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Isolation and identification of *Limosilactobacillus fermentum* ARD2 strain from local yogurt and evaluation of its probiotic, antimicrobial and safety properties

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ABSTRACT

The crucial role of food in life has prompted a shift towards products that not only offer good quality and nutritional properties but also ensure the overall health of consumers. Probiotics, which are non-pathogenic microorganisms, can restore intestinal microbial balance when consumed in sufficient quantities. In this study, Limosilactobacillus fermentum ARD2 isolated from local yogurt was investigated for its probiotic properties. Various aspects of this strain were evaluated, including acid resistance (at pH levels of 2, 3 and 4), resistance to bile salts (at concentrations of 0.3, 0.5 and 0.7 %), antimicrobial activity using disk diffusion agar and well diffusion agar methods, resistance to common therapeutic antibiotics, evaluation of antioxidant activity, cell surface hydrophobicity, DNase enzyme activity, hemolytic activity, and biogenic amine and cholesterol absorption. The results of the acid resistance tests indicated that the viability of L. fermentum ARD2 increased as pH levels rose from 2 to 4, with a decrease observed over time at a constant pH over 3 hours. Growth was inhibited with increasing concentrations of bile salts. Antimicrobial testing revealed that the acidic and neutralized cell-free supernatant (aCFS and nCFS) had no significant antimicrobial effect on Escherichia coli, while nCFS showed no antimicrobial effect on Shigella dysenteriae. L. fermentum ARD2 exhibited resistance to Ciprofloxacin and was semi-sensitive to Penicillin and Nitrofurazone. The L. fermentum ARD2 showed considerable antioxidant activity, with DPPH and ABTS free radical inhibition rates of 41.40% and 43.60%, respectively. Additionally, it demonstrated the ability to reduce cholesterol absorption by 38.60%. The strain tested negative for DNase and hemolytic activities, and biogenic amine production was not observed. Based on these findings, L. fermentum ARD2 exhibits promising probiotic characteristics and could be utilized as a probiotic bacterium in food products.

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1- Introduction

Food plays an important and fundamental role in improving health, and with the correct consumption of food, it is possible to prevent all kinds of diseases. On this basis, the change in lifestyle, increasing people's awareness, and their desire to have a healthy lifestyle have led to a turn towards foods that, in addition to having good quality and nutritional properties, have properties that guarantee human health and lead to reduced treatment costs caused by the occurrence of diseases [1]. Probiotic is derived from the word "prolife" meaning "for life." The World Health Organization proposed a general definition for this term, introducing probiotics as living and non-pathogenic microorganisms that, if consumed in sufficient and specific amounts (Colony Forming Unit/mL 10^7), will have favorable effects on the health of the host. In the past, probiotics were referred to as substances secreted by a microorganism that stimulate other microorganisms [2] and were somehow isolated from the host's native microbiota, but today they do not belong to the host's microbiota and are extracted from other sources such as fermented foods and fruits [3].

Resistance to stomach acid, digestive enzymes, processing and production steps, bile salts, being non-pathogenic and non-invasive, the ability to preserve and maintain genetic stability, the ability to bind to intestinal epithelial cells, and the ability to deal with pathogenic agents are some of the many characteristics of ideal probiotics. They also reduce the risk of diarrhea in children (caused by the use of antibiotics) and protect babies and infants from diseases such as inflammation, intestinal necrosis, and sepsis, and sometimes the occurrence of some respiratory diseases such as colds is prevented by consuming probiotic microorganisms [4]. In addition to the mentioned cases, improving and increasing the digestibility of milk in people with lactose intolerance, regulation of the body's immune system, production of group B vitamins, producing antimicrobial peptides, increasing body immunity, and preventing cells from becoming cancerous are the effects of using probiotics [1].

Microorganisms that are mainly considered as probiotics belong to the family of lactic acid bacteria and genus *Bifidobacterium*, as well as genera such as Streptococcus, *Lactococcus*, *Lactobacillus*, and yeasts like *Saccharomyces cerevisiae* and *Saccharomyces boulardii*. There are other microorganisms that are less commonly used as probiotics [1]. Lactic acid bacteria are the most common probiotic microorganisms, which are classified as safe microorganisms and are one of the desirable and important members of the digestive tract microbiota [3]. *Lactobacillus* are one of the types of lactic acid bacteria, which are gram-positive, non-sporing, catalase-negative, and usually non-motile. Most of them are seen in the form of rods, but other forms such as *coccobacillus*, *carbonaceous*, and filamentous have also been observed in them. The optimal growth temperature of this species is 30-40 degrees Celsius, and it has a small percentage of organic bases, guanine, and cytosine. Generally, they are found in traditional fermented foods as well as in the digestive system, and they constitute the majority of the microbial flora of the small intestine. Among these microorganisms, *Lacticaseibacillus casei*, *Limosilactobacillus fermentum*, *Levilactobacillus brevis*, *Lactiplantibacillus plantarum*, and *Lactiplantibacillus pentosus* can be pointed out [2].

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As mentioned, lactic acid bacteria are one of the most important groups of bacteria used to produce and process a variety of dairy, meat, vegetable, and grain products. They have the ability to metabolize the materials that make up the food matrix and produce compounds such as organic acids, aldehydes, alcohols, esters, peptides, amino acids, and fatty acids, which have an important role in determining the aroma, taste, texture, and shelf life of fermented food products. In addition, these bacteria are often used as starter cultures during food production processes. In general, the technological potential of lactic acid bacteria includes features that are involved in the life and production of essential aroma-creating compounds and microbial metabolites. One of the important technological features of this group of bacteria is autolytic proteolysis, lipolysis, and citrate decomposition [5].

Today, due to the increasing use of industrial dairy products, it is possible to reduce or eliminate probiotic bacteria. Also, the foods that are consumed daily contain different amounts of microorganisms, among which the contribution of probiotics may be insignificant. Therefore, according to the importance of probiotics and their relationship with maintaining and increasing people's health, it is necessary to isolate and identify them in traditional dairy products and use them for the mass production of dairy products [4]. Accordingly, in the present study, the probiotic, antimicrobial, and immune properties of the strain *L. fermentum* ARD2, which is separated from local yogurt, were investigated.

2- Materials and methods

2-1- Strain isolation and identification *L. fermentum*ARD2

According to the study by Saboktakin et al. (2021), the isolation and identification of the strain were done. Samples were randomly collected from the local market (Tashan, Behbahan, Khuzestan, Iran) and then transported to the laboratory under refrigerated conditions. In the first step, 5 grams of samples were added to peptone water (0.1%, 45 ml) and the samples were homogenized (Seaward, Germany). From the prepared dilutions (10^{-1} to 10^{-6}), MRS agar was cultured. Then, the strain isolated from the culture medium was exposed to gram and catalase staining. Using extraction kits for Genomic DNA isolation VI (Dana Bio Asia, Iran), DNA extraction was performed. The culture medium MRS broth was cultured overnight, and after sedimentation in microtubes containing microbial suspension and dissolving in 200 microliters of phosphate buffer and adding the appropriate enzyme solution to reach the final volume of 50 microliters, the procedure was followed according to the manufacturer's protocol. Universal primers based on the conserved regions of the 16S rRNA genes were designed and used. PCR reactions were performed in a final volume of 25.15 microliters, and the microtubes containing the mixture of PCR reactants were placed in a thermocycler. The results showed that the isolate with catalase-negative and Gram-positive properties, with a similarity rate of 98%, belongs to the strain *L. fermentum* ARD2 [6].

2-2- Probiotic characteristics of the strain *L. fermentum*ARD2

2-2-1- Resistance test to acid and bile salts

The acid resistance test was performed according to the method of Barzegar et al. (2021). To check acid resistance, first, the desired strain was inoculated in 5 ml of MRS broth medium for 18-24 hours and kept in an incubator at 37°C under anaerobic conditions. Bacterial cells grown were

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isolated from the culture medium using high-speed centrifugation at 5000 rpm for 10 minutes at 4°C. Then, the pellets were washed twice and resuspended in sterile phosphate buffer solution (PBS) (Sigma-Aldrich). After that, the pH of the solution was set to 2, 3, and 4, and the samples were placed in an incubator at 37°C for 0, 1, 2, and 3 hours. Finally, using the serial dilution method on MRS agar medium, the number of surviving bacteria was counted [6].

To test resistance to bile salts, the desired strain, after activation in the MRS broth medium (incubation at 37°C for 24 hours), was cultured in the amount of 100 microliters on MRS agar containing 0.3%, 0.5%, and 0.7% bile salt. The plates were subjected to anaerobic conditions in the incubator at 37°C for 24 hours, and after the incubation period, the results were visually checked [3].

2-2-2- hydrophobicity of the cell surface[1]

Surface hydrophobicity is considered an indicator of the tendency of bacteria to bind to non-polar solvents. High hydrophobicity gives them the potential to move from an aqueous environment to an organic or non-polar environment, causing the bacteria to adhere to hydrocarbon particles on cell or mucosal surfaces. First, the bacteria are cultured overnight and then placed in a high-speed centrifuge at 6000 rpm for 12 minutes. The bacteria are then washed with sterile, cold phosphate buffer, and the suspension is prepared again. The optical density (OD) is set to 0.6 at 600 nm (A1). Four milliliters of each bacterial suspension are mixed with 2 ml of n-hexadecane and vortexed for 2 minutes. The mixture is left at ambient temperature for 1 hour to allow the bacteria to transfer between the two phases. Finally, the absorbance of the aqueous solution is read at a wavelength of 600 nm (A2) [8]. Hydrophobicity is calculated using the following equation:

$$\%\ \textbf{Hydrophobicity} = \left[\frac{A_1 - A_2}{A_1}\right] \times 100$$

2-2-3- Test DNase, Hemolytic activity and biogenic amine

According to the extensive research of Vasiee et al. (2020), DNase activity was determined. Accordingly, in the first stage, *L. fermentum* ARD2 was cultivated on the environment linearly. After 48 hours of incubation at 37°C, enzyme production was checked [9].

Hemolytic activity of strains through linear culture on Tryptic Soy Agar medium (Merck, Germany) was performed with 7% (v/v) sheep blood. The plates were placed in an incubator at 37°C for 24 hours. The color changes created were investigated. The formation of a clear halo, a green halo, or no halo formation around the colonies, respectively, indicates β-hemolysis, α-hemolysis, and γ-hemolysis. In this test, *Staphylococcus aureus* and *Escherichia coli bacteria* were used as control samples for β-hemolysis and α-hemolysis [9 and 10].

The culture medium containing precursor amino acids including L-histidine monohydrochloride, tyrosine disodium salt, L-ornithine monohydrochloride, and L-lysine monohydrochloride designed by Barzegar et al. (2021), was used to detect the ability of the strain to produce biogenic amine by decarboxylation of amino acids. *L. fermentum* ARD2 was cultured on MRS Broth medium containing 0.1% of each precursor amino acid and 0.005% pyridoxal 5-phosphate. Then the strains were identified on MRS agar medium with and without amino acids containing 0.06% bromocresol

[1] Cell surface hydrophobicity

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violet (Sigma). After 2 to 5 days of incubation, the formation of purple color in the surrounding colonies was considered as a positive result [7].

2-2-4- Measurement of antioxidant activity

According to the method of Alizadeh Behbahani et al. (2023), the effect of the desired strain on radical scavenging activity DPPH and ABTS was checked. After culturing the strain in MRS medium, the agar was kept at 37°C for 24 hours. Samples were washed 2 times with phosphate buffered saline (PBS) and centrifuged at high speed for 5 minutes at 5000 rpm.

To evaluate the antioxidant activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2 ml of methanolic DPPH solution (0.14 mm) and 2 ml of bacterial sample were combined and kept for 30 minutes in the dark at 37°C. The absorbance at 571 nm wavelength indicates the capacity to neutralize DPPH radicals [10].

For the ABTS procedure, an ABTS solution was prepared by mixing 7 mM ABTS (Sigma, Aldrich) and 2.45 mM potassium persulfate (Daejung Chemical and Metals, Siheung, Korea). Then, 600 microliters of the solution and 300 microliters of the sample were combined and kept in the dark for 30 minutes. The absorbance was read at a wavelength of 734 nm, and the radical scavenging activity was calculated from the following relationship [11].

$$\text{percentage of inhibition of free radicals} = \left(1 - \frac{A_{Sample}}{A_{\text{Control}}}\right) \times 100$$

2-2-5- Cholesterol absorption

Ox bile (Oxgall) (0.3% bile salt) along with polyoxyethanol-cholesterol subcategory MRS broth was added. The medium contained 100 µl of cholesterol. 1% of the culture was inoculated and kept in an incubator at 30°C for 24 hours in an anaerobic environment. The broth control sample had no culture [10].

$$\%\text{Cholesterol absorption} = \frac{C - T}{C} \times 100$$

2-2-6- Resistance to antibiotics and antimicrobial activity

To detect and assess the level of resistance or sensitivity of *L. fermentum* ARD2 compared to common therapeutic antibiotics, a suspension equivalent to half of McFarland from the solid culture medium of the microorganism was prepared, and 100 microliters of it was cultured on MRS agar medium. Then, antibiotic discs including Imipenem, Vancomycin, Nitrofurantoin, Chloramphenicol, Ciprofloxacin, Penicillin, Nalidixic acid, and Gentamicin were placed on the culture medium. The plates were placed in an anaerobic jar at a temperature of 37°C in a greenhouse, and after 24 hours, the diameter of the growth halo around the antibiotic discs was measured using a ruler, and the results were reported in millimeters [3].

To evaluate the antimicrobial activity of the strain *L. fermentum* ARD2 against common pathogenic pathogens, diffusion methods with agar wells (Well Diffusion Agar) and disk diffusion (Disk Diffusion Agar) were used. In the test, three gram-positive pathogenic strains including *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* ATCC 19115, *Bacillus cereus* ATCC 14579, and three gram-negative pathogenic strains including *Shigella dysenteriae* PTCC 1188, *Salmonella typhimurium* PTCC 1609, and *Escherichia coli* ATCC 25922 were used. In the first step, the desired strain was cultured on MRS agar medium and placed in an anaerobic jar in a greenhouse for 24 hours. Then, from the colonies grown on MRS agar medium, MRS broth was inoculated and kept

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in an anaerobic jar for 24 hours at 37°C. After that, centrifugation for 10 minutes at 5000 rpm was done at 4°C. A part of the supernatant without cells, with the original pH resulting from the activity of microorganisms and the production of organic acids, was used (aCFS), and the other part of the supernatant was adjusted to pH 5.5 using sodium hydroxide and was used to measure the antimicrobial activity (nCFS). To ensure that both types of supernatants were free from cells, they were lyophilized using a syringe head filter and a freeze dryer (freezing at -45°C and heating under a vacuum of 0.38 mbar at 32°C for 40 hours). Using 2 ml of distilled water, lyophilized powders from both types of sterilized supernatants and their antimicrobial potential were evaluated by the diffusion method in agar. From each of the mentioned pathogen strains, a concentration equivalent to half of McFarland was prepared and applied to the MHA (Merck, Germany) medium. Then, wells were created on the medium using the end of a sterile pipette with a diameter of 6 mm, and 100 microliters of acidic and neutralized bacterial supernatant were poured into the wells. It was kept in a greenhouse at 37°C for 48 hours, and the diameter of the growth halo around the wells was measured in millimeters using a ruler [8].

To check the antimicrobial activity by the agar disk diffusion method, the method of Fallah et al. (2018) was used with a slight modification. In this method, first, the lactic strain was inoculated in MRS Broth culture medium, and pathogenic indicator bacteria were inoculated in MHB culture medium for 24 hours at 37°C. Then, a suspension was prepared from lactic acid bacteria and standard McFarland of the indicator pathogens. Paper discs (Padten Teb, Iran) with a diameter of 6 mm were immersed in the bacterial extract for 15 minutes and placed on the MRS agar plates cultured with pathogens, and the diameter of the non-growth halo after 24 hours of incubation at 37°C was measured in millimeters using a ruler [2].

2-3- Statistical analysis

Data analysis using software SPSS Version 27 and was done by one-way analysis of variance. Duncan's multi-range test and 95% confidence level were used to compare the means and to draw graphs from Excel 2013 used.

3-Results and Discussion

3-1- Resistance test to acid and bile salts

In Figure 1, the survival rate of *L. fermentum* ARD2 at different pH percentages is shown. According to Figure 1, lower pH values and increasing storage time affect the number of viable cells. The acid resistance results showed that the number of live cells of the strain *L. fermentum* ARD2 increased with increasing pH from 2 to 4, and a constant decreasing trend in pH was observed with the increase in time from zero to 3 hours.

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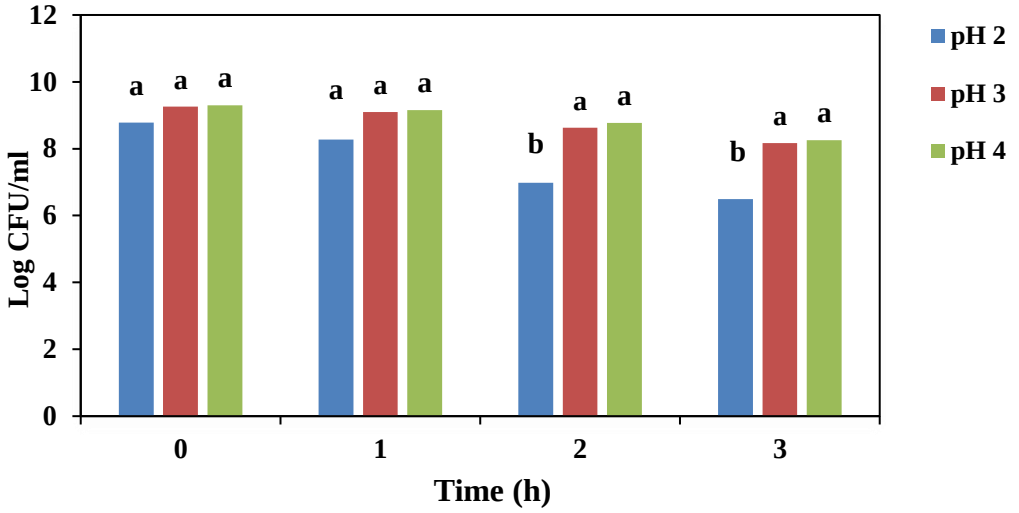

Fig. 1. The survivability of L. *fermentum*ARD2under acidic pHs. The presence of different superscript letters indicates that there are significant differences ($p<0.05$) between the means.

In the present study, the resistance of strain *L. fermentum* ARD2 to bile salts was investigated at different concentrations (0.3%, 0.5%, and 0.7% by weight/volume). According to Table 1, *L. fermentum* ARD2 showed growth and resistance at low concentrations of bile salts. However, after increasing the concentration to 0.7%, the growth stopped.

Table 1. The effect of different concentration of bile salt on the viability of the strain *L. fermentum* ARD2

Survivability	0.3%	0.5%	0.7%	Control
	Growth	Growth	Not grown	Growth

Determining the viability of probiotic microorganisms in food and the digestive system (exposure to the acidic environment of the stomach (pH 1.5-3.5) and bile salts) is an essential feature in the selection of species considered for the production of probiotic foods. The production of some polysaccharide compounds by bacteria contributes to the resistance of probiotic bacteria to acidic conditions, preventing the effect of acid on the cell membrane. Additionally, there are several mechanisms involved in the regulation of acid resistance in lactic acid bacteria, including central metabolic pathways, proton pumps, changes in cell membrane composition and density, DNA and protein damage repair, as well as neutralization processes.

Bile salts play an important role in the specific defense of the intestine by destroying microorganisms, so it is necessary to investigate the viability of the strain in different concentrations of bile salts to demonstrate probiotic potential. Strains belonging to the genus *Bifidobacterium* and *Lactobacillus* in the intestine have bile salt hydrolase, which converts primary bile acids to relatively less toxic secondary bile salts.

Momenzadeh et al. (2021) investigated the effect of different pH levels on the survival percentage of *L. fermentum* and reported that this bacterium did not have the ability to survive at pH 2.5, but at pH 3.5 and pH 5.5,

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it exhibited survival rates of 92% and 99%, respectively. Moreover, more growth was observed at low concentrations of bile salts (0.2% concentration). Panicker et al. (2018) and Tavakoli et al. (2015) also studied different pH levels (2, 2.5, 3.5, and 6.5) and bile salts of different *L. fermentum* strains. The results indicated that the strains were able to tolerate low pH and bile concentrations. The reported results were consistent with the present study, indicating that with an increase in pH and low percentages of bile salts (0.3%), the survival of the strain was higher. Therefore, it can be concluded that the tested strain has a greater ability to survive at pH levels close to neutral, while a decrease in growth was observed at pH 2.5 for 4 hours. Nad Alizadeh Tabari and colleagues (2018) examined *Lactobacillus* isolates from rainbow trout intestine and reported the resistance of *Lactobacillus* strains to bile salt, where *L. fermentum* showed relatively resistance to bile salts at 0.3% concentration. The level of resistance among different bacterial strains is attributed to differences in their ability to reduce the detergent effects of bile salts. Garcia et al. (2017) also stated that all different strains of *L. fermentum* were sensitive to pH 2, but exhibited the ability to survive at pH 3 after 24 hours of incubation. Other researchers also obtained similar results.

3-2- hydrophobicity of the cell surface

The capacity of strains to bind to non-polar sources is explained through hydrophobicity or hydrophobicity, which is created by the hydrophobic components in the outer membrane of bacteria. Sources such as n-hexadecane, chloroform, and ethyl acetate are used to investigate this feature. Based on mixing non-polar solution and microbial suspension and measuring O.D600, the water level is calculated before and after adding the solution. If the absorption of the suspension decreases after exposure to hydrocarbons, the hydrophobic property of the bacteria is recognized. Regarding *L. fermentum* ARD2, a hydrophobicity rate of 50.60± 0.5 was obtained.

Hydrophobicity, one of the essential factors for bacteria to adhere to different surfaces and impact host tissue, is influenced by various factors such as surface electric charge, gravitational force, van der Waals force, and Brownian motion. This characteristic arises from compounds on the cell surface like polysaccharides, fatty acids, and proteins, enabling the microorganism's connection to intestinal cells. Additionally, the covalent attachment of cells to the intestinal mucosa depends on the physical and chemical properties of the cell membrane, along with compounds like protein compounds, teichoic acids, polysaccharides, and certain fatty acids, which affect the organism's hydrophobicity. Teichoic acid, present on the cell wall of lactic acid bacteria in the form of lipoteichoic acid, possesses poly-electrolytic properties. Furthermore, the presence of non-covalent bonding of protein layers in Lactobacillus contributes to this property. Hydrophobicity is crucial in the food industry, particularly for bacteria widely used as co-cultures or starters in the dairy industry, as their affinity for binding to milk fat and aromatic compounds can alter emulsion stability [2 and 8]. Haghshenas et al. (2023) reviewed five *Lactobacillus* strains isolated from curd and found that all strains exhibited hydrophobic properties [21]. In another study, Tabatabai Yazdi et al. (2019) reported hydrophobicity of 43% for different *L. fermentum* strains [17]. Garcia et al. (2017) examined the strain *L. fermentum* UCO-979C and found it to

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have a high capacity to adhere to the cell surface [15].

3-3-DNase, hemolytic activity and production of biogenic amines

The test results indicated that *L. fermentum* ARD2 did not produce DNase, as no clear area was observed around the colonies on the culture medium. The negative hemolytic activity test result suggests that the strain does not break down blood, indicating its non-pathogenic nature. Biogenic amines, organic bases with biological activity primarily produced by the decarboxylation of amino acids, are found in various foods such as meat, seafood, dairy, vegetables, fruits, and nuts. Identifying and controlling these compounds are essential for reducing poisoning risks. The research conducted did not observe the production of biogenic amines [22]. Therefore, the strain *L. fermentum* ARD2 did not exhibit hemolytic properties, DNase production, or biogenic amine production. Consequently, this strain of bacteria holds potential for improving public health. The absence of hemolytic activity is crucial, as hemolysis increases the organism's access to iron, potentially leading to anemia [11]. Moreover, the production of amines can cause adverse effects such as increased blood pressure, nausea, diarrhea, headaches, respiratory issues, and skin problems [15]. Omidvar et al. (2018) conducted research on probiotic properties of *L. fermentum* isolated from honey and concluded that the strains lacked hemolytic activity [16]. Similarly, *Lac. fermentum* UCO-979C did not produce biogenic amines and lacked hemolytic activity, consistent with the results of the present study [15]. Lee et al. (2023) found that all studied *Lactobacillus* strains isolated from kimchi lacked hemolytic activity [23]. Bagheri et al. (2019) also achieved similar results regarding safety properties of *Lactobacillus* [24].

3-4- Evaluation of antioxidant properties DPPH and ABTS

The results of free radical inhibition by *L. fermentum*ARD2 it is shown in Figure 2.

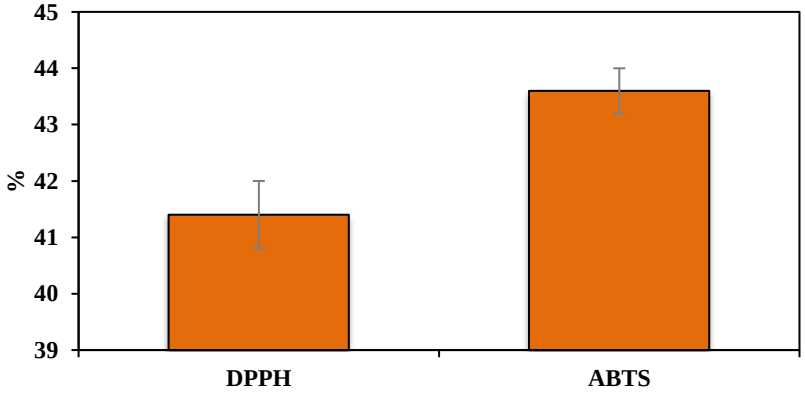

Fig. 2. The antioxidant activity (DPPH & ABTS) of *L. fermentum* ARD2.

Oxidative stress arises from the imbalance between oxidant and antioxidant functions in the body. The human body possesses a defense mechanism against oxidants, but when reactive oxygen species (ROS) accumulate beyond the inherent antioxidant capacity, excessive oxidation of lipids, proteins, and nucleic acids occurs, leading to cellular damage and various diseases such as cancer, inflammation, cirrhosis, and cardiovascular diseases. Therefore, exogenous antioxidants are required to

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mitigate oxidative stress or enhance individual antioxidant capacity. Lactic acid bacteria and their metabolites have been shown to play a significant role in maintaining human health and preventing certain diseases by acting as antioxidants. Antioxidant components found in lactic acid bacteria include bacterial exopolysaccharides, bioactive peptides, antioxidant enzymes, and manganese ions. Additionally, the natural intestinal microflora can produce bioactive food antioxidants through enzymatic reactions [11 and 25]. The strains of different L. fermentum ARD2 exhibited free radical scavenging activities of 40.41% and 43.60% for DPPH and ABTS, respectively. Choruk et al. (2017) reported that *L. fermentum*, *L. casei*, and *L. rhamnosus* showed DPPH activity exceeding 60%. Similarly [26]. Kim et al. (2022) demonstrated that *Lactobacillus* strains derived from food exhibited DPPH radical inhibition ranging from 2.55% to 6.88% and ABTS radical inhibition ranging from 19.69% to 86.26% [27]. Hu et al. (2022) concluded that *L. fermentum* HDY02 obtained from fermented soybean milk possessed antioxidant abilities [28].

3-5- Cholesterol absorption

Cholesterol is a vital component of the body's tissues, but excessive intake and accumulation can lead to elevated cholesterol levels and related diseases such as cardiovascular diseases and certain types of cancer, ultimately increasing mortality rates. While drug treatments are commonly used to manage and reduce cholesterol levels, long-term use of these drugs can cause digestive issues and have adverse effects on overall health. Consuming probiotic-rich foods is a popular method to mitigate these risks, as lactic acid bacteria improve intestinal function, maintain microbial balance, boost the body's immune system, and reduce cholesterol levels [29 and 30]. The results of this study demonstrated that *L. fermentum* ARD2 was able to reduce cholesterol levels by 38.60% ± 0.54% [21].

3-6- Resistance to antibiotics and antimicrobial activity

The resistance level of *L. fermentum* ARD2 to common therapeutic antibiotics is summarized in Table 2. The findings of this study revealed that L. fermentum ARD2 was sensitive to Vancomycin, Gentamicin, Chloramphenicol, Nalidixic acid, and Imipenem, exhibited resistance to Ciprofloxacin, and showed semi-sensitivity to Nitrofurazone and Penicillin. Assessing the sensitivity of lactic acid bacteria to various antibiotics is crucial for ensuring their safety. Antibiotic resistance can pose risks as resistance genes may transfer to harmful bacteria in the digestive system, compromising antibiotic effectiveness during illness. Certain *Lactobacillus* species act as probiotics, offering numerous health benefits such as immune system modulation and pathogenic bacteria control. The antimicrobial activity of lactic acid bacteria can extend product shelf life and may involve the production of compounds like bacteriocins, which inhibit Gram-positive pathogens. While antimicrobial activity against Gram-negative bacteria is linked to organic acids, hydroxy fatty acids, diacetyl, phenols, protein compounds, and hydrogen peroxide. Different strains of *Lactobacillus* have been investigated for producing antimicrobial compounds against spoilage bacteria, aiding in infection control similar to antibiotics. This activity is primarily associated with organic acid synthesis, such as lactic acid and phenyllactic acid [5 and 31]. Results from Garcia et al. (2017) demonstrated that *L. fermentum* UCO-979C

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exhibited susceptibility to Penicillin, indicating lack of plasmid resistance. Additionally, it showed sensitivity to Amoxicillin, Ciprofloxacin, Chloramphenicol, and Gentamicin [15]. *L. fermentum* isolated from kimchi showed sensitivity to antibiotics Ampicillin, Gentamicin, Chloramphenicol, and Clindamycin [23]. Abid et al. (2022) found *L. fermentum* isolated from buffalo milk to be sensitive to Erythromycin, Clindamycin, and Ampicillin, and resistant to Gentamicin, Ampicillin, and Chloramphenicol [19]. Momenzadeh et al. reported sensitivity of *L. fermentum* to Chloramphenicol,
Tetracycline, and Penicillin, and semi-sensitivity to Gentamicin [3]. Strains *L. fermentum* MA-7 and *L. fermentum* MA-8 exhibited moderate sensitivity to Chloramphenicol, resistance to Gentamicin and Nalidixic acid, and sensitivity to Penicillin [18]. Boricha et al. (2019) reported similar results on antibiotic resistance of *L. fermentum* strains from food and human origin [32].

The results of the antimicrobial effect of the strain *L. fermentum* ARD2 on several pathogenic bacteria, as determined by the agar disk diffusion method and agar well method, are presented in Figures 3 and 4. In both methods, it was observed that the strain *L. fermentum* ARD2 did not exhibit any antimicrobial effect against *E. coli*.

Table 2. Effect of common antibiotics on the growth of *L. fermentum* ARD2

Antibiotic	***L. fermentum*ARD2**
Vancomycin	**Sensitive**
Gentamicin	**Sensitive**
Chloramphenicol	**Sensitive**
Nitrofurazone	**Intermediate**
Nalidixic	**Sensitive**
Penicillin	**Intermediate**
Imipenem	**Sensitive**
Ciprofloxacin	**Resistance**

In the investigation using both the disc and well methods for the antimicrobial effect of *L. fermentum* ARD2, it was found that the acidic supernatant without cells (aCFS) did not exhibit any antimicrobial effect on *E. coli* in the well method. Similarly, the neutralized supernatant without cells (nCFS) did not show any antimicrobial effect on E. coli, *S. typhimurium*, and *S. dysenteriae*. However, the aCFS showed the most antimicrobial]
effect on *L. monocytogenes* with a halo diameter of 9 mm and on *S. aureus* with a halo diameter of 7.8 mm. Conversely, the neutralized supernatant without cells showed a larger halo diameter (1.8 mm) on *L. monocytogenes* compared to the other pathogens, indicating a stronger effect. In the disk method, similar to the well method, the aCFS showed no antimicrobial effect on *E. coli* and *S. typhimurium*, while the nCFS did not exhibit antimicrobial effects on *E. coli*, *S. typhimurium*, and *S. dysenteriae*. The most significant impact of both aCFS and nCFS was observed on *L. monocytogenes* with halo diameters of 8.6 mm and 7.5 mm, respectively. Furthermore, the growth of *S. aureus* was limited by aCFS with a halo diameter of 2.8 mm.

Previous studies have shown the antimicrobial effects of *L. fermentum* on pathogenic bacteria, with gram-positive strains being more susceptible than gram-negative strains. The antimicrobial

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mechanisms vary depending on the bacterial type, with organic acids, hydrogen peroxide, hydroxy fatty acids, and carbon dioxide being involved in the destruction of Gram-negative bacteria, while protease-sensitive bacteriocins are effective against Gram-positive bacteria.

In conclusion, *L. fermentum* ARD2 exhibited resistance to pH and bile salt concentrations, along with good antimicrobial activity against pathogenic bacteria. It also showed sensitivity to conventional therapeutic antibiotics, indicating no concerns regarding the transfer of antibiotic-resistant genes to pathogenic bacteria. Furthermore, it did not exhibit hemolytic activity, DNase production, or the production of biogenic amines. Additionally, it demonstrated the ability to inhibit free radicals and absorb cholesterol. Therefore, *L. fermentum* ARD2 holds promise as a natural flora of the digestive system and can be utilized in dairy and fermented products as a probiotic and natural preservative. However, further tests are necessary to validate its potential as a probiotic and preservative in food products.

The results of the antimicrobial effect of the strain *L. fermentum*ARD2 on some pathogenicity index bacteria by agar disk diffusion method and agar well in Fig.3 and 4 are shown. In both methods the strain *L. fermentum*ARD2 No antimicrobial effect on zinc *E. coli* was.

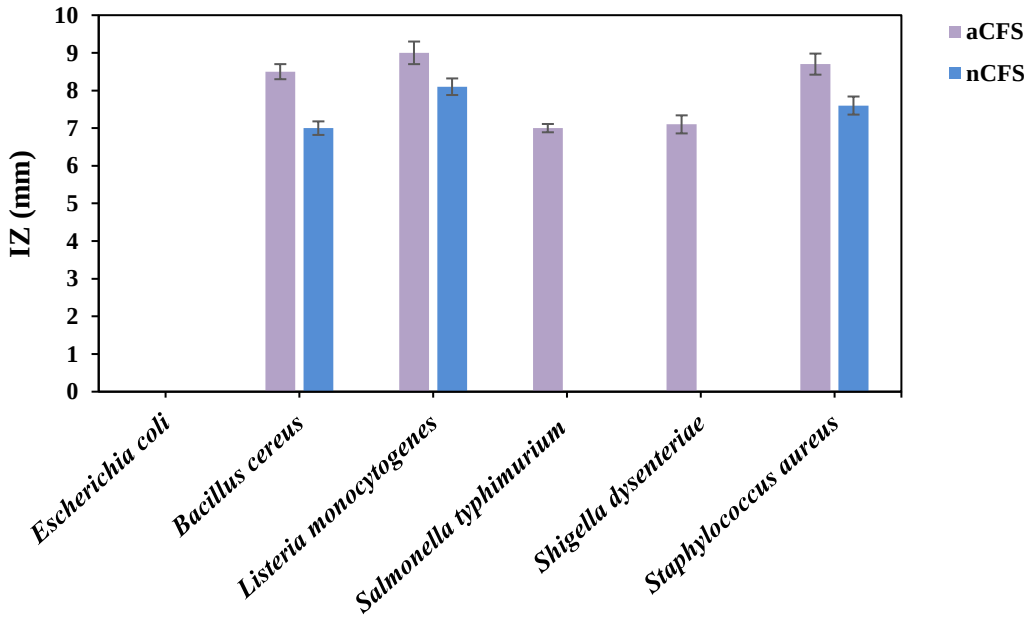

Fig. 3. Antimicrobial activity of *L. fermentum*ARD2 based on well diffusion agar method. Cell-free supernatants (CFS); acid and neutralized CFS (aCFS and nCFS).

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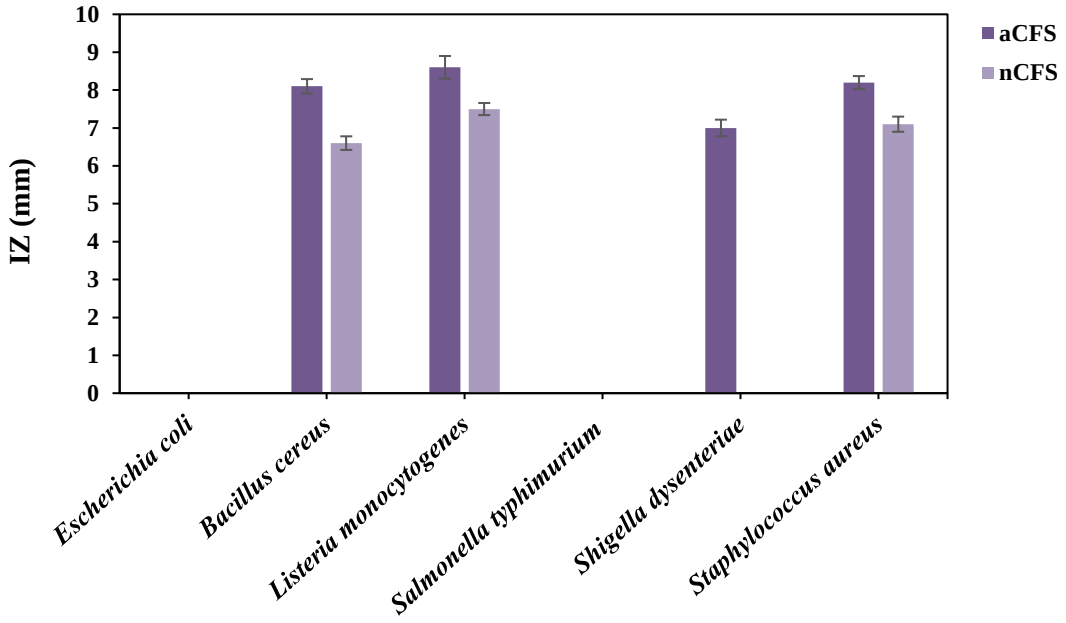

Fig. 3. Antimicrobial activity of *L. fermentum*ARD2 based on disk diffusion agar method. Cell-free supernatants (CFS); acid and neutralized CFS (aCFS and nCFS).

Using disc and well method of antimicrobial effect *L. fermentum*ARD2 was investigated and the results showed that in the well method, the acidic supernatant without cells (aCFS) has no antimicrobial effect *E. coli* was, the neutralized supernatant without cells (nCFS) in addition *E. coli*, on *S.typhimurium* and*S. dysenteriae* No effect, the most antimicrobial effect aCFS Regarding *L. monocytogenes* with a halo diameter of 9 mm and *S. Auerus* with a halo diameter of 7/8. On the other hand, the neutralized supernatant does not contain cells *L. monocytogenes* it formed a larger halo than the others (1.8 mm), which meant more effect. In the disk method, like the well method, aCFS No antimicrobial effect *E. coli* and *S. typhimurium* did not have nCFS In addition to the mentioned pathogens, it has no antimicrobial effect on *S. dysenteriae* was. The most impact aCFS and nCFS on *L. monocytogenes* it was observed with the halo diameter of 8.6 and 7.5 mm, respectively aCFS growth *S. auerus* also limited (diameter of halo 2.8 mm). Tabatabai Yazdi et al. (2019) Antimicrobial effect *L. fermentum* 4-17on pathogenic bacteria *E. coli*, *S. auerus*, *P. aeruginosa* and *S. typhi* investigated and found the highest and lowest antimicrobial effects of zinc respectively *S. auerus* and *E. coli* it was observed and showed that the antimicrobial effect of probiotics on gram positive strains is more than gram negative ones. In Gram-negative bacteria, the mechanism of bacterial destruction is based on the production of organic acids, hydrogen peroxide, hydroxy fatty acids and carbon dioxide, and in Gram-positive bacteria, it is related to protease-sensitive bacteriocins [17]. Abid et al. (2022) stated *L. fermentum* isolated from buffalo milk significantly against similar pathogens *P. aeruginosa*, *S. auerus*, *L. monocytogenes*, *B. cereus* and *E. coli* showed resistance [19]. The research of Haghshenas et al. (2023) also shows the antimicrobial effect of strains *Lactobacillus* separated from the curd showed [21]. According to Ebrahimi et al.'s study (2017), bacteriocin-like compounds produced by *L. fermentum* they were isolated from Chal (a traditional fermented product of

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camel milk) and had an acceptable antimicrobial activity [33]. In general, it can be said that probiotics and metabolites produced by them have a high potential for food and medicinal use [34-51].

4 - Conclusion

Based on the findings of this current research, *L. fermentum*ARD2 having the ability to bear pH and percentages of bile salts. It also had a good ability to inhibit pathogenic bacteria and showed sensitivity to conventional therapeutic antibiotics; therefore, there are no concerns related to the transfer of antibiotic resistant genes to pathogenic bacteria. In addition to the above hemolytic activity, DNase and the production of biogenic amine was not observed and it had the ability to inhibit free radicals and absorb cholesterol; therefore, lactic acid bacteria are important as the natural flora of the digestive system, and dairy and fermented products can be introduced as useful sources of them. Accordingly, the strain in question had good functional characteristics and antimicrobial activity, however, its use in beneficial food products requires other tests to be used as a probiotic and natural preservative in the said products.

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مقاله علمی_پژوهشی

جداسازی و شناسایی سویه *Limosilactobacillus fermentum* ARD2 از ماست محلی و ارزیابی ویژگی‌های پروبیوتیکی، ضدمیکروبی و ایمنی آن

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چکیده

نقش مهم و اساسی غذا در زندگی، سبب روی آوردن به سمت محصولاتی شده است که علاوه بر دارا بودن خواص کیفی و تغذیه‌ای مطلوب، سلامت کلی مصرف‌کننده را نیز تضمین می‌کنند. پروبیوتیک‌ها میکروارگانیسم‌های غیربیماری‌زایی هستند که در صورت مصرف مقادیر کافی سبب برقراری تعادل میکروبی روده شده و سلامتی و ایمنی بدن را افزایش می‌دهند. در این پژوهش، ویژگی‌های پروبیوتیکی، ضدباکتریایی و ایمنی سویه Limosilactobacillus fermentum ARD2 جدا شده از ماست محلی بررسی شد. مقاومت به اسید (۲، ۳ و pH=٤)، مقاومت به نمک‌های صفراوی (۰/۳، ۰/۵ و ۰/۷ درصد)، فعالیت ضد میکروبی به روش دیسک و چاهک، مقاومت نسبت به آنتی‌بیوتیک‌های رایج درمانی، ارزیابی فعالیت آنتی‌اکسیدانی، هیدروفوبیستی سطح سلول، آنزیم DNase، فعالیت همولیتیک و آمین بیوژنیک و جذب کلسترول بررسی گردید. نتایج مقاومت به اسید نشان داد که تعداد سلول‌های زنده سویه L. fermentum ARD2 با افزایش pH از ۲ به ٤، افزایش یافته و با افزایش زمان از صفر به ۳ ساعت در pH ثابت روند کاهشی مشاهده گردید. با افزایش غلظت نمک‌های صفراوی، رشد کاهش یافت. نتایج حاصل از اثر ضدمیکروبی نشان داد که در هر دو روش چاهک و دیسک سوپرناتانت اسیدی فاقد سلول (aCFS) و سوپرناتانت خنثی شده فاقد سلول (nCFS) روی Escherichia coli اثر ضد میکروبی نداشتند. nCFS فاقد اثر ضدمیکروبی بر Shigella dysenteriae بود. L. fermentum ARD2 به Ciprofloxacin مقاوم و نسبت به Penicilin و Nitrofurazone نیمه حساس بود. میزان مهار رادیکال آزاد DPPH و ABTS به ترتیب معادل ٤۱/٤۰ و ٤۳/٦۰ درصد بوده و این سویه توانست جذب کلسترول را به میزان ۳۸/٦۰ درصد کاهش دهد. نتیجه تست‌های DNase و فعالیت همولیتیکی منفی بود و تولید آمین بیوژنیک مشاهده نگردید. مطابق با نتایج به دست آمده L. fermentum ARD2 قابلیت پروبیوتیکی قابل قبولی داشته و می‌توان از این سویه در محصولات غذایی به عنوان یک باکتری پروبیوتیک استفاده نمود.

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