identification of lactic acid bacteria

From 10 local Dough samples, 13 catalase and oxidase negative isolates were obtained, which were coded from D1 to 13 for the convenience of reporting the results. Based on the morphological study, the colonies of

isolates D1, D7, and D12 were milky, and the other isolates were white. All isolates were gram-positive bacilli, except D2, D10, and D5, which were observed as cocci (Figure 2).

Figure 2. A) Milky Colony B) Bacilli form C) Cocci form of one isolated lactic acid bacterium.

The results of sugar fermentation by isolates showed that all of them had the ability to ferment glucose, sucrose, galactose, and lactose. Among the isolates, only D12 did not have the ability to ferment arabinose, xylose, mannitol, and sorbitol sugars simultaneously. Isolated lactic acid bacteria D3, D4, D7, and D10 were able to ferment all sugars. D1and D9 isolates only lacked the ability to ferment sorbitol sugar (Table 1).

Table 1. Fermentation of sugars by isolated lactic acid bacteria

Isolates / Sugars	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	-	+	+	+	+	-
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	-	+	+	+	+	-	+	-	-	+	+	-	+
Dextrin	+	+	+	+	-	-	+	+	+	+	+		+
Xylose	+	-	+	+	+	-	+	+	+	+	-	-	+
Mannitol	+	+	+	+	-	+	+	+	+	+	-	-	-
Arabinose	+	+	+	+	-	+	+	+	+	+	-	-	-
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+
Trehalose	+	-	+	+	+	-	+	-	+	+	-	-	+

The growth results of the isolate at 15 and 45 °C showed that the isolates are different in terms of growth at these two temperatures, and among the isolates, only D11 was not able to grow at temperatures of 15 and 45 °C, and its growth was observed between 35 and 40 °C. In terms of hemolytic activity, none of the isolates were able to hemolysis red blood cells in the blood agar culture medium. According to the results of the acid tolerance test, all isolates were able to grow at three pH levels with a microbial population equal to and greater than 10^6, except for D2, D6, D11, and D12, which were unable to grow and tolerate a pH equal to 2.5 and 4.5, and therefore These were excluded from the group of lactic acid bacteria to conduct antagonistic and anti-biofilm tests (Table 2).

Table 2. Temperature and pH tolerance and hemolytic activity of isolated bacteria

Isolates / Tests	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13
Growth at 15°C	-	+	+	-	-	+	-	+	-	+	-	+	+
Growth at 45°C	+	-	-	+	+	-	+	-	+	+	-	-	+
pH 2.5	+	-	+	+	+	-	+	+	+	+	-	-	+
pH 4.5	+	-	+	+	+	-	+	+	+	+	-	-	+
pH 6.5	+	+	+	+	+	+	+	+	+	+	+	+	+
Hemolytic activity	-	-	-	-	-	-	-	-	-	-	-	-	-

Antagonistic property of supernatant of isolates

The results of MIC and MBC of isolates supernatants showed that isolate D4 had the most inhibitory and lethal effect against *E. faecalis*. The least inhibitory and lethal effects were related to D5, and no inhibitory and lethal effects were observed in D9. The effect of MIC and MBC of isolated lactic acid bacteria supernatant against *E. faecalis* has been shown in Figures 3 and 4.

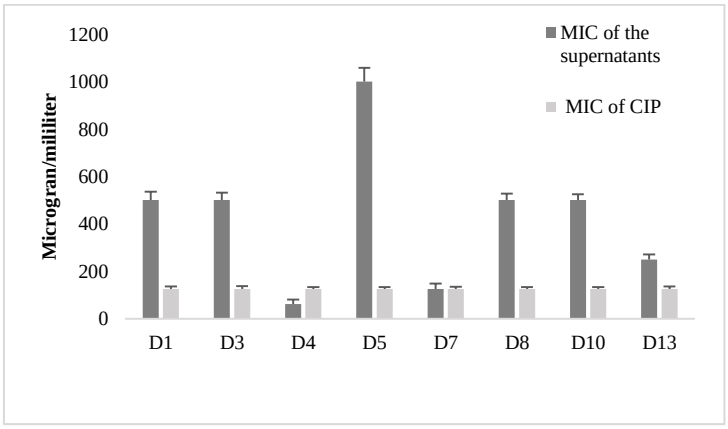

Figure 3. MIC of the supernatant of lactic acid bacteria against *E. faecalis.*

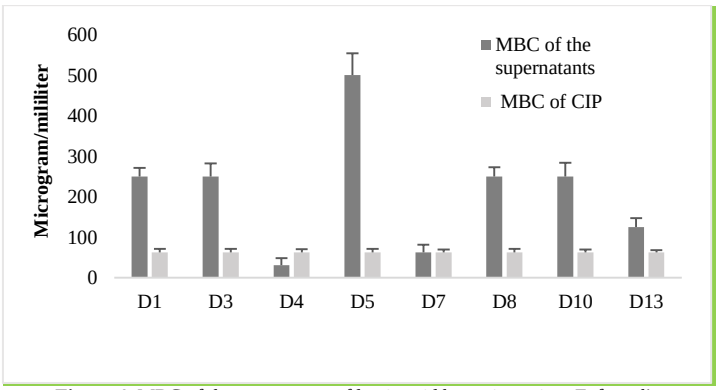

Figure 4. MBC of the supernatant of lactic acid bacteria against *E. faecalis.*

The effect of isolated lactic acid bacteria against E. faecalis biofilm formation

According to the results, all isolates had an anti-biofilm effect against 12 E. *faecalis* with a strong biofilm. The highest anti-biofilm effect was obtained by isolates D4 with an average of (57%) and D13 with an average of (49%), respectively, and the least anti-biofilm effect was obtained by D3 and D9 with an average of (30%) respectively (Figure 5).

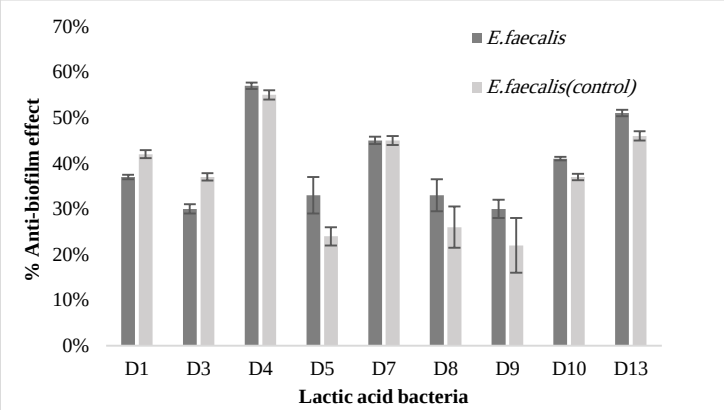

Figure 5. Anti-biofilm effect of isolated lactic acid bacteria against *E. faecalis.*

Molecular Identification

Lactic acid bacterium D4 with the most anti-biofilm effect against *E. faecalis* was molecularly identified using the ribotyping method (Figure 6).

Based on the comparison of similar sequences with the sequence of isolate D4 in NCBI and drawing a phylogeny tree, it was determined that D4 is *Lactobacillus fermentum* (Figure 7).

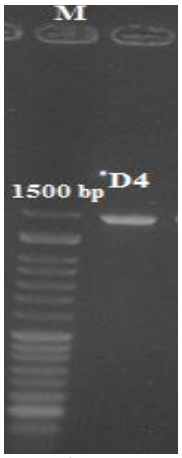

Figure 6. PCR product of 16S rRNA gene of isolate D4.

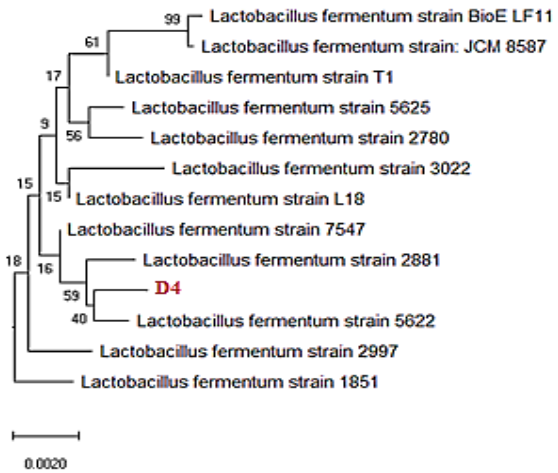

Figure7. Phylogenetic tree of nucleotide sequence of isolate D4.

DISCUSSION

Biofilm production is one of the most important virulent factors in pathogenic bacteria such as *E. faecalis.* The study revealed that 40% of the *E. faecalis* samples exhibited strong biofilm formation. This suggests that these strains possess robust mechanisms for adhering to surfaces and aggregating into structured communities. Strong biofilm formation is often associated with increased resistance to antimicrobial agents and host immune responses, making these strains potentially more virulent and difficult to eradicate [17]. Medium biofilm formation was observed in 33.33% of the samples. While not as robust as strong biofilm formation, medium biofilm-forming strains still possess the ability to adhere and form cohesive communities. These strains may exhibit intermediate levels of resistance and pathogenicity compared to those with strong biofilm formation. Weak biofilm formation was observed in 23.33% of the samples. Strains with weak biofilm-forming capabilities may have reduced adherence and aggregation abilities, potentially making them more susceptible to clearance by the immune system or antimicrobial treatments [17]. A small proportion (3.3%) of the samples were unable to form biofilms. This could be due to genetic variations or other factors that affect the expression of biofilm-related genes in these strains. These non-biofilm-forming strains may have different mechanisms of survival and pathogenicity compared to their biofilm-forming counterparts [18]. Understanding the distribution of biofilm-forming capabilities among *E. faecalis* strains is crucial for several reasons. Firstly, it provides insights into the potential virulence and persistence of these strains in clinical and environmental settings. Secondly, it can aid in the development of targeted strategies to prevent or disrupt biofilm formation, which could help in the management of *E. faecalis* infections. Lastly, these findings contribute to the broader knowledge of biofilm biology and its implications for bacterial pathogenesis [19].

Lactic acid bacteria play an important role in the production of fermented dairy products, as they are responsible for the conversion of lactose into lactic acid, which gives these products their characteristic sour taste and texture. Identifying LAB from local buttermilk samples is important for understanding the microbial diversity of these products and for ensuring their safety and quality [20]. In this study Out of 13 bacteria isolated from buttermilk, 9 acid bacteria were identified according to several experiments [21] that can be used to identify LAB from local buttermilk samples. Two important diagnostic tests for lactic acid bacteria include fermentation of carbohydrates and tolerance to acidic conditions equivalent to stomach acid. In our study, all isolates had the ability to ferment all or some sugars, but all of them had the potential to ferment glucose, sucrose, galactose, and lactose. LAB are known for their ability to ferment sugars, particularly lactose. However, they can also ferment other sugars such as glucose, fructose, and sucrose. Different LAB species have different sugar fermentation profiles, which can be used to identify those [22]. Lactic acid bacteria are divided into two groups: homofermentative and heterofermentative lactic acid bacteria. The first group of glucose fermentation produces only lactic acid, such as *Lactococcus* and *Streptococcus* genera. The second group of glucose fermentation, in addition to lactic acid, also produces carbon dioxide, ethanol and acetic acid, such as *Leuconostoc* and *Lactobacillus* genera [12]. In the present study all of the lactic acid bacteria had the ability to tolerate acidic pH 2.5, except for for D2, D6, D11, and D12. The high resistance of lactic acid bacteria to low pH is due to the production of compounds such as exopolysaccharides which prevents the effect of acid on the cell membrane. The pH range is of great importance in the survival of lactic acid bacteria, it is the reason for the superiority of these bacteria compared to bacteria that do not know this ability [16].

The results of the antagonistic properties of the supernatant of the isolates showed that isolate D4 had the highest inhibitory and lethal effects and isolate D5 had the lowest inhibitory and lethal effects against *E.faecalis*. Antimicrobial compounds that are produced individually or in combination by lactic acid bacteria, such as fatty acids, organic acids, hydrogen peroxide, diacetyl, acetone, and bacteriocins, by affecting the competition of energy resources and chemical compounds, the reproduction of pathogenic microorganisms causes their

growth or death [23]. The antagonistic properties of the supernatant of bacterial isolates have been widely studied due to their potential use as natural antimicrobial agents. In particular, the supernatant of bacterial isolates has been shown to exhibit antagonistic properties against a variety of pathogenic bacteria, including *Enterococcus faecalis* [24]. A study by Elshikh et al; 2016 [25], exhibited a strong inhibitory effect of *lactobacillus plantarum* supernatant against *E. faecalis*, with MIC of 6.25% (v/v) and MBC of 12.5% (v/v) which was similar to the findings of our research. Reuben et al; 2020 announced that isolated lactic acid bacteria from Dough had antagonistic activity against *E. faecalis.* These studies demonstrate the potential of the supernatant of bacterial isolates against *E. faecalis* provide valuable information for the development of new antimicrobial agents. However, further research is needed to fully understand the mechanisms underlying the antagonistic properties of the supernatant of bacterial isolates and to optimize their use as antimicrobial agents [18].

Since attachment is the first step in the relationship between pathogenic bacteria and the target cell and is considered the most important stage of disease initiation and biofilm production, if it is possible to prevent the attachment of bacteria to the target cell, the disease can be easily overcome [16]. Since biofilms can cause persistent infections and are often resistant to antibiotics, making them a significant challenge in healthcare settings [17]. In the present study, all isolates had anti-biofilm effect against *E.faecalis*. The highest effect was related to D4 and D13 isolates and the lowest effect was related to D3 and D9 isolates which was silamr to the finding of Alipor et al; 2021[26]. Some studies indicating their potential as alternative therapies for biofilm-related infections [27, 28]. The results of this study are significant because they suggest that certain bacterial isolates may have the ability to disrupt biofilms and potentially serve as a treatment for biofilm-related infections. This is particularly important given the increasing prevalence of antibiotic-resistant infections and the need for alternative therapies.

CONCLUSIONS

Our findings highlight the potential of lactic acid bacteria, particularly strain D4, in combating biofilm formation by *E. faecalis* and suggest *Lactobacillus fermentum* strain as a promising therapeutic option for UTIs caused by this pathogen. Further research should be focused on exploring the underlying mechanisms of anti-biofilm activity and evaluating the efficacy of this strain in clinical trials.

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Conflict of interests

The authors declare no conflict of interest.

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