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Research Article

Isolation and Identification of Lactobacillus Acidophilus and Bifidobacterium from Different Types of (Traditional) Cheese and Study Their Antibacterial Properties

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ABSTRACT:

Probiotics are dietary supplement from live microorganisms when exist in appropriate values in gastrointestinal would have healthy benefits on the host body. Among the bacteria, lactic acids are the most common type of them that have been introduced as probiotics. The aim of this study was to isolate and identify bacteria with probiotic potential by examining different types of traditional cheese such as Lavark, jars and cheddar cheeses and assessing their inhibitory effects on the growth of Escherichia coli and Listeria monocytogenes. To achieve this goal, lactic acid bacteria were isolated by phenotypic methods (e.g. stain colouring, biochemical and physiological tests) and their basic probiotics indicators (i.e. resistance to acid, bile salts) were evaluated. Finally, five strains of Lactobacillus and one Bifidobacterium strain as natural microbial flora from Lavark samples, 4 strains of lactobacillus from jug cheese and one Lactobacillus strain from cheddar cheese were identified. The results of select screening would include Antibiogram test showed resistance to bile, acid and inhibition test that Lactobacillus reuteri and Lactobacillus casei have potentiality to use as probiotics in dairy products.

Keywords: Lactobacilli - Escherichia Coli - Listeria Monocytogenes, Traditional Cheese, Lavark, Jug Cheese, Cheddar

[I] NTRODUCTION

It's been for a long time that the importance of consumption of dairy products has been proven for human health. Today, lots of researches are done to add probiotic bacteria in dairy products and the effect of such bacteria on the health of people. Probiotics are live microorganisms that if sufficiently exist in the host body would have positive impacts on the health of the individual. The term probiotics was used from the 1960s and is meant "for life" [1]. Probiotic bacteria isolation from dairy products cannot only lead to the isolation of probiotic bacteria with special properties, but would provide a suitable perspective for mass production of dairy products containing naturally probiotic bacteria [2]. Lactic acid bacteria are used as starter for production of dairy products. They are the main cause of fermentation and preservation of food. They also have a significant role in making odor, taste and texture of fermented dairy products. Among probiotic microorganisms, lactic acid and Bifidobacterium bacteria have been identified as the most important group of them. Another important characteristic of Lactobacillus is the production of bacteriocin. Lactobacillus used to improve the smell, taste and texture of foods in the industry [3]. Lactobacillus strains strengthen the intestinal mucosal barrier which will help to maintain and enhance the immunity system of individual, reduce the movement of bacteria through the intestinal mucosa, and also have a crucial role in reducing the incidence of inflammatory bowel disease and irritable bowel syndrome [4]. With establishment of probiotics in intestinal environment, it would improve microbial balance in order to increase their usefulness in a way that by their activity inhibits the activation of non-useful pathogens microorganisms [5].

[II] MATERIALS AND METHODS

In this study, jug cheese, traditional cheddar cheese (by the local name of Selk) and Lavark cheese with the number of 15 samples of each 100 grams were randomly prepared from different shops of Mahabad city, Iran. They were transferred in sterilized containers for sampling to the Islamic Azad University of Mahabad microbiology laboratory. The cheese samples were stored at 4 °C there. It should be noted that the cheese samples were collected from 5th April to July 2014. Ayad et al' method (2004) was used in order to isolate bacteria that produce lactic acid. Firstly, 25 grams of each cheese were separately added to 225 ml culture peptone water. Then were mixed by Stomaker device for 2 minutes and finally serial dilutions from 10^{-1} to 10^{-5} were prepared. Then 100 ml of each dilution were transferred separately to the solid medium MRS agar environment by the help of sampler and cultured on the surface. Then, the plates were incubated for 24 to 48 hours under aerobic and non-aerobic conditions by the help of Pack A gas at 83 °C. It should be noted that the experiment was repeated twice. After the appearance of colonies, 2 and 3 different types of colonies were randomly selected and were purified by passage over the previous environment. Then according to the method of Greco et al. (2005) first, by preparing and hot colouring, then catalyzing and doing biochemical tests and also fermentation of carbohydrates identifying separated bacteria have been done.

Antimicrobial properties of isolates

To do this, lactic acid bacteria isolated from cheese samples in 10 ml at MRS environment for 24 hours at 37 °C were cultured. Then, from each of the bacteria which growth at mentioned environment, were cultured point-wise on the MRS agar surface and were incubated at 37 °C for 24 to 48 hours at non-anaerobic conditions. To evaluate the ability of bacteria to inhibit the growth of E. coli and Listeria monocytogenes standardized strains were used. Thus, 24 hour culture for both bacteria by the density of $5*10^8$ CFU / ml was prepared. Then, microbial sensitivity testing of isolates and sensitivity tests against acid and bile were performed. Statistical analysis of data, before comparing the means, have been evaluated and integrated using Kolmogorov-Smirnov in order to ensure the normality of data. To analyze the data, ANOVA (One –Way Analysis of Variance), SPSS (version 19), and Tukey test (abbreviation: HSD) were used. In all studies, the significance level of tests was considered as P < 0/05. Also, drawing the diagrams was conducted by Excel version 2010.

[III] RESULTS

The results showed that culturing various cheese samples on MRS agar medium are resulted in the isolation of yeasts, Lactobacillus and Bifidobacterium. Results related to the cultivation of jug, cheddar and Lavark cheese is mentioned at Table 1-3. As the results of this chart shows the total isolated microorganisms from Lavark cheese (11 types of microorganisms) were more than the sum of isolated microorganisms from two type of jar (8) and cheddar (3) cheeses. Of 15 samples of jars cheese and also 15 samples of cheddar cheese no Bifidobacterium have been isolated; however, one type of Bifidobacterium was isolated from Lavark cheese. The most isolated Lactobacillus species was related to Lavark cheese (3 different species).

Table 1: microbiological analysis of 100 grams of different cheeses after cultivation at MRS agar medium

15 samples of cheddar cheese	15 samples of jar cheese	15 samples of Lavark cheese	isolated microorganisms
2 different species	4 different species	5 different species	Yeast
1	*4*	5 different species	Lactobacillus
-	-	*1* species	Bifidobacterium
3	*8*	*11*	Sum

Then, identifying Lactobacillus and Bifidobacterium species were taken using common methods of bacteriology including morphology, hot colouring, biochemical tests and fermentation of carbohydrates based on diagnostic characteristics.

Table 2: identified species of isolated Lactobacillus from 15 samples of Lavark cheese

Row	Microorganisms	fr (in 15 samples of faeces)	fr
1	Lactobacillus rhamnosus	*10*	*37*
2	Lactobacillus acidophilus	*7*	*26*
3	Lactobacillus reuteri	*5*	*18/5*
4	Lactobacillus fermentum	*2*	*7/4*
5	Lactobacillus delbrueckii	*2*	*7/4*
6	Bifidobacterium lactis	*1*	*3/7*
Sum		*27* subjects	*100*

Bacteriology studies of jar cheese samples showed that of total 15 different samples 4 different species were isolated from the genus of Lactobacillus. Table 3 shows that Lactobacillus acidophilus has the most frequency (about % 44/48) and Lactobacillus casei has the lowest (11/12 %) frequency.

Table 3: identified isolated species of Lactobacillus and Bifidobacterium from 15 samples of jar cheese.

Row	Microorganisms	fr (in 15 samples of faeces)	fr
1	*Lactobacillus acidophilus*	*8*	*44/48*
2	*Lactobacillus lactis*	*5*	*27/8*
3	*Lactobacillus fermentum*	*3*	*16/6*
4	*Lactobacillus casei*	*2*	*11/12*
Sum		*18* subjects	*100*

Bacteriology studies of cheddar cheese samples showed that of total 15 different species, one type was different from isolated Lactobacillus genus, i.e. it was Lactobacillus paracasei.

The results of bacteriological studies of these species are mentioned in Table 8 which was including morphology, biochemical tests and fermentation of carbohydrates.

Table 4: results of biochemical studies about isolated Lactobacillus and Bifidobacterium from samples of Lavark , jar and cheddar cheese

Test	L. rhamnosus	L. acidophilus	L. reuteri	L. fermentum	L. delbrueckii	L. lactis	L. casei	L. paracasei	B. lactis
Growth at ºC*15*	-	-	+	+	-	-	+	+	-
Growth at ºC*45*	-	-	+	+	+	+	+	-	-
VP	-	-	+	-	-	+	-	-	-
instauration of nitrate	-	-	-	-	-	-	-	-	-
Gas production of glucose	-	-	+	+	-	+	+	-	+
Movement	-	-	-	-	-	-	-	-	-
Catalyze	-	-	-	-	-	-	-	-	-
Arabinose	-	+	+	+	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-
Inulin	-	-	-	-	-	-	-	-	-
Raffinose	+	-	-	+	-	+	-	-	+
Rhamnosus	-	-	-	-	-	-	-	-	-
Cellobiose	+	+	-	+	-	-	+	+	-

Sorbose	-	-	-	-	-	-	-	-	-
Sorbitol	+	+	-	+	-	-	+	+	-
Fructose	-	-	-	-	+	-	+	+	-
Galactose	-	-	-	-	-	-	+	+	-
Wood sugar	-	-	+	-	-	-	-	-	-
Lactose	+	-	+	+	+	+	+	+	+
Mannose	+	+	-	+	-	+	+	+	-
Mannitol	+	+	-	+	-	-	+	+	-
Melezitose	+	+	-	-	-	-	+	+	-
Melibius	+	-	+	+	-	-	-	-	-

Table 5: Obtained results from antimicrobial properties of isolated bacteria from different cheeses

Cheese Sample	Row	Microorganisms	*E. coli*	*Listeria monocytogenesis*
Lavark	***1***	***L. rhamnosus***	***13 ± 0/12***	*13 ± 0/2*
	2	***L. acidophilus***	***11 ± 0/9***	*15 ± 0/4*
	3	***L. reuteri***	***22 ± 0/2***	*24 ± 0/6*
	4	***L. fermentum***	***0***	*3 ± 0/4*
	5	***L. delbrueckii***	***0***	*0*
	6	***Bifidobacterium lactis***	***7 ± 0/23***	*12 ± 0/11*
Jar	***1***	***L. acidophilus***	***0***	*0*
	2	***L. lactis***	***5 ± 0/1***	*7 ± 0/3*
	3	***L. fermentum***	***0***	*0*
	4	***L. casei***	***15 ± 0/4***	*10 ± 0/7*
Cheddar	***1***	***L. paracasei***	***5 ± 0/24***	*9 ± 0/87*
Enrofloxacin			***34 ± 0/4***	*29 ± 0/6*

Results of antimicrobial activity of the strains

As mentioned in Materials and Methods section, in this study, dual culture method were used in order to assess the antibacterial effects of isolated Lactobacillus and Bifidobacterium from different cheeses. Related results to this test are mentioned in Table 5.

Data were diameters of inhibition growth zone that were measured in millimetres and have been expressed as S.D. ± Mean. According to data from Table 8, isolated bacteria from Lavark cheese samples in comparison with isolated bacteria of jar and cheddar cheese, it could possible to inhibited two standard strains of E. coli and Listeria monocytogenes. Related results of antimicrobial sensitivity of bacteria producing isolated lactic acid from Lavark, jar and cheddar cheeses are shown in tables 6 and 7. According to theses tables, the most antimicrobial sensitivity were related to two antibiotics of streptomycin and erythromycin in isolated Lavark cheeses. About other antibiotics, antimicrobial susceptibility varies from sensitive to resistant.

Table 6: Test results of determining antimicrobial susceptibility of isolated bacteria from Lavark cheese

L. delbrueckii	*L. fermentum*	*L. reuteri*	*L. acidophilus*	*L. rhamnosus*	Antibiotics
resistant	sensitive	sensitive	normal	sensitive	Vancomycin
sensitive	normal	normal	sensitive	normal	Bacitracin
normal	sensitive	normal	resistant	normal	Ampicillin
sensitive	sensitive	sensitive	sensitive	sensitive	Streptomycin
sensitive	sensitive	sensitive	sensitive	sensitive	Erythromycin
sensitive	normal	sensitive	sensitive	sensitive	Enrofloxacin
sensitive	sensitive	normal	normal	normal	Polymyxin B

Almost similar pattern have been observed for jar cheese isolates, it means the most drug sensitivity was relate to two antibiotics of streptomycin and erythromycin.

Table 7: The results of determining the antimicrobial susceptibility of isolated bacteria from jar cheese

L. casei	*L. fermentum*	*L. lactis*	*L. acidophilus*	Antibiotics
sensitive	sensitive	sensitive	normal	Vancomycin
normal	normal	sensitive	normal	Bacitracin
sensitive	normal	normal	Resistant	Ampicillin
sensitive	sensitive	sensitive	sensitive	Streptomycin
sensitive	sensitive	sensitive	sensitive	Erythromycin
normal	sensitive	sensitive	sensitive	Enrofloxacin
sensitive	sensitive	sensitive	normal	Polymyxin B

Bile tolerance test results for samples of various isolated cheeses are shown at tables 8 and 9. According to the results, the most relevant bile tolerance was for Lactobacillus acidophilus bacteria isolated from Lavark cheese (table 8) and also the least amount of bile tolerance was for Lactobacillus casei isolated from jar cheese (table 9).

Row	Microorganisms	T1	T2	T2-T1
1	***Lactobacillus rhamnosus***	***[c] 0/79 2/45 ±***	***[c] 0/34 2/89 ±***	*[d] 0/08 0/44 ±*
2	***Lactobacillus acidophilus***	***[a] 0/21 4/33 ±***	***[a] 0/82 7/49 ±***	*[a] 0/11 3/16 ±*
3	***Lactobacillus reuteri***	***[d] 0/74 1/36 ±***	***[d] 0/19 1/78 ±***	*[d] 0/04 0/42 ±*
4	***Lactobacillus fermentum***	***[d] 0/28 1/15 ±***	***[d] 0/22 2/05 ±***	*[c] 0/13 0/9 ±*
5	***Lactobacillus delbrueckii***	***[b] 0/12 3/09 ±***	***[b] ± 0/7 5/16***	*[b] 0/06 2/07 ±*
6	***Bifidobacterium lactis***	***[c] 0/77 2/89 ±***	***[c] 0/49 3/51 ±***	*[d] 0/07 0/62 ±*

Table 8: the results of determining resistance of isolated Lactobacillus from Lavark cheese against % 0.3 Exgal solution

The numbers are expressed as mean ± SD.

Same letters in each column showed no statistically significant differences in the level of P < 0/05.

T1: required time to increase 0.3 light absorption of bacteria in broth MRS medium.

T2: required time to increase the absorption of 0.3 of light for each bactera in the environment of MRS broth containing 0.3 percent of exgal.

Table 9: results of determining the resistance of isolated Lactobacillus from jar cheese against 0.3 percent of exgal solution

Row	Microorganisms	T1	T2	T2-T1
1	***Lactobacillus acidophilus***	***[a] ± 1/1 5/31***	***[a] ± 1/9 7/16***	*[a] ± 0/09 1/85*
2	***Lactobacillus lactis***	***[c] ± 0/9 2/98***	***[c] ± 0/23 3/68***	*[d] ± 0/11 0/7*
3	***Lactobacillus fermentum***	***[d] ± 0/61 1/29***	***[d] ± 0/7 2/03***	*[d] ± 0/06 0/74*
4	***Lactobacillus casei***	***[b] ± 1/09 3/44***	***[b] ± 0/46 4/98***	*[b] ± 0/12 1/54*

The numbers are expressed as mean ± SD.

Same letters in each column showed no statistically significant differences in the level of P < 0/05.

T1: required time to increase 0.3 light absorption of bacteria in broth MRS medium.

T2: required time to increase the absorption of 0.3 of light for each bactera in the environment of MRS broth containing 0.3 percent of exgal.

Acid tolerance test results are given for isolates of cheese samples in tables 10 and 11. According to the results, the most relevant bile tolerance was for Lactobacillus fermentum isolated bacteria from samples of Lavark cheese (table 10) and the least amount of bile tolerance was for isolated Lactobacillus casei from jar cheese (table 11).

Table 10: results of determining susceptibility to isolated bacteria acid from the samples of Lavark cheese.

Row	Microorganisms	The number of bacteria at the time of zero Log10 CFU / ml	The number of bacteria after 24 hours Log10 CFU / ml
1	***Lactobacillus rhamnosus***	***7/11 ± 0/11***	*7/11 ± 0/11*
2	***Lactobacillus acidophilus***	***6/88 ± 0/9***	*8/24 ± 0/89*
3	***Lactobacillus reuteri***	***7/02 ± 0/54***	*7/57 ± 0/72*
4	***Lactobacillus fermentum***	***7/23 ± 0/89***	*7/09 ± 0/43*
5	***Lactobacillus delbrueckii***	***6/91 ± 0/44***	*7/48 ± 0/81*
6	***Bifidobacterium lactis***	***7/29 ± 0/68***	*7/54 ± 0/84*

The numbers are expressed as mean ± SD.

Table 11: results of determining susceptibility to isolated bacteria acid from the samples of jar cheese.

Row	Microorganisms	The number of bacteria at the time of zero Log10 CFU / ml	The number of bacteria after 24 hours Log10 CFU / ml
1	***Lactobacillus acidophilus***	***6/93 ± 0/44***	*8/04 ± 0/69*
2	***Lactobacillus lactis***	***7/52 ± 0/56***	*7/73 ± 0/22*
3	***Lactobacillus fermentum***	***7/21 ± 0/81***	*7/25 ± 0/87*
4	***Lactobacillus casei***	***6/29 ± 0/43***	*6/8 ± 0/45*

The numbers are expressed as mean ± SD.

[IV] DISCUSSION

Probiotics are live microbes that are primarily used in the treatment and prevention of many infectious diseases. If possible to deploy useful and harmless organisms it can prevent from colonization of different bacterial infections (De Roos and Katan, 1998; Gary et al., 1996) [6]. Many studies showed that Lactobacillus has positive role in the prevention and treatment of Intestinal disorders. Lactic acid producing bacteria are used as the most effective bacteria with the least harm in the treatment of antibiotic diarrhea, acute diarrhea during infancy, preventing from Clostridium difficile re-infections and other gastrointestinal diseases [7]. Lactobacillus has the power to connect the epithelial cells of the gastrointestinal tract of humans and animals [8]. In a study conducted by Fazli et al. (2007), it was found that Lactobacillus casei has the power to live and stability in the gastrointestinal tract of mice and is a good candidate as a probiotic factor against enterotoxigenic Escherichia coli. The first phase of enterotoxigenic Escherichia coli colonization in the gastrointestinal tract is to connect to the intestinal epithelial cells. This connection has done through the different colonization factors that this bacterium has. The bacteria have the power to colonize and settle in the digestive tract of mice [9]. In another study, Akalin et al (1997) gave yogurt containing Lactobacillus Acidophilus to the mice and concluded that the faecal among mice that ate yogurt containing Lactobacillus acidophilus were less than mice that had received Lactobacillus acidophilus.

Goldin showed in a study that Lactobacillus might has effective in the treatment and prevention of diarrhea and also in making vaccines for rota-virus and could involved as adjuvant [10]. Aiba et al (1998) are proved Lactobacillus role in inhibiting Helicobacter pylori colonization in the gastrointestinal tract. In another study, Tajabadi et al. (2010) suggested that lactobacilli with potential probiotic have been isolated from Iranian traditional fermented dairy products and showed thier probiotic properties such as their ability to survive in the digestive system, decreasing cholesterol and inhibiting the growth of pathogenic bacteria in "in vitro" conditions.

Various studies showed that probiotics after taking as foods and linking with recievers and occupying them, were compete with pathogens such as Staphylococcus aureus, Salmonella, Shigella, Escherichia, Enterobacter, Vibrio cholerae and Helicobacter pylori and prevent them from connecting and colonization of these bacteria [11]. In another study, Zhao et al. (1998) noted to the effects of pregnancy reduction of Escherichia coli O157: H7 in cattle by probiotics. They concluded that eating probiotics reduce Escherichia coli among pregnant cows and prevent the colonization of E. coli in the digestive tract of cattle.

Antimicrobial susceptibility testing results showed that isolated lactobacilli sensitivity against antibiotics was led to the relative resistance to ampicillin and perfect resistant to vancomycin, respectively. It should be noted that resistance to vancomycin is one of the indicators of lactobacilli.

CONCLUSION

This study just proved that different Lactobacillus by the sources of cheese has the power to reduce the colonization of E. coli and Listeria monocytogenes in vitro conditions, and the inhibitory effect and functional mechanism of probiotics was not evaluated which is suggested to be addressed in future studies. Also, using Lactobacillus with strong antimicrobial properties in diet, creating recombinant probiotics and designing recombinant strains with new probiotical features will be some cases that should be investigated.

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