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Scientific Research

Evaluation of pathogenicity indicators and technological properties of *Enterococcus faecium* isolates obtained from traditional Iranian cheeses

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ABSTRACT

Recently, special attention has been paid to enterococci for use as probiotics in dairy products. All these desirable features are a stimulus for the producers of dairy products to use enterococci isolated from dairy products such as Liqvan cheese. Despite having all these features, enterococci are not recognized as GRAS and their presence in food products is a sign of fecal contamination. The purpose of this research is to investigate enterococci isolated from Liqvan and Koze cheese in terms of having pathogenic indicators in order to confirm that they are safe for consumers and finally to investigate the possibility of using them as starters or starter aids in dairy products. Especially cheeses. Based on this, 57 isolates of Enterococcus faecium from traditional Iranian cheeses were examined for the presence of pathogenic genes, and finally 23 isolates did not have any pathogenic genes. Then the technological properties of these isolates such as acidification, proteolytic, lipolytic, autolytic, heat and acid resistance and exopolysaccharide production were investigated. The results showed that among the 23 investigated strains, 19 isolates had antimicrobial activity against pathogenic bacteria, 16 strains were able to produce exopolysaccharide and 20 isolates had moderate acidification properties. The highest proteolytic and lipolytic activity was related to strains c18 and c16, respectively, and strain LR78 showed the highest acid and heat resistance.

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1. Introduction

Gram-positive and catalase-negative Enterococcus bacteria are present everywhere and are often found in a large number of vegetables, plant materials, and food, especially of animal origin, such as dairy products [1]. These bacteria are often present in a large number of dairy products and other fermented foods. They are also part of the natural microbial flora of the gastrointestinal tract of some mammals, including humans. The presence of enterococci in dairy products has long been considered as an indicator of inappropriate sanitary conditions during milk production and processing. However, enterococci have a long history of safe use in food. In addition, a significant number of different species of Enterococcus genus have interesting biotechnological features such as bacteriocin production and probiotic properties [2]. In 2002, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) recommended that antimicrobial resistance patterns and viral opportunism characteristics be used to ensure the safety of Enterococcus species used as primers or Probiotics should be tested [3]. *Enterococcus faecalis*، *Enterococcus faecium* And*Enterococcus durans* There are species that have the most abundance among other species and are abundantly found in dairy products [4]. Enterococcus has important uses in the dairy industry and plays a role in the development of organoleptic properties during the ripening of many cheeses. It is also used as the main component in the starter culture medium [5]. Although in Iran, cheeses made from raw sheep's milk are not as diverse as European countries, but famous cheeses such as Liqvan cheese and Koze cheese have been produced in East and West Azarbaijan provinces and even neighboring countries for a long time and are highly sought after by consumers. The production of these cheeses is done without adding commercial starters and with old and traditional cheese making techniques [6]. The presence of enterococci in Liqvan cheese has been proven, which leads to the creation of a desirable aroma and taste, but despite having all these characteristics, enterococci are not recognized as GRAS, and their presence in food products is a sign of fecal contamination [7 and 8].

1-1-Indicators of pathogenicity

In the past, pathogenicity indicators in enterococci were unknown, but today, these indicators have been identified to a large extent.

Aggregation factor (asa1) is a surface protein factor induced by pheromones in enterococci, which leads to the firm attachment of bacteria that intend to transfer plasmids to each other [9, 10]. This factor also acts as a super Antigen facilitates the attachment of bacteria to eukaryotic cells such as human macrophages, intestinal epithelial cells and extracellular protein matrices such as fibronectin, thrombospondin and collagen type one [11, 12]. It has been proven that adhesion to the matrix of extracellular proteins plays an important role in causing infection in wounds, wounds and also bacterial endocarditis [13 and 14].

Gelatinase (gel E) is an extracellular metalloendopeptidase that plays a role in the hydrolysis of gelatin, collagen, hemoglobin and other peptides [17]. Gelatinase enzyme production*Enterococcus vulcanis*Food is also common [15 and 16]. Eaton and Gasson also proved that even in cases where the gelatinase gene is present in the bacterium, it is possible to observe the opposite phenotype, so that none of the*Enterococcus vulcanis*In these studies, they did not produce gelatinase enzyme [15].

Cell surface protein (esp) was first introduced by Shanker and colleagues, in*Enterococcus vulcanis* Clinical signs were identified. Studies on the prevalence of this surface protein showed that the majority*Enterococcus vulcanis* Infectious agents have this factor. ESP probably plays a role in adhesion and suppression of the host's immune system [18].

Sex pheromones (cpd) are the breakdown products of 21-22 amino acid peptides that are related to surface lipoproteins [9]. This agent, which is actually a type of surface protein in the form of an antigen, can lead to pathological changes such as acute inflammation in the host. Pheromones in humans and rats show chemotoxic properties by producing superoxide and releasing lysosomal enzymes [19].

Collagen-binding factor (ACE) is structurally very similar to CNA (collagen-binding protein) in*Staphylococcus aureus* has it. Similar to the

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mechanism proposed by Shanker and colleagues for ESP, this factor can lead to the suppression of the immune system. Ace not only binds to collagen, but also to laminin. In fact, enterococci express ace when causing infection in humans[20].

1-2- Technological characteristics of Enterococcus I see

Proteolytic activity by enzymes produced by enterococci has been observed in the hydrolysis of milk proteins such as casein, alphalactalbumin, betalactaglobulin and albumin. In fact, the ability to degrade proteins depends on the species and strain [21]. Enterococci may have lipase or esterase, but the presence of enzymes depends on the type of strain and substrate [22]. Enterococci show higher sterolytic activity than other genera of lactic acid bacteria.*Enterococcus faecium* It is the most astrolytic species in enterococci [1 and 23]. Estradiol activity with lipolysis of milk fat leads to the production of methyl ketones and thioesters from fatty acids, which improves the taste of the product. Lipolysis does not have a direct effect on cheese rheology, but some glycerides produced by having surface activity affect cheese texture [1].

Autolytic activity becomes important due to the release of intracellular enzymes such as proteases and lipases, which are effective in cheese ripening and flavor production. The presence of intact cells is necessary to perform physiological reactions such as lactose fermentation, oxygen removal and other reactions to create flavor, but on the other hand, the presence of autolyzed cells in cheese helps to accelerate peptidolytic reactions [24].

It is important to create high acidity and rapid decrease in pH in the early ripening period of fermented products such as cheese, and it prevents the growth of foreign bacteria and rapid coagulation. Lactic acid bacteria ferment carbohydrates and produce lactic acid. The lactic acid produced decreases the pH and causes the milk to curdle and the whey to come out. Also, acid production has an important effect on the formation of texture, aroma and taste [25].

Exopolysaccharide produced by lactic acid bacteria during the fermentation process has a great effect on the texture of fermented dairy products. These compounds are either attached to the cell wall in the form of capsules or they are released into the extracellular environment [28]. Exopolysaccharides have several different roles, such as protecting bacteria against desiccation, phagocytosis and phage attack, providing higher oxygen pressure, participating in the absorption of metal ions, and acting as adhesive agents [29]. Exopolysaccharide compounds as natural thickeners or stabilizers improve the rheological properties of fermented dairy products and reduce water retention by retaining water. Although exopolysaccharide materials have no taste, they help to perceive and receive its taste by increasing the viscosity of the product and consequently increasing the duration of residence in the mouth and the duration of contact with the taste receptors [30].

The dual properties of enterococci as a beneficial microorganism or an opportunistic pathogen have prompted researchers today to carefully investigate the intrinsic enterococci isolated from food and their effect on consumer health and the possibility of using them as starters in food products. Examine dairy products. The aim of this research is to evaluate the pathogenicity indicators and technological properties of the isolates*Enterococcus faecium* The result of traditional Iranian cheeses is to confirm that they are safe for the consumer, and finally, it is possible to use them as a starter or starter aid in dairy products, especially cheeses.

2- Materials and methods

2-1- The investigated bacterial strains

In this research, 57 isolates*Enterococcus faecium* The results of Ligvan cheese and Koze cheese were evaluated [7].

Table 1 Bacterial strains tested

Row	Bacteria code	Row	Bacteria code	Row	Bacteria code
1	M1	20	LF44	39	KR25
2	M9	21	LF50	40	KR26

3	M13	22	LF54	41	KR27
4	Q15	23	LR59	42	KR30
5	C16	24	LR61	43	KR31
6	C17	25	LR66	44	KR32
7	C18	26	LR67	45	KR34
8	C19	27	LR68	46	KR35
9	C20	28	LR73	47	KR36
10	C21	29	LR74	48	KR37
11	C31	30	LR75	49	KR38
12	C32	31	LR76	50	KR39
13	C33	32	LR77	51	KR40
14	LF36	33	LR78	52	KR41
15	LF37	34	KR17	53	KR42
16	LF39	35	KR18	54	KR43
17	LF40	36	KR19	55	KR45
18	LF41	37	KR20	56	KR46
19	LF42	38	KR21	57	KR48

2-2- Activation of isolates

Isolates*Enterococcus faecium* It is removed from the negative 80 freezer and then it is cultured linearly in the specific solid culture medium of Enterococcus (M17). Incubation will be done at a temperature of 37 degrees Celsius.

2-3-Confirmation tests

2-3-1- Warm coloring

First, the desired colony is harvested and a dry spread is prepared on the slide. The range is fixed by passing the flame three times on the slide. Then it is covered with crystal violet for one minute so that the dye is absorbed by the cells. After washing with water, Lugel's solution is used for washing for 1 minute. Next, alcohol-acetone solution is used for 13 seconds to wash the slide (decolorizing role for crystal violet) and finally the slide is washed with water. In order to complete the staining, the surface of the area is covered with safranin dye for 43 seconds. The sepsis is washed once more and after drying, in order to observe the bacteria, the slide is placed under the electron microscope with 100x magnification and the Gram positive or negative bacteria is determined [31].

2-3-2-catalase test

Using a drop of hydrogen peroxide on each of the desired strains and the formation or non-formation of gas bubbles, respectively, determines whether the test result is positive or negative [31].

2-4- Checking the presence of pathogenic genes withUsing the multiplex PCR technique

At this stage, the multiplex PCR technique was used to check the presence of pathogenic genes, and this goal was achieved by using three separate reactions. Item primers use in The table below is mentioned.

Table 2 Primers

first	sequence
Forward Van A	5' - CATGAATAGAATAAAGTTGCAATA -3'
Reverse Van A	5' - CCCCTTTAACGCTAATACGATCAA -3'
Forward Van B	5' - GTGACAAACCGGAGGCGAGGA-3'
Reverse From B	5' - CCGCCATCCTCCTGCAAAAAA -3'
Forward esp	5’ - AGATTTCATCTTTGATTCTTGG-3’
Reverse esp	5' - AATTGATTCTTTAGCATCTGG-3'
Forward gelE	5’ - TATGACAATGCTTTTTGGGAT-3’
Reverse yellowE	5' - AGATGCACCCGAAATAATA-3'
Forward asa1	5’ - GCACGCTATTACGAACTATGA-3’
Reverse asa1	5' - TAGAAGAACATCACCACGA-3'
Forward cpd	5’- TGGTGGGTTATTTTTCAATTC-3’
Reverse cpd	5’ - TACGGCTCTGGCTTACTA-3’
Forward ace	5' - GGAATGACCGAGAACGATGGC-3'
Reverse ace	5’ - GCTTGATGTTGGCCTGCTTCCG-3’

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2-4-1- Checking the presence of Van A and van B genes

Atmultiplex PCR First, primers whose purpose was to check the presence of Van A and Van B genes were used.

The PCR reaction was performed in a dry PCR kit in a final volume of 20 microliters, which included the following:

10 microliters of Red master mix, 3 microliters of molecular biology grade deionized distilled water, 3 microliters of DNA and 1 microliter of each of the Forward and Reverse Van A primers, 0.5 microliter of each of the Forward and Reverse Van B primers.

After adding each component of the reaction mixture, the microtubes were placed inside the thermocycler and the temperature program was set as follows [32].

Activation: 95°C temperature for 5 minutes, one cycle.

Deposition: temperature of 94°C for one minute, annealing: temperature of 51°C for one minute, extension: temperature of 72°C for one minute, 30 cycles.

Final development: temperature of 72 degrees Celsius for 10 minutes, one cycle.

2-4-2- Checking the presence of asa1, gel E and esp genes

In the second multiplex PCR, primers were used whose purpose was to investigate the presence of asa1, gelE and esp genes.

The PCR reaction was performed in a dry PCR kit in a final volume of 20 microliters, which included the following:

10 microliters of Red master mix, 3.5 microliters of molecular biology grade deionized distilled water, 2.5 microliters of DNA, 1 microliter of each one of the Forward and Reverse esp primers, 0.5 microliter of each of the Forward and Reverse gelE primers 0.5 µl of each one of the forward and reverse asa1 primers.

After adding each component of the reaction mixture, the microtubes were placed inside the thermocycler and the temperature program was set as follows [33]:

Activation: 95°C temperature for 15 minutes, one cycle

Deposition: 94°C temperature for one minute, Annealing: 49°C temperature for one minute, Extension: 72°C temperature for one minute, 30 cycles.

Final development: temperature of 72 degrees Celsius for 10 minutes, one cycle.

2-4-3- Examining the existence of cpd and ace genes

In multiplex somase PCR, primers whose purpose was specifically to investigate the presence of cpd and ace genes were used.

The PCR reaction was performed in a dry PCR kit in a final volume of 20 microliters, which included the following:

10 microliters of Red master mix, 3 microliters of molecular biology grade deionized distilled water, 2 microliters of DNA, 1 microliter of each one of the Forward and Reverse cpd primers, and 1.5 microliters of each of the Forward and Reverse ace primers.

After adding each component of the reaction mixture, the microtubes were placed inside the thermocycler and the temperature program was set as follows [33].

Activation: 95°C temperature for 15 minutes, one cycle.

Deposition: 94°C temperature for one minute, Annealing: 50°C temperature for one minute, Extension: 72°C temperature for one minute, 30 cycles.

Final development: temperature of 72 degrees Celsius for 10 minutes, one cycle.

After conducting PCR reactions, electrophoresis was performed to see the results and bacteria with pathogenic genes were identified.

2-5- Review of technological features

2-5-1- lipolytic activity

The lipolytic activity of the isolates was evaluated based on halo formation in the tributyrin agar culture medium. The light halo created around the cells indicates lipolytic activity. To check the lipolytic activity, the isolates were cultured on tributyrin agar medium and kept in a greenhouse at 37°C for 7 days. The halo radius around the colonies was measured in millimeters, which indicates the intensity of lipolytic activity [34].

2-5-2-autolytic activity

The cell sediment obtained from the overnight culture centrifuge with OD600 = 1-0.8 was diluted with potassium phosphate buffer to

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OD600 = 0.6-0.8 and kept in a greenhouse at 37°C for 48 hours [35]. Finally, the amount of autolytic activity was measured in the form of a decrease in the percentage of Jadb at 600 nm based on the following formula:

$(A0-At)/A0\times100$

A0 = initial absorption

At = secondary uptake after greenhouse period

According to this formula, enterococci are graded as follows in terms of autolysis [36]:

Good = 33-66, relatively good = 24-34 and poor = 0-22

2-5-3- proteolytic activity

Enterococcus suspension was prepared in potassium phosphate buffer. 2 microliters of this suspension was spotted on the surface of skimmed milk agar culture medium (2% agar and 10% skim milk) and kept in a greenhouse at 30°C for 4 days. The formation of a bright halo around the colonies indicates proteolytic activity [37].

2-5-4-acidification activity

To check the acidification activity, first, 1% by volume of the strain activated in the M17 culture medium was inoculated into tubes containing 10 ml of sterile skimmed milk (10% by weight and volume) and incubated at 37 degrees Celsius for 24 hours. A greenhouse was installed. pH was measured immediately after inoculation (time zero), 24 hours after inoculation. Then the degree of acidification was calculated using the following formula [36].

$$\Delta pH = pH24 - pH0$$

2-5-5- thermal resistance

In order to check heat resistance, the strains inoculated into M17 Broth culture medium were placed in a hot water bath at temperatures of 60, 65, 70, 75, 80 and 85 degrees Celsius for 15 and 30 minutes. Then they were kept in a greenhouse at 37°C for 16 hours and the absorbance of the strains was read at a wavelength of 600 nm [38].

2-5-6- acid resistance

First, the pH of the M17 Broth culture medium was adjusted using normal 0.1 hydrochloric acid at pHs of 3, 3.5, 4, 5, and 6, and then the strains were inoculated into the mentioned culture medium. The strains were incubated at 37°C for 16 hours and then the absorbance of the strains was read at a wavelength of 600 nm [38].

2-5-7- exopolysaccharide production

Activated strains in M17 agar culture medium on Ruthenium Red Milk Agar culture medium containing yeast extract (5%), skimmed milk (10%), sucrose (1%), agar (1.5%) and Ruthenium Red (08 0.0%), were cultivated linearly. The production of exopolysaccharides will be confirmed by the formation of white colonies by the strains [39].

2-5-8- Antimicrobial activity

First, Enterococcus faecium isolates were cultured linearly on M17 Agar culture medium and then kept in a greenhouse at 37°C for 24 hours. Then the strains were transferred to M17 Broth culture medium and kept at 30°C for 24 hours. After creating turbidity, 5 microliters were dotted on BHI Agar culture medium and kept in a greenhouse at 30°C for 24 hours [7]. After the growth of the strains, the surface of the culture medium was covered by a layer of soft agar (about 10 cc) which was inoculated with 0.25% of indicator microorganisms. Again, the plates were placed in a greenhouse for 24 hours at the growth temperature of the indicator microorganisms. The presence of a clear halo around the points inoculated with Enterococcus faecium indicated the lack of growth of the indicator microorganism and, as a result, the antibacterial properties of the strains [40].

6-2-Statistical analysis

The tests were conducted in the form of completely randomized block design and at least in three repetitions using one-way and two-way analysis of variance and with the help of SPSS software (USA, version 23). Averages were compared with Duncan's test method and all data analysis was done at 95% confidence level.

3. Results and Discussion

3-1- Examining the presence of pathogenic genes

The first multiplex PCR was performed for simultaneous detection of van A and van B genes. Among the 57 investigated isolates, none contained the van B gene and only 4 isolates contained the van A gene. In the second multiplex PCR, which was performed for the simultaneous detection of gel E, esp and asa1 genes, among the 57 investigated isolates, 6 isolates contained the esp gene and 19 isolates contained the asa1 gene, and all isolates lacked the gel E gene. The third

multiplex PCR for simultaneous detection cpd and ace genes were also tested, and among the 57 isolates examined, 20 isolates contained the cpd gene and 22 isolates contained the ace gene.

Table 3 Examining the presence of pathogenic genes

pathogenic genes							Bacteria code	Row
ace	Cpd	so1	come E	esp	From B	Van A		
-	-	+	-	-	-	-	M1	1
-	-	+	-	-	-	-	M9	2
-	-	+	-	-	-	+	M13	3
-	-	-	-	-	-	+	Q15	4
-	-	-	-	-	-	-	C16	5
-	-	+	-	-	-	-	C17	6
-	-	-	-	-	-	-	C18	7
-	-	+	-	-	-	-	C19	8
-	-	-	-	-	-	-	C20	9
-	-	-	-	-	-	-	C21	10
+	+	-	-	-	-	-	C31	11
+	+	+	-	-	-	-	C32	12
-	-	-	-	-	-	-	C33	13
+	+	+	-	-	-	-	LF36	14
-	-	+	-	-	-	-	LF37	15
+	+	+	-	-	-	-	LF39	16
-	-	-	-	-	-	+	LF40	17
-	-	-	-	-	-	+	LF41	18
-	-	+	-	-	-	-	LF42	19
+	+	-	-	+	-	-	LF44	20
+	+	+	-	-	-	-	LF50	21
-	-	-	-	-	-	-	LF54	22
-	-	-	-	-	-	-	LR59	23
-	-	+	-	-	-	-	LR61	24
+	+	+	-	-	-	-	LR66	25
+	+	+	-	-	-	-	LR67	26
+	+	-	-	-	-	-	LR68	27
+	+	+	-	-	-	-	LR73	28
+	-	+	-	-	-	-	LR74	29
+	-	+	-	-	-	-	LR75	30
+	+	+	-	-	-	-	LR76	31
+	+	+	-	-	-	-	LR77	32
-	-	-	-	-	-	-	LR78	33
+	+	-	-	+	-	-	KR17	34
-	-	-	-	-	-	-	KR18	35
-	-	-	-	-	-	-	KR19	36
+	+	-	-	-	-	-	KR20	37
-	-	-	-	-	-	-	KR21	38
-	-	-	-	-	-	-	KR25	39
-	-	-	-	-	-	-	KR26	40
+	+	-	-	+	-	-	KR27	41
+	+	-	-	-	-	-	KR30	42
-	-	-	-	-	-	-	KR31	43
-	-	-	-	-	-	-	KR32	44
-	-	-	-	-	-	-	KR34	45
+	+	-	-	+	-	-	KR35	46
-	-	-	-	-	-	-	KR36	47
-	-	-	-	-	-	-	KR37	48
-	-	-	-	-	-	-	KR38	49

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-	-	-	-	-	-	-	KR39	50
+	+	-	-	-	-	-	KR40	51
+	+	-	-	-	-	-	KR41	52
-	-	-	-	-	-	-	KR42	53
+	+	-	-	+	-	-	KR43	54
-	-	-	-	+	-	-	KR45	55
-	-	-	-	-	-	-	KR46	56
-	-	-	-	-	-	-	KR48	57
22	20	19	0	6	0	4	Total	

Finally, out of 57 strains, only 23 strains did not have any pathogenic genes, and these 23 samples went to the next stage to check the technological properties.

2-3-Evaluation of autolytic activity

Autolytic activity is the ability of strains to lyse cells and release intracellular enzymes (such as lipase and protease) during cheese ripening, which is one of the important characteristics of lactic acid bacteria. In this regard, Franciosi et al., in a study on the technological and biological potential of lactic acid bacteria isolated from cow's milk, showed that the maximum speed and amount of autolytic activity belonging to the species*Enterococcus faecalis* was and species*Enterococcus durans* And*Lactococcus lactis* also had good autolysis activity [37]. Ayad et al., in a study on lactic acid bacteria isolates, reported that the autolytic activity of lactic acid bacteria strains is different and they are divided into three groups: good, medium and weak. Lactobacilli showed a higher degree of autolysis than Enterococci and Lactococcus strains [36]. In the evaluation of the autolytic activity of enterococci, Asprey et al observed that among the 57 strains of enterococcus examined, only 10 strains of enterococci had high autolytic activity. Therefore, according to the above studies, autolytic activity to release some intracellular enzymes is known as a desirable feature in the production of some dairy products such as cheese [41].

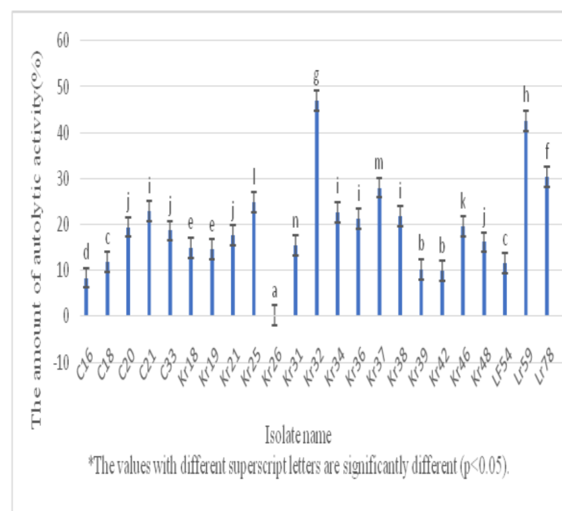

Fig 1 Investigation of autolytic activity

One of the ways to speed up the production of dairy products is to add co-culture, and the selection of co-culture strains should be based on the profile of enzymes (such as protease enzyme) and autolytic characteristics [42]. Also, the results of the study by Dawati et al. on the autolytic activity of Enterococcus species showed that Enterococcus faecium and Enterococcus faecalis had the highest degree of autolysis, respectively [43].

In this study, isolates Lr59 and kr32 have good autolytic activity, isolates kr25 and Lr78 have relatively good autolytic activity, and other isolates have weak autolytic activity.

3-3-Evaluation of acidification activity

For the production of fermented dairy drinks, the amount of acid production is an important factor that comes from the metabolism of milk lactose and plays a role in creating the flavor of fermented products. A decrease in pH prevents the growth of undesirable bacteria, including spoilage and pathogenic bacteria. A suitable mesophilic bacterium accelerates acid production

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by a starter culture in milk by lowering the pH [44]. In this study, strains LF54 and c21 showed the highest decrease in pH (1.9 units) and strain Kr25 showed the lowest decrease in pH (1.35 units).

According to the classification of Nito Aribas et al., the isolates are divided into three groups according to their ability to reduce pH after 24 hours in the greenhouse: 1) those with high acid capacity with a pH reduction of more than 2 units; 2) those with medium acid capacity with the ability to reduce pH in the range of 2-1.5 units and 3) those with low acid capacity with the ability to reduce pH less than 1.5 units [45].

Based on this classification, isolates Kr19, Kr25 and Lr78 have low acid capacity and other isolates have medium acid capacity.

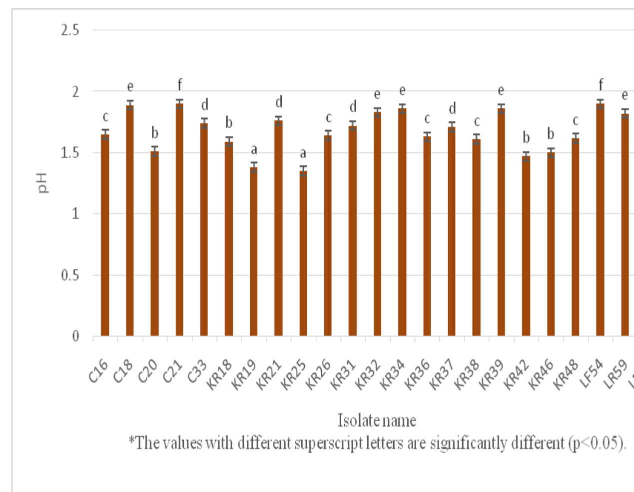

Fig 2 Acid production capability of the isolates

According to the researchers' study, enterococci have moderate acid activity with a decrease in pH in the range of 2-1.5 units. strains*Enterococcus faecalis* It has the lowest pH value and the highest acid activity, followed by strains*Enterococcus faecium* have been [23 and 46]. The study by Dawati et al. on the acidification activity of camel milk isolates showed that the highest decrease in pH was related to*Enterococcus durans*، *Enterococcus mondetti*، *Enterococcus faecium* And*Enterococcus durans* has been [43].

3-4- Evaluation of lipolytic activity

Co-cultures are also selected according to other characteristics such as proteolytic and lipolytic activity, which lipolytic activity is an important process in the development of flavor and texture of dairy products such as cheese [4]. This process by the lipase enzyme hydrolyzes triglycerides and leads to the production of flavor-producing compounds such as methyl ketone, esters and lactones, and reports indicate that Enterococcus faecalis has the most lipolytic activity, followed by It has been Enterococcus faecium and Enterococcus durans [23, 47]. The results obtained from the study of researchers in the field of lipolytic evaluation using tributrin agar medium have shown low lipolytic activity in enterococci [34, 4, 48 and 41]. On the other hand, in the study of Masedu and Malkata, a strain of*Enterococcus faecium* able to reduce milk fat to a greater extent than*Lactococcolactis* Slow hydrolysis [49]. The study by Dawati et al. showed that the isolates had good lipolytic activity and Enterococcus faecalis showed the highest lipolytic activity [43]. Lipolysis is desirable in Italian and blue cheeses, as a small hydrolysis of milk fat results in improved flavor without imparting a bitter aftertaste. On the other hand, lipolysis is unfavorable in fermented milks [50].

In this study, two isolates, c16 and c33, had no lipolytic activity, and the highest and lowest lipolytic activity was related to isolate c21 and isolate c20, respectively.

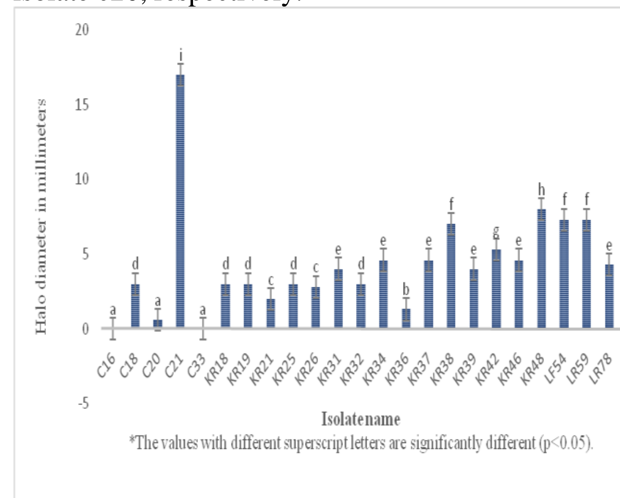

Fig 3 Assessment of lipolytic activity

5-3- Evaluation of proteolytic activity

According to the researchers, the degradation of casein in relation to the proteolytic and peptidolytic activity of microorganisms plays an indicator role in cheese ripening and cheese

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texture [51, 27, 47]. In addition, some peptides help to form the desired aroma and flavor in the product, and some other peptides lead to undesirable aroma and flavor. In addition to native proteolytic enzymes of milk and renin enzyme, which play a role in protein coagulation, protease and intracellular peptidases are released in the curd after lysis of the cell wall of lactic acid bacteria, which plays an important role in the hydrolysis of casein during cheese preparation. do [52]. In this regard, the level of protease and peptidase activities in enterococci is low, so that the most active of them is Enterococcus faecalis [23]. According to the researchers, proteolytic activity depends on the type of strain and time, on this basis, a strain with proteolytic activity can have higher activity over time [53].
Franciosi et al., in examining the technological characteristics of lactic acid isolates from cow's milk, reported the low proteolytic activity of lactic acid bacteria, so that only 8 strains (*Lactococcolactis* subspecies*Lactis NS38*, Staphylococcus aureus V96, V98 and V99, Enterococcus faecalis N4 and P343 and Enterococcus durans V25 and V1) were able to create a halo in the milk culture medium [37]. Asprey et al. observed that 78% of the examined Enterococcus strains were able to create a halo in the culture medium of colostrum, and 22% of the strains were unable to have proteolytic activity due to the lack of halo formation in the medium [41]. Berr C. Dawati et al showed that enterococci isolated from camel milk did not have visible proteolytic activity capable of creating a halo around the colony [43].
In this study, isolate Kr32 had no proteolytic activity, isolate c18 had the highest and isolate Kr42 had the lowest amount of proteolytic activity.

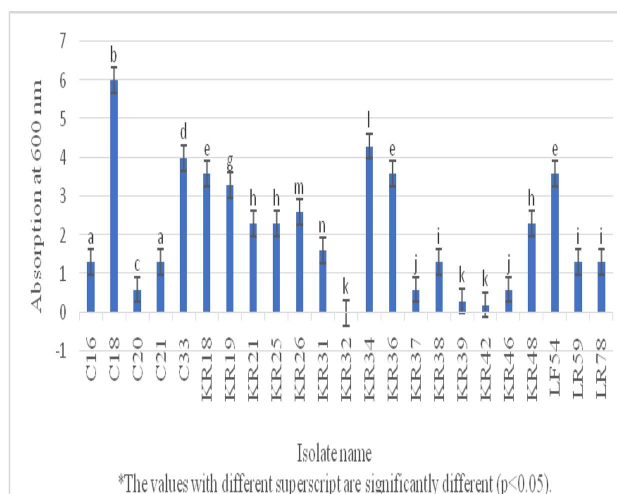

Fig 4 Evaluation of proteolytic activity

6-3-Evaluation of exopolysaccharide production

According to Asprey et al.'s study, 36% of the studied Enterococcus strains had the ability to produce exopolysaccharide, and they introduced it as an advantage, because it leads to a better mouthfeel (such as better viscosity, soft texture, and creaminess). For example, exopolysaccharide-producing strains have been used as starter cultures in the production of Aso-Scandinavian cheeses in order to create a better texture (rope texture). In addition, exopolysaccharide-producing bacteria can be substituted for hydrocolloids to help reduce costs in the production of fermented products. Apart from their role in improving the rheology of fermented dairy products, exopolysaccharide-producing lactic acid bacteria may play a protective role against environmental factors such as drying, phagocytosis, phage attack, osmotic stress, antibiotics, or toxic compounds. slow [54]. Also, the production of exopolysaccharide is a desirable trait for probiotic bacteria to colonize in the digestive tract [41].
Among the 23 investigated strains, 16 isolates were able to produce white colonies on ruthenium red milk (RRM) culture medium, so they have the ability to produce exopolysaccharide.

Table 4 Examination of exopolysaccharide production

Exopolysaccharide production	Bacteria code	Exopolysaccharide production	Bacteria code
+	KR34	-	C16

+	KR36	-	C18
+	KR37	+	C20
+	KR38	+	C21
+	KR39	-	C33
+	KR42	-	KR18
+	KR46	-	KR19
+	KR48	+	KR21
-	LF54	-	KR25
+	LR59	+	KR26
+	LR78	+	KR31
16	Total	+	KR32

7-3- Assessment of acid resistance

Riham et al. in evaluating the resistance of strains*Enterococcus faecium* isolated from a Tunisian fermented dairy product to acidic conditions at pH 1 to 5 reported the survival of strains*Enterococcus faecium* It was more in pH 3, 4 and 5 and in contrast, the survival of strains in higher acidic conditions of pH 1 and 2 decreased with increasing time and greenhouse storage [55]. Also, Ahmadova in her study on Roy*Enterococcus faecium* isolated from cheese, reported that the studied strain grew well in Broth MRS culture medium with pH values between 7 and 11, growth was slower at pH = 13 and no growth was observed at pH 3 and 4 [56]. Resistance to acidic conditions (low pH) is considered as one of the important characteristics of lactic acid bacteria, because it shows their ability to survive as well as grow in the small intestine [57, 58]. Resistance strains*Enterococcus faecium* The investigated case at pH 3, 5, 3, 4, 5 and 6 showed that the growth of the strains decreased with the decrease in the pH of the culture medium. At pH 5 and 6, the growth has been done well, but with the decrease in pH, the growth trend has decreased and slowed down a lot. Also, isolate Lr78 has the highest resistance to acid, followed by Kr36 and Lr59, showing resistance to acidic conditions.

Table 5 Examination of acid resistance at different pH

PH=3	PH=3.5	PH=4	PH=5	PH=6	Bacteria code	Row
0.086 and	0.168 c	0.310 b	0.404 a	1.309 a	C16	1
0.076 d	0.094 b	0.201 a	0.456 a	1.139 a	C18	2
0.085 and	0.093 b	0.202 a	0.506 b	1.189 b	C20	3
0.053 c	0.060 a	0.136 c	0.499 a	1.393 a	C21	4
0.077 d	0.111 and	0.154 c	0.524 b	1.483 c	C33	5
0.051 c	0.070 b	0.079 d	0.515 b	1.263 a	Cr18	6
0.093 b	0.140 d	0.237 a	0.574 b	1.159 b	Kr19	7
0.080 and	0.097 b	0.284 a	0.406 a	1.352 a	Kr21	8
0.050 c	0.063 b	0.175 b	0.583 b	1.293 a	Kr25	9
0.077 d	0.182 c	0.185 a	0.510 b	1.083 d	Kr26	10
0.106 a	0.114 and	0.207 a	0.455 c	1.247 a	Kr31	11
0.105 a	0.113 and	0.304 b	0.416 a	1.273 a	Kr32	12
0.093 b	0.095 b	0.267 b	0.440 c	1.221 a	Kr34	13
0.145 f	0.182 c	0.297 b	0.680 d	1.321 c	Kr36	14
0.097 b	0.112 and	0.268 a	0.690 d	1.324 a	Kr37	15
0.102 a	0.113 and	0.239 a	0.404 a	1.211 a	Kr38	16
0.118 f	0.184 c	0.206 a	0.505 and	1.199 b	Kr39	17
0.089 and	0.114 and	0.229 c	0.369 f	1.174 b	Kr42	18
0.057 c	0.091 b	0.212 d	0.384 f	1.219 c	Kr46	19
0.067 b	0.160 c	0.259 b	0.507 and	1.160 b	Kr48	20
0.085 d	0.153 f	0.310 and	0.484 and	1.189 b	LF54	21
0.126 f	0.137 g	0.292 a	0.657 g	1,249 and	Lr59	22
0.158 g	0.168 c	0.197 b	0.640 g	1.265 a	Lr78	23

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*The values with different superscript letters in a same column are significantly different (p<0.05)

3-8- Evaluation of thermal resistance

The presence of enterococci in pasteurized milk is due to their resistance at 62.8 degrees Celsius for 30 minutes. The spread and persistence of enterococci during the growth of dairy products in the growth temperature range (10-45), heat resistance, pH resistance in the range of 9.6– 4 and survival in sodium chloride is attributed to 6.5% [47]. Enterococci may be naturally present in starter cultures of dairy products. The starter culture usually consists of thermophilic lactic acid bacteria, the presence of enterococci is natural in the starter culture due to its high heat resistance and thermophilic nature. In fact, the starter culture is created by a traditional process by pasteurizing raw milk of suitable quality and keeping it in a greenhouse at 42-44°C for 12-15 hours; Therefore, the natural selection of lactic acid bacteria to*Streptococcus thermophilus*and thermophilic and heat-resistant enterococci are limited [59]. Investigation by Kearns et al. in evaluating the thermal resistance of strains*Enterococcus faecium* And*Enterococcus faecalis* at temperatures of 65, 71, 75 and 80 °C for 30 minutes reported that all strains*Enterococcus faecium* at 60°C for 20 minutes and some strains survived at temperatures of 71, 75 and 80 degrees Celsius for three minutes. This is while all the isolates*Enterococcus faecalis* also survived at 65°C for 10 minutes and 71°C for 3 minutes [24].

T=85 (30min)	T=85 (15min)	T=80 (30min)	T=80 (15min)	T=75 (30min)	T=75 (15min)	T=70 (30min)	T=70 (15min)	T=65 (30min)	T=65 (15min)	T=60 (30min)	T=60 (15min)	bacteria code
0.207a	0.228 a	0.577 c	0.971 a	1.496 a	1.560 b	1.589 b	1.701 b	1.736 a	1.762 a	1.963 a	2.249 a	C16
0.153d	0.222 a	0.227 c	0.242 a	0.264 b	0.485 a	1.546 b	1.694 b	1.762 a	1.845 a	1.987 a	2.149 b	C18
0.225a	0.236 a	0.247 b	0.307 b	1.580 a	1.689 c	1.764 c	1.895 c	1.987 b	2.082 b	2,498 and	2,635 and	C20
0.240 a	0.282 b	0.300 a	0.336 a	0.399 b	1.639 c	1.791 c	1.859 c	1.929 b	2.096 b	2.221 c	2,648 and	C21
0.156d	0.28 a	0.289 a	0.302 a	0.349 b	1.653 b	1.722 c	1.853 c	1.898 a	2.033 b	2.358 d	2,650 and	C33
0.175e	0.242 c	0.249 b	0.257 a	1.653 b	1.684 c	1.759 c	1.793 b	1.973 b	1.981 a	2.257 c	2.402 d	Cr18
0.228a	0.254 c	0.278 c	0.282 b	0.366 a	0.378 a	1.852 d	1.896 c	1.922 b	2.091 b	2.172 b	2.217 a	Kr19
0.133d	0.158 d	0.215 a	0.246 a	0.269 a	0.285 b	1.490 b	1.820 c	1.937 b	2.221 d	2,499 and	2,608 and	Kr21
0.107f	0.142 d	0.222 a	1.298 c	1.584 a	1.691 c	1.789 d	1.841 c	2.100 d	2.242 d	2.288 c	2.525 d	Kr25
0.142d	0.154 d	0.184 d	0.210 a	0.224 b	0.286 a	0.320 a	1.799 b	1.806 a	2.193 c	2.242 c	2.281 a	Kr26
0.163e	0.186 a	0.235 b	0.260 a	0.284 b	1.545 b	1.825 d	1.981 d	2.032 c	2.102 c	2.271 c	2.302 c	Kr31
0.162e	0.252 a	0.267 b	0.281 b	0.286 b	1.756 b	1845 and	1.891 c	1.796 b	2.200 d	2.226 c	2.282 a	Kr32
0.206a	0.255 a	0.298 a	0.313 b	0.319 c	0.500 a	1.806 c	1.899 c	2.037 c	2.158 c	2.266 c	2.314 c	Kr34
0.234b	0.253 b	0.261 d	0.271 a	0.278 c	0.304 b	1.789 c	1.848 c	1.879 a	2.041 b	2.398 c	2.540 d	Kr36
0.241b	0.251 b	0.261 d	0.267 a	0.279 a	0.284 c	0.300 a	1.859 c	1.984 b	2.023 b	2.222 c	2.285 a	Kr37
0.241b	0.264 c	0.284 a	0.297 a	0.322 d	1.697 c	1.818 d	1.858 c	1.965 b	2,997 and	2.117 b	2.282 a	Kr38
0.232b	0.267 c	0.299 b	0.322 b	1.794 d	1.869 d	1887 and	2013 and	2.037 c	2.126 c	2.195 b	2.293 a	Kr39
0.244b	0.254 b	0.261 c	0.266 a	0.272 a	0.283 a	0.293 a	1.871 c	1.946 b	1.986 a	2.037 a	2.224 a	Kr42
0.273c	0.284 a	0.313 b	1.521 d	1.644 b	1.807 d	1928 and	1.961 d	1.995 b	2.063 b	2.093 a	2.149 a	Kr46
0.199g	0.23 b	0.570 c	1.106 d	1.190 b	1.400 b	1.781 c	1.862 c	1.892 a	1.926 a	2.209 c	2.272 a	Kr48
0.250 c	1,234 and	1,522 and	1,666 and	1.744 d	1.895 d	1.835 c	1.855 c	1.933 b	1.956 a	1.994 a	2.098 b	LF54
0.151d	0.181 d	0.245 c	0.251 a	0.273 a	0.286 a	1.260 b	1.422 a	1.893 a	1.930 a	1.946 a	2.114 b	Lr59
1.688h	1,755 and	1,779 and	1.834 f	1866 and	1955 and	1.999 f	2,043 and	2.054 c	2.229 d	2.366 d	2.414 d	Lr78

Table 6 Evaluation of thermal resistance

*The values with different superscript letters in a same column are significantly different (p<0.05(

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In a study on heat resistance of enterococci sensitive and resistant to vancomycin, Bradley and Friesi reported that only one type of clinical strain showed the highest resistance, such that at 65°C for 10 minutes, 71°C for 3 minutes and the temperature was 80°C for one minute. In case, all the clinical strains showed different resistance except vancomycin sensitive strains. Three of the strains survived at 80°C for more than three minutes, and these strains were resistant to vancomycin [60]. According to the researches, enterococci have good thermal resistance, so that they survive in pasteurization of milk, and enterococci can be used as a co-culture for the fermentation of dairy products.

9-3- Assessment of antimicrobial activity

The presence of bacteriocin-producing enterococci in fermented products has an inhibitory effect on spoilage and pathogenic bacteria, as well as enterococci in particular.*Enterococcus faecium* And*Enterococcus durans* In terms of lipolytic, proteolytic and citrate decomposition activities, they play a significant role in creating the flavor of fermented products, but enterococci lead to diseases such as endocarditis, bacteremia, and urinary infections. Their resistance to several antibiotics, including vancomycin, as well as the presence of aggressive factors, have made it necessary to investigate the negative effects of enterococci on the health of the consumer in case of use of enterococci in fermented products [61 and 62].

Table 7 Evaluation of antimicrobial activity

Indicator microorganisms					Bacteria code	Row
Listeria	*Escherichia coli*	*Staphylococcus aureus*	*Lactococcus lactis*	*Lactobacillus plantarium*		
-	-	-	-	-	C16	1
-	+	+	-	-	C18	2
-	-	+	-	-	C20	3
-	+	-	-	-	C21	4
-	-	+	+	-	C33	5
+	-	+	+	+	KR18	6
+	+	-	+	+	KR19	7
-	-	+	+	-	KR21	8
-	+	+	-	+	KR25	9
+	-	+	+	+	KR26	10
-	-	-	+	+	KR31	11
-	+	-	-	+	KR32	12
+	+	-	-	+	KR34	13
-	-	-	-	-	KR36	14
+	+	-	-	+	KR37	15
-	-	-	-	-	KR38	16
-	+	+	+	-	KR39	17
+	-	-	-	+	KR42	18
+	+	-	-	+	KR46	19
-	+	+	-	+	KR48	20
-	+	+	-	+	LF54	21
-	+	-	+	-	LR59	22
-	-	-	-	-	LR78	23
7	12	10	8	12	Total	

Bel Jassim et al. reported that in evaluating the antimicrobial activity of strains*Enterococcus faecium*Isolated from Tunisian fermented meat, strains of Enterococcus faecium against several spoilage and pathogenic bacteria including*Listeria*، *Enterococcus* And*Staphylococcus aureus* showed antimicrobial activity as well as one of the strains against*Escherichia coli* has been active [63].

The results of the study of Enterococcus strains isolated from kefir by Kargarari et al. showed that the strains*Enterococcus durans* They were able to

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inhibit Gram-positive and Gram-negative pathogens [64]. Morandi et al., in the study of genotypic, phenotypic and technological characteristics of lactic acid bacteria isolates, reported that only*Enterococcus faecium*able to restrain*Listeria monocytogenes* and created a halo with a diameter of 14 mm and five isolates (one strain of*Streptococcus* , 3 strains of*Lactobacillus delbruecki* and a strain of*Lactobacillus fermentum*) antagonistic activity against*Staphylococcus aureus* (8-11 mm) showed [4]. In this research, only 4 isolates did not have any antimicrobial activity and other isolates have antimicrobial activity against bacteria.*Lactobacillus plantarium*، *Lactococcus lactis*، *Staphylococcus aureus*، *Escherichia coli* And*Listeria* They were.

4 - Conclusion

In this research, 57 isolates*Enterococcus faecium* The result of traditional Iranian cheeses firstly in terms of the presence of pathogenic genes including vancomycin resistance gene (van A and van B), gelatinase (gel E), enterococcus surface protein (esp), aggregation factor (asa1), sex pheromones (cpd)) and collagen binding factor (ace) were screened using multiplex PCR method. 23 isolates did not have any pathogenic genes and their technological properties were investigated. All strains studied in this research, except for 4 strains LR78, KR38, KR36 and C16, have antimicrobial activity against pathogenic bacteria.*Lactobacillus plantarium, Lactococcus lactis*، *Staphylococcus aureus, Escherichia coli, Listeria* They were. Among 23 strains, 16 strains were able to produce exopolysaccharide. LR59 and KR32 strains had good autolytic activity. The highest proteolytic and lipolytic activity is related to C18 and C21 strains, respectively. Except for the three strains KR19, KR25 and LR78, which have low acid capacity, other isolates have moderate acidification activity. Also, the studied isolates had good heat resistance and acid resistance. Especially, LR78 strain showed the highest resistance to acid and the highest heat resistance. Therefore, in general, it can be said that these isolates have favorable technological characteristics.

5- Resources

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مقاله علمی_پژوهشی

ارزیابی شاخص های بیماری زایی و خواص تکنولوژیکی جدایه های *انتروکوکوس فاسیوم* حاصل از پنیر های سنتی ایران

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چکیده

اخیرا به انتروکوکوس ها برای استفاده به عنوان پروبیوتیک در فراورده های لبنی توجه خاصی شده است. تمامی این ویژگی های مطلوب محرکی برای تولید کنندگان فرآورده های لبنی جهت استفاده از انتروکوکوس های ذاتی جدا شده از فرآورده های لبنی نظیر پنیر لیقوان، می باشد.علی رغم داشتن تمامی این ویژگی ها انتروکوکوس ها به عنوان GRAS شناخته نمی شوند و حضور آن ها در فراورده های غذایی نشانه ای از آلودگی مدفوعی می باشد. هدف از این پژوهش بررسی انتروکوکوس های جدا شده از پنیر لیقوان و کوزه به لحاظ دارا بودن شاخص های بیماری زایی به منظور تایید بی خطر بودن برای مصرف کننده و در نهایت بررسی امکان به کارگیری آنها به عنوان آغازگر و یا کمک آغازگر در فرآورده های لبنی به ویژه پنیرها می باشد. بر این اساس، ۵۷ جدایه *انتروکوکوس فاسیوم* از پنیر های سنتی ایران از نظر وجود ژن های بیماری زایی بررسی شدند که در نهایت ۲۳ جدایه فاقد هرگونه ژن بیماری زایی بودند. سپس خواص تکنولوژیکی این جدایه ها از قبیل خاصیت اسیدیفیکاسیون، پروتئولیتیک، لیپولیتیک، اتولیتیک، مقاومت حرارتی و اسیدی و تولید اگزوپلی ساکارید مورد بررسی قرار گرفت. نتایج به دست آمده نشان داد که از بین ۲۳ سویه مورد بررسی ۱۹ جدایه دارای فعالیت ضد میکروبی در برابر باکتری های بیماری زای شاخص، ۱٦ سویه قادر به تولید اگزوپلی ساکارید و ۲۰ جدایه دارای خاصیت اسیدیفیکاسیون متوسط بودند. بیشترین فعالیت پروتئولیتیک و لیپولیتیک به ترتیب مربوط به سویه های c18و c16 بود و سویه LR78 بیشترین مقاومت اسیدی و حرارتی را از خود نشان داد.

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