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A STUDY OF IRANIAN TRADITIONAL DAIRY BEVERAGE (RICHAL SHIRI) AND INVESTIGATION INTO SOME PROPERTIES OF ITS ISOLATED LACTIC ACID BACTERIA

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Abstract

Richal is a traditional fermented dairy beverage that has a long history in the Iranian encyclopedia. This beverage contains whole milk, natural starter, herbs and salt. It can be produced in three different forms, Shiri (from milk), Masti (from yogurt), and Dooghi (from drinking yogurt). The present study was an investigation into the characteristic features of the lactic acid bacteria isolated from Richal Shiri.

After preparation, the traditional fermented drink was investigated and tests were carried out, such as the determination of lactic acid and pH. Lactic acid bacteria and yeasts were isolated from a traditional fermented beverage sample and cultured in various media such as fermented whole or skimmed milk at various temperatures and test cultures.

Some strains had anti-bacterial activity against sporogenous microflora S. typhimurium G-38, B. subtilis 17-89, B. Thuringensis 69-6. Eleven Lactic acid bacteria strains were isolated; ten of them were Gram-positive, cocci, and one of them was Gram-positive, Bacillus. Ten strains were catalase-negative and one was catalase positive; the lactic acid bacteria had antibacterial activity by the determination which measuring zone diameters of bacterial growth inhibition. The functions of strains were different but the differences were not significant ($p \geq 0.3$). Between the 3 groups, however, B. subtilis 17-89 had the best function.

According to the results, Richal Shiri has some lactic acid bacteria strains with anti-bacterial activity and the ability to ferment milk as a starter culture. The isolated fermented milk, according to its properties, may introduce a new functional food product for development in the food industry. These bacteria will be used in future investigations.

Keywords: *Richal shiri, antibacterial properties, isolated strains, Iranian traditional food.*

Introduction

Fermentation is a natural biological process in raw food materials such as milk, vegetables, and meat. The aroma, taste, texture, and nutritional value of fermented foods (FF) are often enhanced during fermentation. In addition, FF have a longer shelf-life than their raw and unprocessed form [*Karimpour F, 2014*]. Fermented milk products have been consumed throughout the history of civilized or uncivilized humanity and have improved since. Although different kinds of dairy products

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were known to many cultures and people from different regions in ancient times, fermentation had not been defined properly at times. The roots of yogurt, *Mast, and Matson* return to the Middle East [*Pashapour N, Iou S, 2006; Swidan N, 2010*]. The products and microbiological analysis have been carried out in countries such as Armenia, Indonesia, Zimbabwe, South Africa, Morocco, Tanzania, Burkina Faso, Sudan, Nigeria, Ethiopia, Iraq, Turkey, Lebanon, Cis countries, and Iran [*Tamine A, Robinson R, 1999; Mortazavian A, 2006*]. Researchers have shown that a specific population of microflora can be found in traditional fermented milk [*Tamine A, Robinson R, 1999; Afrikian E, 2012*]. Local indigenous microflora and the climatic conditions of the regions affects the natural types of dairy products in different locations [*Aly Savadogo A et al., 2004*]. In cold climate conditions, mesophilic bacteria such as *Leuconostoc spp.* and *Lactococcus* are the dominant microflora of the products. Thermophilic bacteria mostly consist of *Lactobacillus* and *Streptococcus*. [*Tamine A, Robinson R, 1999; Kafili T et al., 2009; Afrikian E, 2012; Tkhruni F et al., 2013; Karimpour F, 2014*]. Traditional fermented milk has had several applications in food and beverages. Fermented dairy products are excellent sources of B vitamins. Moreover, digestion of the milk proteins, casein, and whey, occurs during fermentation [*Akabanda F et al., 2010; Karimpour F, 2014*]. Fermented foods contain starter cultures called natural lactic acid bacteria (LAB). Lactic acid bacteria are natural starter cultures in FF such as dairy products. Propagation and viability of LAB are done in the gastrointestinal tract. The groups of LABs are called "probiotics". Research has shown that LAB has huge probiotic capability including antimicrobial, antiviral, antifungal, antimutagenic, antiplatelet, and antioxidant activity [*Kazemipoor M et al., 2012; Flora T et al., 2015*].

To overcome it is possible, due to the uniting the knowledge and will of all doctors in the world

Lactic acid bacteria have been considered natural food preservatives due to their natural compounds and they are generally recognized as safe. The preservative properties of LAB are mainly related to the production of organic acids (for instance, lactic acid), which results in a lowered pH. They also produce antimicrobial compounds, including hydrogen peroxide, CO2, diacetyl, acetaldehyde, D-isomers of amino acids, reuterin, and bacteriocins [*Denisa L et al., 2017*]. The bacteriocins are ribosomally synthesized peptides that have antimicrobial activity against other bacteria [*Yang S et al., 2014*]. The antimicrobial bioactive peptides may be produced by both gram-positive and gram-negative bacteria [*Al-Sahlany S et al., 2020*]. The LABs are normally regarded as "food grade" organisms and selective organisms, and their performance is regarded as protective cultures used in nutraceuticals. Using the LAB bacteriocins in recent years has raised significant interest because of their generally recognized as safe status and potential use as safe additives for food preservation [P*al V et al., 2005; Yang E et al., 2012; Ishola R, Adebayo-Tayo B, 2012; Husain A et al., 2017*]. Yogurt, drinking yogurt (*Doogh*), Kashk, Gharaghooroot, different kinds of cheese, etc., have been produced in Iran for many years. The source of these traditional dairy products is sheep, goat, and cow milk. In comparison to the commercial species, the composition of lactic acid bacteria in these products is more varied. Richal is a traditional fermented dairy beverage that has a long history in the Iranian encyclopedia. This beverage contains whole milk, natural starter, herbs and salt. All of the ingredients are processed in tanned sheep and goat skin, called Mashk. It can be produced in three different forms, *Shiri* (from milk), *Masti* (from yogurt), *and Dooghi* (from drinking yogurt). *Richal Shiri* (RS) consists of whole milk, special local herbs, traditional or local starter cultures, and salt. Natural processing, it is kept in environmental condition for several days. The main target of the present study is the isolation and characterization of some properties of LAB gained from RS in order to constitute an original collection of LAB strains of the Kohgiloyeh region.

MATERIALS AND METHODS

Preparing Richal Shiri: First, heated milk with 3.5% fat, containing local starter culture, is put into the *Mashk*, and then, salt and herbs (such as chicory, mint, shallot and Celeriac (*Kelussia odoratissima Mozaff*)) are added to it. Ripening of the content of

Mashk takes place during 3-4 days while placed in shadow (out of sun exposure). The *Mashk* containing RS was transferred from Iran to the *ArmBiotechnology* Institute of Armenia for investigation.

Culture media: The media as culture used in this study were **De Man**, **Rogosa** and **Sharpe agar** (MRS), M17, Lacto agar (HiMedia, India); MRS broth (Merk, Germany; HiMedia, India); Nutrient agar (HiMedia, India); Dry skim milk powder - production by Ashtarak Kat (Armenia); Fat milk content of 3.2% fat by Ashtarak Kat (Armenia); Sabouraud agar and Sabouraud broth (HiMedia, India); Violet Red Bile Agar (Oxoid Ltd, Basingstoke, Hampshire, England); Pure Agar (Merk, Germany or HiMedia, India); Broth (Kenner Fecal) Agar (Merk, Germany). The culture was maid according to the company's label's and international food microbiology protocols.

Determination of lactic acid: The production of lactic acid was determined by titrating 10 *ml* of the homogenized sample of RS containing 1 *ml* of phenolphthalein indicator (0.5% in 50% alcohol) with 0.25 *mol/l* NaOH. The titrated acidity was calculated as the percentage of lactic acid (*v/v*). Each milliliter of 1 *N* NaOH is equivalent to 9.008 *mg* of lactic acid.

Determination of pH: The pH change of the fermenting samples was monitored by use of an Oakton pH meter (Kent Ind. Measurements Ltd. Survey), model 700, with a glass electrode.

Isolation of LAB and yeasts: The samples were weighted and homogenized in sterile conditions. For each sample, a 1:10 dilution was subsequently made using peptone water, followed by making a 10-fold serial dilution. A 0.1 *ml* from each dilution was then subcultured, in duplicate, into the MRS, M17, Lacto, and Milk agar (medium with hydrolyzed milk containing 1.2% pure agar) used for isolating LAB [*Badis A et al., 2004*]. For yeast isolation, the same dilution was used but the dilutions were cultured on Sabouraud Agar. The plates were incubated in an anaerobic system at 25*ºC*, 30*ºC*, 37*ºC*, and 42*ºC* for 3 days in order to provide an optimal temperature for growing thermophilic and mesophilic lactobacilli and yeasts. Yeasts or molds were enumerated on spread plates of Sabouraud agar and they were followed for strain characterization and subsequent identification by standard methods. Enterobacteriaceae were enumerated on plates of Violet Red Bile Agar incubated at 37*ºC* for 24 hours [*Golubev W, 2006; Akabanda F et al., 2010*]. The LAB strains were maintained as frozen stocks: 1 *ml* of each grown culture was transferred to the plastic tubes (Eppendorf, Hamburg, Germany) containing 40% glycerol, and then the cultures were stored in the freezer at 20. Before use, LAB strains were transferred twice into the appropriate medium and incubated according to the respective growth conditions. After growth, cell concentration reached 7±2×108 *CFU/ml*. As a criterion for the preliminary selection of the isolated LAB and yeast, the ability of strains to ferment milk was taken.

Definition of LAB sensitivity to bile, pH and acidic proteases: The isolated strains of LAB were inoculated in a nutrient media MRS broth with the content of a definite amount of growth LAB in the conditions (range of bile, pH) [*Thornton G, 1996*]. The survival of the LAB was estimated by the change of the optical density at wavelength 590 *nm* on the 2800 *WV/VIS* spectrophotometer (Cole Parmer, Illinois, United States).

Physiological characteristics of LAB strains: All strains were initially tested for gram staining, catalase production and spore formation [*Azadnia P, Khan Nazer A, 2009*]. Colonies were morphologically characterized by growth on MRS and M17 agar. Strains with gram-positive and catalase negative reactions were finally used for further investigation [*Azadnia P, Khan Nazer A, 2009*].

Obtaining supernatants: The liquid from the culture, obtained from growing microorganisms in the appropriate media for 48*h* at 37*ºC*, was centrifuged for separation of the biological mass at 4,000 *rpm* for 30 minutes. The antimicrobial activity of obtained supernatants was determined by measuring the zone diameters of bacterial growth inhibition of the test cultures on solid nutrient agar [*Tkhruni F et al., 2013*].

Test cultures: For investigating antimicrobial properties of the supernatants of LAB and yeasts, the gram-negative conditionally pathogenic bacteria *Salmonella typhimurium* G-38 and gram-positive bacteria *Bacillus subtilis* 17-89 *and Bacillus thuringiensis* 69-6, contained in the collection of the "Laboratory of Microbial preparations" of the Scientific and Production Center *"Armbiotechnology"*, RA, were used [*Golubev W, 2006; Karimpour F, 2014*].

Milk fermentation: To select colonies for investigation of their physiological properties, the milk fermentation by LAB strains from RS was carried out using milk of different fat percentages at different temperatures. As starter cultures, 3 variants of grown colonies were used:

1. Colony from MRS solid media, then immediately cultivated in the milk.
2. Colony grown in MRS broth, then cultivated in the milk.
3. Colony grown overnight in the skim milk, kept in the incubator (starter formation), and the inoculation of the starter and its cultivation to the skim milk [*Pourahmad R, Mazaheri Assadi M, 2005; Azadnia P, Khan Nazer A, 2009; Kafili T et al., 2009; Larsson M, Jacobsson K, 2009; Tkhruni F et al., 2013*].

Statistical analysis: We have used SPSS-22 software for the process and analyzed by ANOVA one-way Dancan. We measured the significant level ($p<0.5$).

RESULTS

The first findings regarding the number (N) of yeasts showed that the molds of "RS" did not exceed standard norms. E. coli and coliform norms were compared with the norms of Iranian Doogh (a kind of fermented milk product). Chemical parameters such as acidity and pH were around the standard ranges. Eleven LAB strains were isolated from RS using standard classical microbiological methods. The isolated strains were investigated morpho-physiologically. Microscopic results showed that the colonies of LAB strains were in both cocci and rod shapes; however, the majority of them were in cocci shape. All strains were gram-positive and most of them were catalase negative (Table 1).

All 11 strains of lactic acid bacteria isolated from Richal Shiri were tested for their ability to grow on MRS agar (from Merk and Himedia) and Lacto (Russia), Bifido (Merk) Kenner Fecal (Merk) broths. The strains were able to grow in different culture media and at different temperatures, including 37 and 42°C, determining the best temperature and. Out of 11 strains, inoculations were positive for agars: MRS (Merck) in 9 (81.1%) strain, MRS (Himedia) in 10 (90%) strains, Lacto, Bifido and Kenner Fecal in 11 (100%) strains. In Broth medium, MRS (Merck) was obtained in 10 (90%) and MRS (Himedia) in 11 (100%) strains.

TABLE 1

Total count, chemical and morphological properties of microflora of LAB strains

Total count of microflora of "RS"					
TC, *(CFU/ml)*	Col *(CFU/ml)*	*E. coli*	M&Y *(CFU/ml)*	pH	Acidity, *°T*
1×10^4	6	Neg	3.0×10^5	3-4	140

Characteristics of the investigated LAB strains											
Microscopy				Gram-staining				Catalase activity			
Cs		Rs		Pos		Neg		Pos		Neg	
N	%	N	%	N	%	N	%	N	%	N	%
10	90	1	10	0	0	11	100	1	10	10	90

Notes: *Abberviations used in the table Tc - Total count, Col -Coliform, M&Y - Molds and Yeasts, Cs - Cocci-shape, Rs - Rod-shap, Pos - Positive, Neg - Negative*

The multivariate analysis indicated significant differences between different bacteria at different time & temperature conditions ($p<0.001$). According to the pairwise comparisons analyses, the best growth was again at 37 °C for 24h. The result at the conditions was significant with a 95% confidence Interval for the difference (Table 2).

The results have shown the LAB strains had antibacterial activity against sporogenous microflora including *S.typhimurium G-38, B. subtilis 17-89, B. thuringensis sp*. The multivariate analysis showed that the comparisons of mean diameters (zone of inhibition) between the LABs on the sporogenous microflora food was not significant ($p\geq0.3$). However, as shown in Table 3, *B. subtilis*

TABLE 2

Growth of LAB by optical density in different condition and inhibition zone, Ø, *mm*

Condition	Mean	St. Deviation	N
37°C/24h	4.3109	0.89672	11
3737°C/2h	2.4273	0.80912	11
4237°C/24h	1.6273	0.25823	11
1537°C/24h	1.3273	0.63418	11

TABLE 3

Antimicrobial activity

	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
S.typhimurium G-38	1.875	8.455	4.277	12.632
B.subtilis 17-89	1.022	10.091	7.814	12.368
B. thuringensis sp. 17-89	1.550	8.273	4.820	11.726

Table 4

Growth of the LAB in skim milk and 3.2% fat content at different conditions

	N	pH	Acidity T^o	N	%
Growth of the LAB in skim milk					
In milk 42^oC	11	5.2	60	2	18.18
from MRS to Milk 42^oC	11	4.9	128	11	100
from milk to milk 42^oC	11	4.9	79	7	63.63
In milk 37^oC	11	5.1	57	6	54.54
from MRS to milk 37^oC	11	4.9	85	11	100
from milk to milk 37^oC	11	4.9	82	8	72.72
Growth of the LAB in fat milk 3.2% fat content					
In milk 42^oC	11	5	63	1	9.09
from MRS to Milk 42^oC	11	4.9	100	11	100
from milk to milk 42^oC	11	5	70	8	72.72
In milk 37^oC	11	4.9	75	3	27.27
from MRS to milk 37^oC	11	4.7	75	11	100
from milk to milk 37^oC	11	4.8	95	7	63.63

17-89 had the best function between groups. Pairwise comparison analysis, T2 Hotelling's, showed that the antibacterial activity of the LAB strains against the food spoilage bacteria, is significantly different ($p<0.001$).

According to the table 4, the strains fermented the milk under different conditions. The variants showed important and different results, but the best results were obtained from variant 2 (the colony was grown in the MRS broth, and then cultivated in the milk). The least growth time was in variant 3, 1 (colony from milk or colony from MRS solid media then immediately cultivated in the milk). The Acidity was different in all conditions. All of the LAB strains were inoculated to MRS broth and they were kept at 15 to 18°*C* temperature; Then, the colonies from their overnight culture were inoculated to milk. The results showed that two of the LABs did not ferment milk and the other 9 LAB strains fermented milk after 86 hours on average.

Discussion

RS, a form of *Richal*, is a traditional dairy beverage in the south of Iran that is prepared in *Mashk*. *Richal* has different ingredients such as heated milk (pasteurized), local herbs, starter culture, and salt. In this study, RS was investigated and the results showed a correspondence between norms of coliform ،E. coli and the national Iranian standard of *Doogh*. Moreover, the N of molds and yeasts was more than the standard range. Although the total count of microorganisms is not the main index in the standard of *Doogh*, we measured it to compare it with the total count in other forms *of Richal Dooghi*, and *Masti*. The total count can show approximately the amount of LAB, yeasts, molds, and other harmful and useful bacteria. Chemical parameters such as pH and acidity of the traditional RS were in standard ranges. These parameters can be with the microbial index. Although not all parameters of RS are in the standard range, nomads and people used it and believed it was a healthy beverage. So, the traditional Richal needs to be adjusted for industrial use [*Fallahi F, Madani M, 2014*]. Eleven strains of lactic acid bacteria were isolated from RS in this study. A hundred percent of them were gram-positive and 90 percent were catalase-negative, and 10 percent were catalase-positive. The gram-positive and catalase-negative strains were similar to the previous results [*Azadnia P, Khan Nazer A, 2009*]. The strains have been cultured on several media and the results have shown that they could grow on different media at incubation temperatures from 37 to 42^oC. The best growth was seen on the MRS Broth (Himedia), Bifido Agar (Merck), Lacto (Russia), and Kenner Fecal (Merck) among all broths and agars. The colonies on the media were respectively of Lacto Agar, Milk Agar, MRS Agar (ISO), MRS Agar (Himedia) 81.81%, 72.72%, 63.63%, 0% Transparent and 18.19%, 27.27%, 36.37%, 100% were white color. On Bifido Agar, the color of 90% of the colonies was white to gold, and 10% was a white and cream color. For future work, have to use the media in the results in accordance with [*Taheri P et al., 2005*]. According to optical density as an index for investigating the propagation of the strains in the media, table 2 has shown that the strains have different behaviors in different conditions (times and temperatures). The best results were at 37^oC for 24*h* and 37 *C* for 2*h*. This can be a good finding; because the temperature of the human body is also 37^oC, so the use of these bacteria as starter cultures in the food industry will be economical. These strains may have probiotic properties and the ability to be applied in supplements. Probiotics must propagate after 2h in the gastrointestinal tract and their viability is an important matter. This part of the study has been done

by examining strains of RS in different condition inoculums in milk. As shown in the table 4, the propagated N of strains in the skim milk at 37^oC was different, while at 42^oC inoculation of strains of fermented milk to milk was better in comparison to 37^oC. Thus, if we want to use these strains as an indirect starter in fermented whole milk in the food industry, the 42^oC temperature will be better. However, the bacteria that functions well at 42^oC, can also have lipolytic properties in specific temperatures which needs attention [*Israyelyan A, 2021*]. We also examined the antibacterial activity of the isolated bacteria strains against test cultures, *S.typhimurium G-38, B.subtilis 17-79,* and *B.thuringiensis* as sporogenous microflora. According to table 3, some of the LAB strains isolated from RS, inhibited the growth of the gram-positive and gram-negative bacteria. The diameters of strains had different zone inhibition (*mm*). The results showed that 36.6% of the strains were capable of growth of inhibition of *S.typhimurium G-38,* 54.54% inhibited the growth of *B. subtilis 17-89,* and *B.thuringiensis* 69-6. The mean of diameters (zone) obtained from one-way ANOVA showed that there is a significant difference in the antibacterial activity of the strains. The zone mean differences in the 3 test cultures were not significant, while the following analysis showed the antibacterial property of B.*subtilis 17-79 is* better than the two other test cultures.

In sum, the supernatant of LAB can be used as a bactericide substance. LAB strains isolated from RS can be used as a starter culture for the production of new functional foods and also as a source for the production of natural bio-preservatives in the food industry. The classification of bactericides by several authors mentioned the ability of some selected strains to inhibit the growth of pathogen bacteria. According to another research, *Richal* can be considered a healthy functional beverage [*Karimpour F, 2014*].

CONCLUSION

According to the results of this study, RS has some strains of the LAB with anti-bacterial activity and the ability to ferment milk as a starter culture. These properties are suggested to be used in producing new functional foods in the food industry.

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