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Potentially Probiotic Bacteria Isolated from Preparation Stages of Kermanshahi Traditional Fat

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ABSTRACT

Background and Aim: Dairy fermented foods such as yogurt, cheese, fermented milk, butter milk, curd, butter and ghee are used as major diet ingredients in west of IRAN, such as Kermanshah province. Ghee (Kermanshahi traditional oil or roghan-e heiwâni) is traditionally produced from butter milk of yoghurt after fermentation. Review of the literature yielded no study on isolating probiotics from Kermanshahi traditional oil preparation stages. Therefore, purpose of this study was just to focus on isolating and identifying lactic acid bacteria in these products using culture and PCR-sequencing methods.

Materials and Methods: Fifteen samples from each dairy products including yogurt, butter and Kermanshahi traditional oil were collected in Kermanshah province, Iran. Each sample was diluted, homogenized, and cultured on selective medium for growing of lactic acid bacteria. *16SrRNA* gene sequences analysis was carried out for final confirmation of these isolates.

Results: After culturing of samples on MRS and M17 under aerobic and anaerobic condition, a total of 78 strains of bacteria were isolated and identified by conventional biochemical tests. The frequency of bacteria in all isolates (78) were 48.71% for *Lactobacillus*, 33.33% for *Streptococcus*, 6.41% for *Enterococcus* and 6.41% *for Bacillus*. *Lactobacillus, Streptococcus, Enterococcus,* and *Bacillus* genus*,* were isolated from 84.44%, 57.78%, 11.11% and 15.56% of all three kind of samples, respectively.

Conclusion: Based on our findings, *Lactic acid bacteria* and other potentially probiotic microorganisms are present in Kermanshahi traditional oil. Of course, the potential probiotic properties of these isolates and impact of consumption of Kermanshahi traditional oil containing those on human health need to be analyzed more. By proving the presence of probiotic bacteria in Kermanshahi oil, it may be falls in the category of functional foods.

Keywords: Dairy, Kermanshahi traditional oil, *Lactic acid bacteria*, PCR sequencing, Probiotics

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Introduction

According to the definition by the world health organization (WHO) and food and agriculture organization (FAO), probiotics are live non-pathogenic microorganisms that have beneficial effects on the health of human if consumed sufficiently **(1)**. A main group of these microorganisms is lactic acid bacteria (LAB) that are used as starter for dairy products. Probiotic strains of LAB have antioxidant, anticancer and antimicrobial potential effects. Moreover, it has been proved that probiotic bacteria can reduce serum cholesterol, relieve lactose intolerance, and stimulate the immune system leading to the stability of the microbiota **(2)**. LAB that are associated with fermented foods include some species of *Carnobacterium*, *Enterococcus Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus*, and *Weissella* genera **(3)**.

Fermentation is a frequently used food processing technique worldwide. There are many locally produced fermented foods **(4)** **(5)**, including various kinds of yogurt, cheese, fermented milk, butter milk, curd, and butter **(5)**. Some evidences suggest traditional butter fat or Ghee is a fermented food and could contain different kind of LAB. Fermentation of milk to yoghurt is traditionally occurred by *Lactobacillus bulgaricus* and *Streptococcus thermophiles* at 40-45°C. These bacteria, are not present in intestinal microbiota, are not s and cannot survive in the gut **(6)**.

Changes that occur during milk fermentation by LAB enhance nutritional value of the product **(7)**. Kermanshahi traditional oil, which is traditionally produced, serves as an imp ortant type of fat in the diet of some areas in Iran and other countries of the South Asia **(8)**. It is known as yellow oil, Kermanshahi oil, Ghee, roghan-e heiwâni, or Kermanshahi traditional oil **(9)**. First, yogurt is produced from milk by traditional methods, and then it is put in a Mashk (a large leather bag/a big waterskin). After this step, butter is separated from the tan (doogh), then transferred to a larger pot, and slowly heated, allowing the components to separate by density and the remaining is called roghan-e heiwâni. Studies have shown that changes traditionally occur in the preparation of butter and Kermanshahi traditional oil in fatty acid. Among the reasons of such changes, bacterial fermentation process for the preparation of products can be pointed out. In products like yoghurt, butter and Kermanshahi traditional oil, due to the function of bacteria, lactic acid is produced and environmental pH is reduced **(10)**. Furthermore, changes occur in the fatty acid of these products. There has been no study on isolating probiotics from Kermanshahi traditional oil preparation stages. Therefore, our purpose in this study was to identify probiotic bacteria from these products using culture and PCR-sequencing. Recognizing and recording of these microorganisms can help to preserve biological sources.

Materials and Methods

The present study was conducted during the period from October to the end of November, 2015. A total of 15 samples from each dairy product including yogurt, butter and Kermanshahi traditional oil were collected from Kermanshah Province. The samples were collected in sterile bottles and kept cold until they arrived at the laboratory, where they were kept at 4°C. Only samples have been prepared using traditional starter were included in the study to ensure the absence of commercial starters. Ten grams of each sample were homogenized with 90 mL sterile normal saline solution (0.85%, w/v) to make an initial dilution (10^{-1}). An amount of 100 µL from each diluted sample was then cultured on MRS Agar (Oxoid, CM0361) and M17 Agar (Oxoid, CM0785) containing 0.05% cysteine, and incubated in both aerobic and anaerobic conditions at 37°C for 48-72 hours. All bacterial isolates were screened using colony morphology and gram staining, and final identification was carried out through PCR sequencing. Then, 4-5 colonies were selected from each plate. After subculture, DNA of each plate was isolated using the DNA extraction kit according to the manufacturers' instruction. The extracted DNA was stored at -20 until PCR implementation. The PCR technique was employed for the amplification of genus-specific target of *Lactobacillus* and *Bifidobacterium* of using specific primers and universal primer of *16S rRNA* gene previously described by Lauren *et al.* The primers used in this study are summarized in Table 1 **(11)**. The PCR was performed in a total volume of 25 µL, containing 2.5 µM dNTP mix, 2.0 mM $MgCl_2$, 0.5 U Taq DNA polymerase, 0.5 µM of each primer, and 2.5 µL of each template DNA and 10X buffer. Standard strains, *Lactobacillus delbrueckii* (PTCC 1737) and *Bifidobacterium bifidum*, (PTCC 1644) were used as a positive control in each reaction. The PCR was performed under the following conditions: Denaturation for 5 min at 95°C; 30 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, as well as a final extension step of 7 min at 72°C. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel. The agarose gel was stained using ethidium bromide and visualized in gel doc. Visualization of 340 bp and 520 bp confirmed the *Lactobacillus* and *Bifidobacterium* genus, respectively. Finally, each sample with single band within 470 bp was selected for *16SrRNA* sequencing. The nucleotide sequences of the *16SrRNA* `gene of all the isolates were analyzed and edited using Bioedit software. Similarity searching was conducted for each edited sequence for exact determination of genus and species of each isolate

(http://www.ncbi.nlm.nih.gov/BLAST). All *16SrRNA* sequences were submitted to the NCBI GenBank under the accession numbers KX951698.1 To KX951735.1, KX926497.1 to KX926536.1.

Table 1. primer sequences for PCR-based detection of probiotic bacteria

Target	Primer Name	Sequence (5′ to 3′)
Universal primers *16SrRNA* gene	341 –F 768-R	CCTACGGGAGGCAGCAG GACTACCAGGGTATCTAATC
Specific primers *Lactobacillus 16SrRNA* gene	Lact-F Lact-R	AGCAGTAGGGAATCTTCCA ATTYCACCGCTACACATG
Specific primers *Bifidobacterium 16SrRNA* gene	Bif-F Bif-R	GGGTGGTAATGCCGGATG CCACCGTTACACCGG

Results

Overall, 45 samples were cultured and 78 colonies were selected according to colony morphology. All the isolates were gram positive catalase and oxidase negative. The isolates were all confirmed using the PCR and sequencing targeted *16SrRNA* gene. The result is shown in Figure 1 and Table 2. Several species were identified including *Lactobacillus, Lactococcus, Enterococcus, Streptococcus* and *Bacillus* from the samples. The result showed that *Lactobacillus* was the dominant flora of the existing probiotic microorganism in the samples. In the yogurt samples, the *L. delbrueckii*, *Lactobacillus helveticus*, *Lactobacillus fermentum, Lactobacillus rhamnosus, Lactobacillus paracasei* with *Streptococcus thermophilus*, *Enterococcus faecium, Brevibacillus formosus* and *Brevibacillus agri* species were isolated. *Brevibacillus* and *E. faecium* were isolated from the yogurt samples only. Also, *Brevibacillus* was isolated from two samples only. However, *L. paracasei* and *L. rhamnosus* were isolated from both the yogurt and butter samples. Several species of *Lactobacillus, Enterococcus, Streptococcus, Lactococcus* and *Bacillus* were also isolated from the butter samples. The most prevalent genus was *L. delbrueckii, L. helveticus, L. paracasei, L. rhamnosus,* and *L. fermentum.* Other bacteria that were isolated from the traditional butter samples with less frequency were *Lactococcus lactis, S. thermophiles, Enterococcus feacalis and Bacillus cereus. Bacillus subtilis and Bacillus licheniformis* were isolated only from the butter samples. The detailed results of the isolates are shown in the following Table 2. Some species of the *Lactobacillus, Streptococcus* and *Enterococcus* genus were present in Kermanshahi traditional oil, but in a small number. The *Lactobacillus* species included *L. fermentum, L. delbrueckii, Lactobacillus casei and L. helveticus. S. thermophiles, Enterococcus feacalis, Enterococcus feacium* and *Enterococcus hirae* were the other isolated species from Kermanshahi traditional oil. *E. hirae*, however, was isolated from a single Ghee sample. The frequency of probiotic bacteria in all the samples including local yogurt, butter and Kermanshahi traditional oil was %84.44 for *Lactobacillus*, %57.78 for *Streptococcus*, %11.11 for *Enterococcus* and %15.56 *for Bacillus*.

Table 2. probiotic species in collected samples

Bacteria	Bacteria isolated Kermanshahi traditional yogurt(n)%	Bacteria isolated Kermanshahi traditional butter(n)%	Bacteria isolated Kermanshahi traditional oil (n)%	Total
L. delbrueckii	(9) 60	(6) 40	(4) 26.67	(19) 42.22
L. helveticus	(1) 6.67	(4) 26.67	(2) 13.33	(7) 15.56
L. fermentum	(2) 13.33	(2) 13.33	(2) 13.33	(6) 13.33
L. rhamnosus	(1) 6.67	(1) 6.67	0	(2) 4.44
L. paracasei	(1) 6.67	(2) 13.33	0	(3) 6.67
L. casei	0	0	(1) 6.67	(1) 2.22
S. thermophilus	(11) 73.33	(4) 26.67	(11) 73.33	(26) 57.78
Bervibacillus	(2) 13.33	0	0	(2) 4.44
E. faecium	(1) 6.67	0	0	(1) 2.22
E. faecalis	0	(1) 6.67	(2) 13.33	(3) 6.67
B. cereus	0	(1) 6.67	0	(1) 2.22

Bacteria	Bacteria isolated Kermanshahi traditional yogurt(n)%	Bacteria isolated Kermanshahi traditional butter(n)%	Bacteria isolated Kermanshahi traditional oil (n)%	Total
L. lactis	0	(2) 13.33	0	(2) 4.44
E. hirae	0	0	(1) 6.67	(1) 6.67
B. subtilis	0	(1) 6.67	0	(1) 6.67
B. licheniformis	0	(3) 20	0	(3) 20
Total	(28) 100	(27) 100	(23) 100	(78) 100

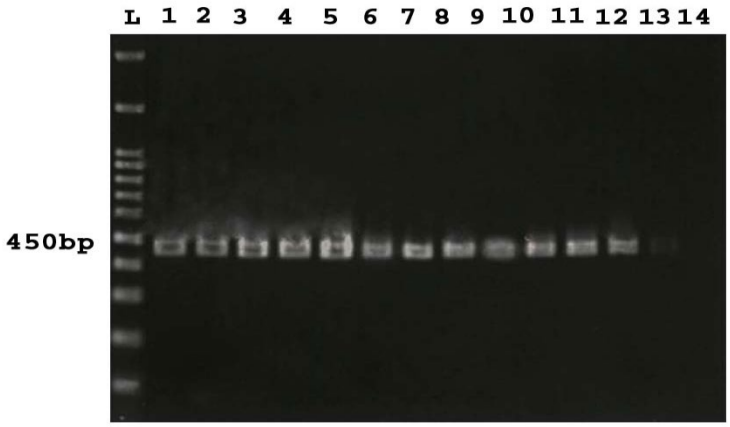

Figure 1. PCR products of *16SrRNA* gene. L: 100 bp DNA Marker, 1-13: positive samples, 14: Negative control.

Discussion

Probiotic bacteria are non-pathogenic microorganisms known for their nutritional value and therapeutic effect as well as anti-microbial and anti-cancer effects on the host health. During the past two decades, these microorganisms have been isolated from variety of foods, especially dairy products. A wide range of dairy products such as fresh milk, yogurt, cheese, fermented butter and drinks containing LAB can provide a good environment to support the growth and durability of probiotic microorganisms (**12, 13**). Many studies have been conducted on the isolation and identification of probiotic bacteria in various traditional dairy and fermented food products from all around the world (**14**). *Lactobacillus plantarum*, *L. casei* and *Lactobacillus brevis* were isolated from (traditional) yogurt and cheese by Ebrahimi *et al.* (**15**). Angmo *et al.* succeeded in isolating *L. plantarum* from Ladakh, a fermented vegetable product in India (**1**). Seven species of *Lactobacillus* and *Bifidobacterium* were isolated and reported studying traditional fermented dairy products in different regions of Russia (**16**). Moreover, numerous studies have indicated that isolated lactic acid bacteria from milk, cheese, fermented olives, kefir, sourdough of wheat and barley flour and other fermented products have the ability to inhibit the progress of some gastrointestinal diseases and have remarkable antibacterial properties compared with commercial strains (**17-23**). The most important lactic acid bacteria in the dairy industry are *Lactobacillus*, *Streptococcus*, *Lactococcus* and *Enterococcus*, and according to many studies, traditional fermented products are mostly dominated by *Lactobacillus* species (**24**). In the present study, *Lactobacillus* was the dominant 84.44% of all lactic acid bacteria. *L. delbruekii* and *S. thermophilus* were reported to be the most abundant probiotic bacteria in a Colombian study of dairy products using biochemical tests and 16SrRNA gene sequencing (**25**). *Lactobacillus acidophilus* and *L. casei* were reported as the most abundant isolate from dairy products of Bhardwaj in India (**26, 27**). *L. paracasei* and *L. rhamnosus*, two species that were isolated from yogurt and butter in present study, were used commonly as non-starter lactic acid bacteriain in dairy products. In some studies, *L. paracasei* has been isolated and identified from cheese and curd samples. *Bervibacillus brevis* and *E. faecium* were isolated from yogurt samples in this study. Although similar results have been reported (**28**), little information is available about their probiotic properties, which might even be regarded as contamination. Several reports have been presented in isolating (**25**) probiotic bacteria from local yogurt and butter separately. However, this study was the first to focus on isolation and identification of LAB from traditional Kermanshahi oil in various stages of development. Kermanshahi traditional oil is a common edible oil in parts of Iran

including Kermanshah Province. It is an important consumable fat in people's diet in particular areas in Iran, and is traditionally prepared from yogurt **(29)**. Bahrami *et al.* showed that fatty acids can change in traditional preparation of butter and Kermanshahi traditional oil. One of the reasons can be the fermentation process and lactic acid production by probiotic bacteria **(10)**. *Lactobacillus*, *Enterococcus*, *Lactococcus* and *Leuconostoc* genera were isolated from traditional butter (Dhan), in a previous study **(30)**. Thus, a probiotic bacterium in the preparation process of fermented products has been isolated according to traditional methods without the use of any commercial starter, which can be a valuable source of probiotic microorganisms. Hence, preparation of fermented products is being industrialized, making isolation and preservation of local strains particularly valuable. In this study, we attempted to isolate bacteria from local traditional yogurt, butter and Kermanshahi traditional oil. The result of this study revealed different isolated groups of probiotic bacteria from the samples of traditional yogurt, butter and Kermanshahi traditional oil, indicating that fermentation conditions were different in the samples. We found that *L. delbruekii, L. helveticus, L. fermentum,* and *S. thermophilus* were the pred-ominant isolates from the samples of traditional yogurt, butter and Kermanshahi traditional oil. Tarhana is a traditional fermented food mixture of wheat flour and yoghurt. During natural fermentation in Tarhana, LAB are dominant strains while *S. thermophiles*, *L. delbrueckii* and *L. fermentum* are dominant isolates **(31)**. In our study, *S. thermophilus* and *L. delbrueckii* were the predominant isolates from the three samples. Fermentation of lactose by LAB produce large amounts of lactic acid. Consuming ferm-ented Ghee with low pH increases absorption of a short chain of fatty acids essential for the optimum performance of the body **(10)**. *L. delbrueckii* as a starter with the *S. thermophiles* species used in dairy industries has shown that all strains have high lactose activities that help to improve lactose digestion and eliminate symptoms of lactose intolerance **(32)**. *Lactobacillus delbrueckii* and *S. thermophiles* elimin-ate cholesterols by binding to released bile acids, which can reduce the risk of cardiovascular diseases **(33)**. *L. helveticus* can hydrolyze milk proteins and release bioactive peptides that are responsible for elevating IgA in the bronchial and intestinal regions and subsequently, for boosting mucosal immunity. As a thermophilic bacterium, *L. fermentum* is a non-starter lactic acid bacterium, which has considerable importance in fermented foods. It is isolated from different environments such as fermented milk. *L. fermentum* is one of the dominant *Lactobacillus* species in the intestinal tract and vagina in humans **(34)**. In contrast to other studies, we isolated the *Brevibacillus* species only from yogurt (13.33% from yogurt), where *Brevibacillus* was also isolated from soil and milk. So far, this species has not been isolated from any food substance in Iran. However, at least in one study, *Brevibacillus* was used successfully as a starter for curd. It has been confirmed that *Brevibacillus* can be used as a suitable candidate in fermented milk production **(28)**. The frequency of isolation was 6.67% for *L. paracasei,* 2.22% for *L. casei* and 4.44% for *L. rhamnosus*. These bacteria are frequently used as non-starter LAB in food and pharmaceutical industries. In addition to different species of *Lactobacillus*, *S. thermophilus*, *L. lactis* and *B. cereus* from traditional butter samples, *E. faecium* was isolated from two samples of traditional butter and oil in the present study. *Bacillus* strains are highly resistant to physical and chemical conditions due to spore formation, and are widely used as probiotics. *L. lactis* isolated from traditional butter in the present study has been indicated as a probiotic bacterium, and its antibacterial effect has been proven against pathogenic bacteria by Garsa earlier in a research **(35)**. The presence of *Enterococcus hirae* and *Enterococcus faecalis* in traditional oil samples was another finding of the present study. While about 50% of the lactic acid isolated from Brazilian cheese belonged to *Enterococcus* species, four strains of *E. hirae* were isolated from fermented cheese by Xinjisng in another study **(27, 36)**. The genus *Enterococcus* has many species, but *E. faecalis*, *E. faecium,* and *E. lactis*, and more recently *E. hirae* and *E. dorans* have been studied as probiotic species. A strain of *Enterococcus*, named *E. faecium* K77D, is used commercially as a starter **(37-40)**. *L. rhamnosus* has been extensively studied. This bacterium can survive even in difficult conditions of the digestive system and urinary tract **(41)**. Non-starter LAB are traditionally used to improve organoleptic properties of products. Non-starter bacteria such as some genus of *Lactobacillus* and *Enterococcus* are resistance to heat and extreme conditions. Guessa *et al.* predominantly isolated *Lactobacillus, Enterococcus, Lactococcus* and *Lecconestoc* species from a *traditional butter* called "Dhan" **(42)**. *Enterococci* are not used as starter in food preparation, but they can be used as dietary supplements in pharmaceutical preparation. Nevertheless, the use of *Enterococci* as probiotics has remained unknown in food safety. It must be noted that *Enterococci* have a positive role in traditionally fermented foods **(43, 44)**.

Conclusion

Various species of *Lactobacillus* and other lactic acid bacteria were isolated and identified from traditional diary products using culture and 16SrRNA gene sequencing, in this study. *L. delbruekii*, *L. helveticus* and *L. fermentum* are a group of thermophilic bacteria

used as starters at high temperatures. Due to their ability to tolerate higher temperatures, in our study, these species were isolated from all three kinds of samples including Kermanshahi traditional oil that receives intense heat in the production process. These species have more suitable proteolytic properties and more beneficial biological effects than other *Lactobacillus* species. *L. fermentum*, was isolated from all three kinds of samples, traditional yogurt, butter and oil and is widely used in the production of fermented food. In this study, a variety of potentially probiotic bacteria were isolated from all three samples, but many are probably killed by high temperatures during the preparation and production of traditional oil. However, findings of the present study showed that some species were able to tolerate high heat and harsh conditions, and were able to survive and replicate in traditional oil. Therefore, isolation and identification of various potentially probiotic bacteria from different traditional dairy products can help preserve these bacteria to be used in fermented and functional food products.

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Conflict of Interest

There is no Conflict of Interest.

Referance

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چکیده

زمینه و اهداف: خوراکی‌های تخمیری مانند ماست، پنیر، شیر تخمیری، کره شیر، کشک، کره دوغ و روغن محلی به‌عنوان یکی از اجزای اصلی رژیم غذایی در شهر های غرب ایران مثل کرمانشاه مصرف می‌شود. روغن محلی کرمانشاهی به صورت سنتی از کره دوغ به دست می‌آید. مروری بر متون منتشرشده نشان داد که تا به حال مطالعه‌ای برای جداسازی باکتری‌های پروبیوتیک از مراحل آماده‌سازی روغن محلی کرمانشاهی انجام نشده است. بنابراین هدف از انجام این مطالعه جداسازی و تعیین هویت باکتری‌ها با تمرکز روی باکتری‌های تولیدکنندهٔ اسید لاکتیک با استفاده از روش‌های کشت و توالی‌یابی بود.

مواد و روش کار: ۱۵ نمونه از هر یک از محصولات ماست، کره سنتی و روغن محلی کرمانشاهی از استان کرمانشاه جمع‌آوری شد. هر نمونه رقیق‌سازی و هموژن شد و سپس روی محیط‌های انتخابی برای رشد باکتری‌های اسید لاکتیک رشد داده شد. توالی‌یابی بر پایهٔ ژن *16SrRNA* برای تایید نهایی هر یک از ایزوله‌ها استفاده گردید.

یافته‌ها: پس از کشت روی محیط‌های MRS و M17 تحت شرایط هوازی و بی‌هوازی، ۷۸ سویه مختلف باکتریایی جداسازی شد و به‌وسیله تست‌های بیوشیمیایی رایج تعیین هویت گردید. فراوانی باکتری‌های جداشده از همهٔ نمونه‌ها شامل ۴۸٪/۷۱ لاکتوباسیلوس، ۳۳/۳۳٪ استرپتوکوکوس، ۶/۴۱٪ انتروکوکوس و ۶/۴۱٪ باسیلوس بود. جنس‌های *لاکتوباسیلوس*، *انتروکوکوس* و *باسیلوس* به ترتیب از ۸۴/۴۴٪، ۵۷/۷۸٪، ۱۱/۱۱٪ و ۵۶/۱۵٪ هر سه نوع نمونه جداسازی شد.

نتیجه‌گیری: بر اساس یافته‌های ما باکتری‌های اسید لاکتیک و سایر میکرارگانیسم‌هایی که پتانسیل پروبیوتیک دارند در روغن محلی کرمانشاهی یافت می‌شوند. خصوصیات پروبیوتیکی این ایزوله‌ها و اثرات مصرف روغن محلی کرمانشاهی حاوی این ارگانیسم‌ها بر سلامت انسان نیاز به مطالعات و آنالیزهای بیشتری دارد. با اثبات حضور این باکتری‌ها ممکن است روغن محلی کرمانشاهی را در دستهٔ مواد غذایی فراسودمند قرار گیرد.

کلید واژه‌ها: پروبیوتیک، اسید لاکتیک باکتری‌ها، لبنیات، روغن محلی کرمانشاهی، توالی‌یابی