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Evaluation of the probiotic potential of *Lactobacillus brevis* and *Lactococcus lactis* isolated from yellow curd native to southeast Iran

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ABSTRACT

Background: Traditional dairy products can be a very suitable reservoir for beneficial microorganisms such as probiotics. This study aims to isolate lactic acid bacteria from yellow curd, a traditional dairy product of Sistan and Baluchestan province in southeast Iran. **Methods:** The samples were cultured in MRS agar and suspicious colonies, gram-positive and catalase-negative, were identified using some biochemical tests including sugar fermentation, growth at 10 and 45°C, and 6.5% NaCl. Antibacterial activity of culture supernatant of isolates against four gastrointestinal pathogens was performed by disk and well methods. Also, the tolerance of isolates to acidic conditions and bile salts was measured. **Results:** Two isolates were identified, and after identification procedures, they were identified as *Lactobacillus brevis* and *Lactococcus lactis*. The antibacterial activity of the two isolates was observed, especially by the well method. The diameter of the inhibition zone of *E. coli*, *S. dysenteriae*, *S. aureus*, and *B. cereus* in contact with *L. brevis* culture supernatant was 16, 12, 12, and 8 mm respectively. *L. brevis* showed more antibacterial activity in both disc and well methods than *Lc. lactis*. The results of the tolerance tests of bile salts and acidic pH for LAB isolates indicated that *L. brevis* was more tolerant than *Lc. lactis*. **Conclusions:** In general, yellow curd produced by traditional methods can be considered beneficial carriers for bacteria with probiotic potential and in addition, the probiotic potential of *L. brevis* isolate was evaluated favorably.

Introduction

Lactic acid bacteria (LAB) are the largest group of probiotics, mainly found in dairy products, vegetables, etc. They are rod or spherical, gram-positive, non-motile, catalase-negative bacteria and lactic acid is their main product of carbohydrate fermentation. Important lactic acid bacteria in the dairy industry belong to the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, and *Enterococcus* [1,2].

Lactic acid bacteria, added to milk as a starter culture, exist naturally in milk and play an essential role in the taste and aroma of dairy products [1-4].

On the other hand, some lactic acid bacteria can prevent the growth of pathogenic bacteria. For

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this reason, these bacteria are essential from a technological point of view [3,4].

Probiotic bacteria are characterized by antibacterial activity against bacteria that cause digestive diseases, the ability to tolerate acidic conditions, and tolerance of bile salts [1-4].

They are living microorganisms with many effects, such as improving the immune system, preventing the establishment and growth of pathogenic bacteria, reducing cholesterol absorption, and reducing the possibility of colon cancer [1-4].

Nowadays, many studies have been done to add probiotic bacteria to dairy products and to study the effect of these bacteria on consumer health. Some types of lactic acid bacteria, such as probiotics, can be used to treat and control some diseases. Probiotics effectively inhibit pathogenic bacteria through several mechanisms, such as the production of enzymes, vitamins and the reduction of pH. In dairy products, including native (traditional) dairy products, the dominant bacteria are lactic acid bacteria. Many lactic acid bacteria create compounds during metabolic processes, such as organic acids, compounds with low molecular weight, such as hydrogen peroxide, carbon dioxide, and diacetyl compounds with high molecular weight, such as bacteriocins, fatty acids, etc., which can have antimicrobial effects against other microorganisms. For this reason, these bacteria are essential from a technological point of view [1-4].

Among the people of Sistan and Baluchistan province, in the southeast of Iran, a traditional dairy-cereal fermented product is widely consumed, which is known as Zaboli yellow curd.

This traditional dairy product is one of the nutritious foods of the Sistan people, which is made of sour buttermilk, wheat flour, and herbal spices such as cumin, dill egg, coriander, black pepper, garlic, turmeric, and onion. This collection is kept in a cotton bag for 5 days to a week, then it is kneaded and powdered. Zaboli yellow curd is cooked as a complete and nutritious meal in a short period. Local people fry it with onion and oil, add water and boil it then eat. For those who people have a cold or diseases such as diabetes, blood fat, constipation, and kidney diseases, it is useful. Due to the presence of buttermilk and wheat flour, the presence of lactic acid bacteria in this fermented product is possible, and fermentation is carried out by the inherent microorganisms in the raw materials.

During this study, the existence of this species was investigated to isolate the dominant lactic acid bacteria in yellow curd native to the southeastern region of Iran (Zabol) and investigate their probiotic potential. The bacteria are among the candidates isolated from an indigenous and unique curd in Iran, called yellow curd, which is native to the southeast of Iran, and it has received more attention due to its specificity and different cooking methods, as well as the studied strains for a specific reason [1-4].

This study aims to isolate and identify lactic acid bacteria from yellow curd and evaluate their probiotic potential. For this purpose, the ability to inhibit four gastrointestinal pathogenic bacteria, including *Escherichia coli*, *Staphylococcus aureus*, *Shigella dysenteriae*, and *Bacillus cereus*, and to investigate the ability of these isolates to tolerate acidic conditions and bile salts isolates were carried out.

Materials and methods

Isolation lactic acid bacteria

Two distinct local samples were randomly selected from Zabol County, Sistan, and Baluchestan province production areas. The samples were to be enriched for bacteria in the Curd samples; a dilution series was prepared using Ringer's solution. One hundred microliters of each dilution were inoculated into 1 ml of De Man–Rogosa–Sharpe agar (MRS) broth containing nystatin (50µ/ml), an antimicrobial agent inhibiting unwanted yeasts' growth. The inoculated tubes were incubated in a candle jar at 37°C for 48 to 72 hours to allow the bacteria to grow and multiply. Gram-staining and catalase tests were performed on white convex and smooth colonies. Gram-positive and catalase-negative bacteria were purified, and identification was performed to keep isolates from MRS broth medium containing 10% glycerol at -70°C [2].

Identification of isolates of lactic acid bacteria

The isolates were identified by different sugar fermentation tests (arabinose, xylose, galactose, sorbitol, fructose mannitol, mannose, rhamnose, raffinose, maltose, lactose, and sucrose) and other tests such as growth at 10 and 45 °C and growth ability at 6.5% NaCl. It was used the MRS broth medium containing the Phenol red reagent and 2% of each of the sugars to investigate the fermentation of sugars. To create an anaerobic condition, the medium was coated with sterile liquid

paraffin, and after 4 days of incubation at 37°C, the fermentation of the sugar was confirmed by observing the color change from red to yellow. To evaluate the growth at 10 and 45°C, each isolate was inoculated in two tubes containing MRS broth medium, and anaerobic conditions. One of the tubes was incubated at 10°C for 7 days and the other at 45°C for 24-48 hours, and after this period, the growth of the isolate was confirmed by investigating turbidity in comparison with the negative control tube. To assay growth at 6.5% NaCl, each isolate was inoculated in MRS broth containing 6.5% NaCl and incubated at 37°C for 48 h under anaerobic conditions. After this period, by investigating turbidity in comparison with the control tube (negative), the growth of isolates was confirmed [2,3,5-7].

Preparation of supernatant for lactic acid bacteria

To prepare the supernatant solution of isolated Lactic acid bacteria cultures, was several colonies of 24 hours' cultivation of the isolates inoculated in the MRS broth medium and then coated with sterile liquid paraffin and incubated for 4 days at 37°C. During this time, isolated lactic acid bacteria produced antimicrobial compounds, then the paraffin was removed, the tubes were centrifuged for 10 minutes at 4000 rpm, and the supernatant was transferred to a sterile tube for antimicrobial testing. To ensure the absence of cells in the supernatant solution, the supernatant was filtered by a syringe filter with a diameter of 0.2 μm [2,3,7].

Pathogenic bacteria

The Pathogenic bacteria were provided from the culture collection of the Iranian Research Organization for Science and Technology (IROST) in a lyophilized form (**Table 1**).

Tests for determination of lactic acid susceptibility to bile salts

To determine the susceptibility of the isolates the method provided by Liong and Shah was used (2005). For this purpose, all isolates were cultured in MRS broth overnight. Then, 100 μl of each bacterium was transferred separately to tubes containing 20 ml of MRS broth medium and 0.3% bile salts or oxgall, and a tube was considered as a control that lacked any oxgall substances. In the next stage, all tubes were cultured in anaerobic conditions in an atmosphere containing 5% carbon dioxide gas. The growth rate of bacteria was measured every half hour to 10 hours using a spectrophotometer at 620 nm wavelength. The tolerance of each bacterium during this process is equal to the time it takes to increase the absorption of 0.3 units of the tube containing oxgall compared to the control tube [8].

Determination of lactic acid susceptibility to Acidic pH

The isolated strains were grown in liquid MRS medium at 37°C for 24 hours. Then, one ml of each bacterial culture was inoculated in 9 mL PBS with a pH of 2.5 and incubated for 3 hours at 37°C **.** To determine the survival percentage, the colony forming unit (CFU) was determined at the moment of inoculation and the end of 3-hour incubation in phosphate-buffered saline (PBS) inorganic medium for each strain separately. For this purpose, at zero time (moment zero) and the end of the initial bacterial culture and inoculated in PBS medium, two cultures were done as pour plate and plates were incubated in low aerobic condition at 37°C for 24 hours. At the end of the incubation period, plates with a countable number of colonies and normally plates with 30 to 300 colonies were counted and the number of colonies per plate was recorded and recorded [8].

Evaluation of the antibacterial activity of isolates against gastrointestinal pathogens

Antibacterial activity of culture supernatant of the isolates against four gastrointestinal pathogens including *E. coli*, *S. dysenteriae*, *S. aureus*, and *B. cereus* was performed based on diffusion in agar and using disk and well methods. A uniform culture was performed from the suspension of 0.5 McFarland (1.5×10^8 CFU/ml) of the studied pathogens, separately by sterile swaps on Muller-Hinton agar medium. Using a sterile Pasteur pipette, wells with a 6 mm diameter were drilled in the medium, and 100 μl of the supernatant solution of lactic acid bacteria isolated were poured into the wells and incubated for 24 hours at 37°C. After this period, the diameter of the inhibition zone was measured and recorded based on millimeter (mm) [9-11].

Results

Isolation of lactic acid bacteria from yellow curd

The results of this study showed that the culture of Zaboli yellow curd on MRS agar medium resulted in two distinct colonies based on morphological characteristics. These two forms of

colonies were purified and identified using morphological and biochemical tests **(Figure 1)**.

The staining of purified colonies on MRS medium indicates the presence of a gram-positive bacillus and a gram-positive cocci species encoded with the names k1 and k2, respectively, and their microscopic image is visible **(Figure 2)**.

Based on sugar fermentation tests and growth at 10 and 45°C and growth in the presence of 6.5% NaCl, isolates, *Lactococcus lactis* and *Lactobacillus brevis* were identified.

Results of antibacterial activity of lactic acid bacteria isolates

Based on the disk method and the results of determining the diameter of the Inhibition zone, it was found that the Lactic acid bacteria isolated had an inhibitory effect on *E. coli* and *S. dysenteriae* pathogens, for the other two pathogens, no inhibitory effect was observed.

Evaluations showed that *S. dysenteriae* was only sensitive to one *Lactobacillus brevis* with an 11 mm diameter of inhibition**.** The diameter of the inhibition zone of *E. coli* when exposed to the culture supernatant of *Lactobacillus brevis* and *Lactococcus lactis* was 10 and 8 mm, respectively. In the well method, it was found that all four pathogens tested were sensitive to culture supernatant of lactic acid bacteria isolates obtained from curd samples.

The antagonistic effects in the presence of lactic acid bacteria showed that in the well method, *E. coli* is the most susceptible bacteria to lactic acid bacteria isolates.

This bacterium with a diameter of inhibition zone 16 mm in the presence of *Lactobacillus brevis* and 13 mm in the presence of *Lactobacillus brevis* was inhibited**.** *S. aureus* showed the diameter of inhibition zone 12, and 10 mm, respectively against the culture supernatant *Lactobacillus brevis* and *lactococcus lactis*. *S. dysenteriae* also showed similar behavior to *S. aureus* and against with culture supernatant of *Lactobacillus brevis* and *lactococcus lactis*, the diameter of the zone of inhibition was 12 and 10 mm, respectively. *Bacillus* cereus was more resistant than other species, with an inhibitory zone diameter of 8 mm for *Lactobacillus brevis* while *lactococcus* did not affect it **(Table 2,3) (Figure 3)**.

Bile salts tolerance of lactic acid bacteria isolates

The results of the bile tolerance test for lactic acid isolates obtained from Zaboli yellow curd are presented in **(Table 4)**. Based on the obtained results, *Lactobacillus brevis* showed more tolerance to bile salts compared to *Lactococcus lactis*.

Acidic pH tolerance of lactic acid bacteria isolates

Based on the observations, it was found that *Lactobacillus brevis* has more and longer resistance in the presence of acidic PBS compared to another probiotic species isolated from the curd, *Lactococcus lactis* in exposure to simulated acidic conditions of the stomach, it has less resistance **(Table 5)**.

For plates inoculated by liquid culture at zero time and before incubation of bacteria-containing PBS, maximum growth in plates (colony count >300) was observed indicating the viability and activity of lactic acid bacteria under investigation before exposure to acidic conditions for three hours. After the 3 hours' incubation of bacteria-containing PBS, their growth rate and reproduction decreased. So, in zero time(T0) was non-countable (colony count >300) but after 3 hours in acidic condition, log CFU/ml for *L.brevis* and *Lc. lactis* was 3.44 and 2.95, respectively.

Table 1. Pathogenic bacteria used in this study

Row	Bacteria standard code	Bacteria name	Morphology and gram stain
1	PTCC 1154	*Bacillus cereus*	Gram positive bacilli
2	PTCC 1112	*Staphylococcus aureus*	Gram-positive cocci
3	PTCC 1338	*Escherichia coli*	Gram-negative bacilli
4	PTCC 1188	*Shigella dysenteriae*	Gram-negative bacilli

Table 2. The diameter of the inhibition zone of pathogenic bacteria in the presence of culture supernatant of LAB isolates in the well method (mm)

Pathogenic Bacteria Isolate LAB	*Bacillus cereus*	*Staphylococcus aureus*	*Escherichia coli*	*Shigella dysenteriae*
Lactobacillus brevis	8	12	16	12
Lactococcus lactis	-	10	13	10

Table 3. The diameter of the inhibition zone of pathogenic bacteria in the presence of culture supernatant of LAB isolates in disk diffusion method (mm)

Pathogenic Bacteria Isolate LAB	*Bacillus cereus*	*Staphylococcus aureus*	*Escherichia coli*	*Shigella dysenteriae*
Lactobacillus brevis	-	-	10	11
Lactococcus lactis	-	-	8	-

Table4. results of determining the The tolerance of lactic acid bacteria isolated from yellow curd against bile salts

Isolate LAB	**T1**	**T2**
Lactobacillus brevis	3.0	5.5
Lactococcus lactis	2.5	3.5

T1: The time required to increase the absorption light of each bacterium by 0.3 in broth culture medium MRS. (control medium)
T2: Time to increase 0.3 light absorption for each bacterium in MRS broth medium containing 0.3% of oxgall.

Table 5. The results of determining the resistance of Lactic acid bacteriaisolated from yellow curd in acidic conditions

Isolate LAB	**Number of bacteria at time T0 log10 CFU/ml**	**Number of bacteria at time T1 log10 CFU/ml**
Lactobacillus brevis	NC	3.44
Lactococcus lactis	NC	2.95

T0: Zero time; Moment of culture from liquid medium to solid before incubation
T1: After 3 hours incubation of broth environment at 37°C
NC: Not Countable (colonies numbered more than 300 per plate)

Figure 1. Lactobacillus purified colony on MRS agar medium

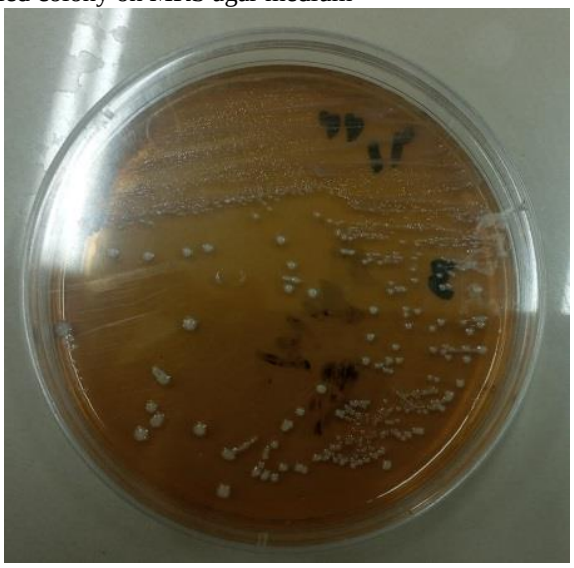


Figure 2. Gram staining results for purified isolates from yellow curd samples

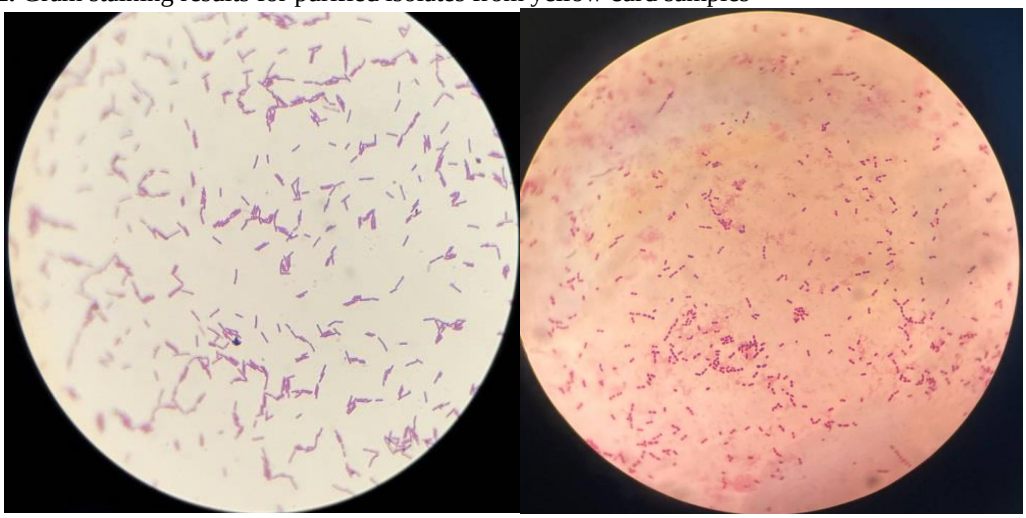
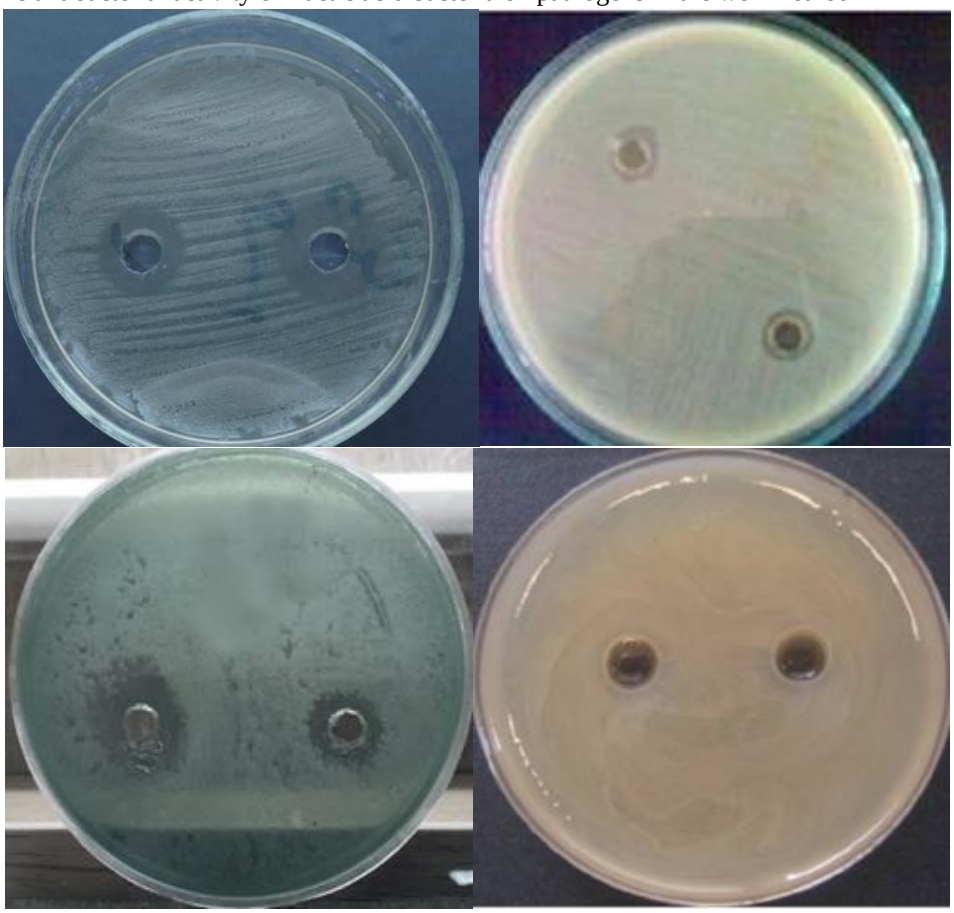


Figure 3. The antibacterial activity of Lactic acid bacteria on pathogens in the well method



Discussion

In this study, the effect of lactic acid bacteria isolated from traditional yellow curd prepared in the Zabol region in Sistan and Baluchestan province was evaluated in vitro environment on the gastrointestinal pathogens of *B. cereus, S. aureus, E. coli, and S. dysenteriae* and then the resistance of probiotic isolates intolerance to acidic pH and bile salts was evaluated.

Novel and colleagues from traditional dairy products identified 18 isolates Of *Lactobacillus plantarum*, *Lactobacillus brevis*, and *Lactobacillus paracasei*, all of which were resistant to gastric acid and showed high antagonistic activity against pathogenic bacteria.

According to the researches carried out by Durlu-Ozkaya et al., it was also found the most important characteristics of probiotic bacteria in each genus and species are acid tolerance and resistance to bile salts determining these characteristics can investigate the differences among probiotic strains. In the present study, both of these characteristics were investigated and *Lactobacillus* was more resistant in the analysis tests performed by *Lactococcus* species, in addition, this species had more antibacterial effects on the studied pathogens [12].

The sensitivity of these Gram-negative bacteria, along with the gram-positive *Staphylococcus aureus*, to the culture supernatant of lactic acid bacteria isolates was also confirmed in the well method, and *Bacillus cereus* was the most resistant bacteria tested against the culture supernatant of lactic acid bacteria isolates in well method [12].

With the aim introduction of *Lactococcus lactis* as a species with probiotic potential in line with the results of the present research, De Chiara et al. in 2024 in Italy during studies investigated the probiotic potential of *Lactococcus lactis* species isolated from whey and the survival of this bacterium in simulated pH stomach, tolerance to intestinal bile salts and inhibition of *E. coli* (EIEC) and *Salmonella typhimurium* were investigated [13].

Their results showed that *Streptococcus lactis* strains are able to compete with pathogens and also the ability to exert a protective effect on cells previously infected with *E. coli* or *Salmonella Typhimurium* [13].

In 2019, Akbar et al. studied fermented milk in Thailand and determined the antibacterial activity of LAB against *Salmonella Typhimurium*, *Staphylococcus aureus*, *Escherichia coli*, and *Listeria monocytogenes*. *Lactococcus lactis* has a wide range antimicrobial spectrum compared to other isolates and the probiotic evaluation showed the survival of this isolate in acidic pH and bile salts [14].

The inhibitory activity caused by lactic acid bacteria is attributed to pH changes and the metabolites produced by these bacteria including antibiotics, bacteriocin, siderophores, lysozyme, protease, and organic acids. Bacteriocins produced by lactic acid bacteria have a killing or growth-inhibitory effect on sensitive bacteria [15].

The antibacterial activity of the culture supernatant of lactic acid bacteria isolated from curd samples can be attributed to acidic pH, as a result of the fermentation process and increasing the content of organic acids, such as lactic acid. Lactic acid penetrates through the cytoplasmic membrane and acidifies the cytoplasm and inhibits the activity of enzymes. At intracellular pH, most acids lead to the production of hydrogen ions, which interfere with important metabolic processes such as oxidative phosphorylation and inhibit aerobic species [16].

In addition to the role of organic acids in antibacterial activity, lactic acid bacteria can play a role in creating antibacterial activity by producing peptides (bacteriocins), carbon dioxide, hydrogen peroxide, ethanol, and diacetyl [17].

The difference between some of the results of this study and the results of other studies can be attributed to the different behavior of different strains and isolates of bacteria and the specific differences of each type of microorganism.

An essential step for antibacterial activity is crossing the outer membrane in gram-negative. The resistance of gram-negative bacteria, including *E. coli*, can be due to the lower permeability of the outer membrane compared to gram-positive bacteria, which limits the entry of antimicrobial agents into the bacterial cell [18].

One of the most important mechanisms of antimicrobial activity in lactic acid bacteria is the reduction of pH through the production of organic acids, including lactic acid, which is one of the most important mechanisms in the antibacterial activity against gram-positive and gram-negative bacteria [19].

Also, the effect of lactic acid produced on the permeability of the cytoplasmic membrane should be considered attributed [20].

The effect of lactic acid on the permeability of the outer membrane of *Escherichia coli* O157: H7, *Pseudomonas aeruginosa*, and *Salmonella enterica* serovar t*yphimurium* studied and showed that lactic acid, in addition to its antimicrobial properties, as a penetrant of the outer membrane of gram-negative bacteria due to the reduction of pH it also may act as an enhancer of the effects of other antimicrobial agents [21].

Chateau et al. tested the effect of bile salts on the growth of 38 isolates of lactic acid bacteria. Half of the isolates tested were mildly affected by 0.3% bile salts and showed a growth delay of less

than one hour to reach an optical absorbance of 0.3 at 600 nm in an MRS liquid culture medium compared to the control culture without bile salts [22].

A study by Soundharrajan et al. was carried out in Thailand in 2021 on traditional rice. According to the results of this research, in addition to antibacterial activity against gastrointestinal pathogens by well method, *Lactococcus lactis* isolates showed the ability to tolerate the acidic pH of the stomach and bile salts of the intestine [23].

Kwon et al also showed the probiotic properties of *Lactobacillus brevis* in the study of fermented dairy products from South Korea. This isolate showed antibacterial activity against foodborne pathogens and was able to survive in the simulated conditions of gastric juice and intestinal bile salts [24].

From the results of the present study, gram-negative bacteria were more sensitive to the culture supernatant of lactic acid bacteria. This has been reported in similar studies [3,25-28].

The difference in the behavior of gram-positive bacteria *S. aureus* and *B. cereus* compared to gram-negative bacteria *S. dysenteriae* and *E. coli* when confronted with the culture supernatant of lactic acid bacteria isolated from the yellow curd sample reminds us that this behavior change can be attributed to the characteristics of the wall structure of these bacteria. Gram-positive bacteria have a different wall than gram-negative bacteria. The main composition of the cell wall in gram-positive bacteria is peptidoglycan, while the amount of peptidoglycan in gram-negative bacteria is very low and most of the cell wall consists of an outer membrane containing lipid compounds, and this structure is not seen in the cell wall of gram-positive bacteria [21,29].

In the study of isolating probiotic bacteria from traditional dairy products, by 16SrRNA sequencing, *Lactobacillus brevis* was introduced, which, in addition to antibacterial activity against *S. aureus* and *E. coli*, also showed tolerance to acid conditions and bile salts [24].

Lactic acid bacteria such as *lactobacilli* and *lactococci* can be candidates as probiotic bacteria due to their digestive origin, antagonistic effects against gastrointestinal pathogens, tolerance to acidic conditions, and bile salts. Research in this field, which began in 1977 and continues to this day, reinforces the belief that probiotics and biotherapy should be used instead of antibiotics in the prevention and treatment of bacterial infections. Antagonistic results of lactic isolates obtained from whey and their resistance to acid and bile showed that, in general, *Lactobacillus brevis* acts better than *lactococcus lactis* isolates. Only visual differences were evaluated and no significant functional differences were observed between these isolates.

Conclusion

In general, the present study showed that traditional yellow curd native to the southeastern region of Iran (known as Zaboli curd), can be considered as a very suitable reservoir for the isolation of lactic acid bacteria with probiotic potential. Considering the significant antibacterial activity of *Lactobacillus brevis* against the examined pathogenic bacteria and the ability to grow in acidic conditions and different concentrations of bile salts, the probiotic potential of this isolate was evaluated favorably. Therefore, the addition of these bacteria as starters to industrial dairy products allows products with desirable characteristics to be marketed. Consumers can be used other benefits of these bacteria such as improved digestive health and boosting the immune system. Of course, more extensive tests are recommended to identify effective compounds and tolerate digestive conditions *in vivo*. Considering the studies and increasing knowledge about the mechanisms involved in interactions between lactic acid bacteria and pathogenic bacteria (food and clinical), a new strategy can be proposed for the biological control of dangerous microorganisms without the use of antibiotics, because this can significantly improve the balance of bacterial ecosystems *in vivo*. New strategies can control the spread of antibiotic-resistant strains. Also, there have been such developments and improvements in the treatment of some cancers of the digestive system, which is recommended to conduct more and more extensive research on this matter.

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Conflicts of Interest:

The authors declare no conflicts of interest.

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