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Original research

Evaluation of Viability of Probiotic Bacteria in some Iranian Probiotic Dairy Products

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ABSTRACT

In this study, the viability of *Lactobacillus bacteria* in probiotic dairy products sold in Qazvin city within two periods was examined. Eighteen samples of dairy products (yogurt, cheese, and kefir drinks) labeled with probiotics from different brands of various companies in Iran were prepared. Dilutions were prepared appropriately from each sample and inoculated into MRS Bile Agar culture medium as a pour plate culture procedure. After incubation, the colonies were counted and checked to see if the standards of the Iranian National Standardization Organization (INSO) (min 10^6 CFU/gr) were met. Then, to perform probiotic confirmation tests, each colony characterized different morphological was examined regarding catalase and Gram staining tests. The average count of live *Lactobacillus* showed that out of eighteen samples of probiotic dairy products, only one sample (5.5%) had the minimum number of bacteria according to INSO. In the yogurt, none of the samples could grow in the bile culture medium. In the cheese, none of the samples had the minimum number of viable bacteria according to the standard. Regarding the kefir drink, only one sample had the minimum number of live bacteria based on the standards, in the first week after the production date. In most of the dairy products labeled as probiotic, the average count of *Lactobacillus* with the ability to grow in bile, is lower than INSO measurements, which is at least one million bacteria per gram of a product.

Keywords: Dairy products; *Lactobacillus*; Microbial count; Probiotics

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1. Introduction

Probiotics are non-pathogenic microorganisms that have beneficial health effects for their host by creating a microbial balance in the intestine, provided they are used in sufficient numbers and in live form. Most of these micro-organisms are part of the lactic acid family of bacteria (Akın et al., 2007). Among the probiotic micro-organisms, lactic acid bacteria are known as the most important group, among which *Lactobacillus* and *Enterococcus* are part of the natural flora of the digestive system and fermented foods (Klaenhammer, 2000). *Lactobacillus*, being a probiotic, has received attention because of its ability in fermentation and its crucial role in human health. It produces compounds including organic acids, diacetyl, hydrogen peroxide, and bacteriocins during lactic fermentation, whose protective effect on food is of particular importance (Mirdamadi & Tangestani, 2011).

Probiotic foods contain at least one million probiotic bacteria per gram of product. This ensures that a sufficient number of bacteria will reach the intestine in a live form after consumption, providing health benefits (Mozafari et al., 2023). It is important that this number of bacteria remains consistent from the production date to the expiration date of the product, as well as throughout its storage period. To meet these requirements, the micro-organism used as a probiotic must be qualified and able to meet the conditions of production and storage (Rezaei et al. 2017).

Dairy products are the most important products usually produced as probiotics, so the different forms of these probiotic products have occupied an important part of the market share (Heydarpour & Mazdarani, 2011; Tajabady Ebrahimi et al., 2009). Yogurt,

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buttermilk, kefir (dough), cheese, and other types of fermented kinds of milk are among the most common probiotic dairy products in the world market (Tajabady Ebrahimi et al., 2009). Yogurt is a fermented product that is produced by the fermentation of milk at present (Lactobacillus bulgaricus and Streptococcus thermophilus) at the temperature of 40 to 45°C (Lourens-Hattingh & Viljoen, 2001). Kefir is a fermented dairy product that is produced by kefir grains from dried starters. This drink originates from the Turkish tribes who lived in the Caucasus mountains and is famous for increasing their lifespan (Esener et al., 2018). Kefir has a mild acidic taste, an alcoholic smell, a relatively thick texture like yogurt, and an elastic consistency (Farnworth, 2005).

Although dairy products are considered a suitable platform for transferring probiotic bacteria to the human body, there are several technological obstacles. These include the lack of suitable probiotic strains, acidity, pH, salt content, type of packaging (presence or absence of oxygen), hydrogen peroxide levels, ripening time, storage conditions, and temperature of cheese. These factors can result in less efficient production and use of these products. (Kadiya et al., 2014; Milanović et al., 2004). This condition should be met during consumption and until the last day of the expiration date (Rezaei et al., 2017). The purpose of this research is to investigate the viability of probiotic bacteria in probiotic dairy products sold in Qazvin city, in the period between the first week of production and the last week of expiration date, and finally check if they meet the ISO standards. Many researchers have studied the viability of probiotic bacteria in different products and reached similar results as the viability of bacteria in probiotic date dessert (Mahdi Zadeh et al., 2021), the viability of *Lactobacillus acidophilus* bacteria in pomegranate juice (Ghazavi et al., 2018), the survival of *Lactobacillus casein* probiotic yogurt containing *Teucrium polium* essential oil (Mahmoudi et al., 2014), the viability of *Lactobacillus acidophilus* in tomato juice during storage (Rasekhi Kazeruni & Hossein, 2017), the survival of *Lactobacillus acidophilus,* and *Lactobacillus delbrueckii* in probiotic cherry juice (Tahmasebian et al., 2020). The results of their research indicated that the number of living cells of probiotic bacteria in all samples decreased significantly during the storage time.

Considering more studies, the number of probiotic bacteria *Lactobacillus casei* and *Lactobacillus acidophilus* in quark cheese increased according to storage time (Kadiya et al., 2014). The survival of *Lactobacillus casei* in probiotic yogurt produced with aloe vera extract was significantly extended after the storage time (Bajalanlou & Pakbin, 2016). In addition, the survival of *Lactobacillus casei* grew significantly during the ripening of probiotic feta cheese, which included *Mentha longifolia L* (Mahmoudi et al., 2013). Another research aimed at investigating the number of live bacteria in probiotic yogurt. It was done at different points of production and processing line, and the result showed that part of the reduction in the number of probiotic bacteria took place during the production procedure and even before the distribution (Jafarpor-Sadegh et al., 2010).

2. Material and Methods

2.1. Preparation of samples

Eighteen samples of dairy products with the probiotic label sold in Qazvin city were randomly chosen, including three samples of probiotic yogurt from brands Y_1, Y_2, and Y_3, four samples of probiotic cheese from brands Ch_1, Ch_2, Ch_3, Ch_4, and two samples of kefir drink from brands K_1, K_2. The samples were taken in two periods: the first week after the production date and the last week before the expiration date.

2.2. Acidity measurement

The acidity of the kefir drink was measured based on the national standard number 11177. The acidity of the kefir drink should not be less than 0.6% in terms of lactic acid (ISIRI, 2022). Yogurt acidity was examined as indicated by the national standard No. 695. The acidity of yogurt should not be less than 0.7 (ISIRI, 2019). The acidity of cheese was tested according to the Iranian national standard number 2852. The acidity of cheese should be between 0.8 and 1.4 (ISIRI, 2006).

2.3. pH measurement

The pH of all samples was measured with a digital pH meter. As mentioned by the national standard number 11177, the pH of a kefir drink should not be more than 4.5 (ISIRI, 2022). According to the national standard number 11325, the pH of probiotic yogurt should not be more than 4.5 (ISIRI, 2008). Moreover, according to the national standard number 6629, the pH of cheese should not be more than 5.2 (ISIRI, 2015).

2.4. Bioavailability of probiotics

To count the probiotic bacteria, the culture was grown using the pour plate method on MRS-Bile Agar medium after preparing the appropriate dilution of the samples. Additionally, it was encapsulated at 37°C for 72 hours (Shahabbaspour et al., 2013). According to ISIRI (2008), bile prevents bacteria from growing in traditional yogurt, and the colonies that grow are representative of the target species. After the encapsulation period, colonies resembling indicator microorganisms were counted on plates containing between 10 and 300 colonies, following national standard No. 9616 (ISIRI, 2007). As mentioned by Ehsani et al. (2011), since the viability of Bifidobacteria is lower than that of Lactobacillus, only the number of living Lactobacillus was considered in this study.

2.5. Probiotic confirmatory tests

The morphology of each sample colony was studied by microscopic examination considering the Gram Staining Method. Catalase test was performed for the colonies of each sample with hydrogen peroxide 3%. According to Halt et al. (1985), While the lack of gas production in the catalase test indicates that the test is negative, the experiment reveals that all the colonies were catalase-negative.

2.6. Statistical analysis

Data analysis was done by using SPSS 22.0 software. One-way analysis of variance (ANOVA) was applied to compare the data. Duncan's test was administered to compare the averages with an error level of 5%. Graphs were drawn with the help of Microsoft Office- Excel software.

3. Results and Discussion

3.1. pH changes in yogurt samples

As illustrated in Table 1, a decrease in pH was observed in all the tested samples during storage, and this decrease was evaluated as significant ($p<0.05$). The highest and lowest pH in both periods were respectively related to Y_2 and Y_1 yogurt samples. In addition, the pH of all samples in both periods was consistent with the ISIRI, which is 4.50 at maximum.

A decrease in pH was observed in all the samples during storage. The decrease in pH can be due to the metabolic activity of lactic acid bacteria and the production of organic acids, especially lactic acid (Perrin et al., 2002). The acidity increases in yogurt with the fermentation of lactose to lactic acid by the activity of starter bacteria, as mentioned in many studies (Bakirci & Kavaz, 2008; Cho et al., 2020; Jeong et al., 2018; Tarakci, 2010; Walstra et al., 2005). According to studies conducted by researchers, the pH of probiotic yogurt samples decreases but acidity increases during storage at 4°C. Results of another study also proved the downward process of yogurt pH after some time (Dave & Shah, 1997). The present results are in agreement with the outcomes of researchers regarding the increase in acidity of probiotic and regular yogurt samples during storage (Bano et al., 2011; Beheshtipour et al., 2013; Papastoyiannidis et al., 2006; Salwa et al., 2004; Tamime & Robinson, 2007; Zamberlin et al., 2011).

3.2. pH changes in cheese samples

As seen in Table 1, a decrease in pH was observed in all the examined samples during storage, which was evaluated as a significant change ($p<0.05$). The highest pH in the first week of production was related to the sample Ch3. The lowest pH level in the last week was related to sample Ch2. In addition, the pH of all the samples in both periods was within the standards, with a maximum of 5.20.

The results of Farahani et al. (2014) revealed a decrease in pH and an increase in acidity of salted white cheese (Golpayegan cheese) during the ripening period. The researchers attributed this to the activity of bacteria during this ripening period. They announced that the results of this research are consistent with current research (Farahani et al., 2014). The decrease in pH during the ripening period is mostly related to the fermentation of lactose. During the long ripening period of traditional cheeses, it is also related to the production of amino acids and fatty acids through proteolysis and lipolysis. However, the important factor for the reduction of pH is the production of lactic acid (Fox et al., 2000).

3.3. pH changes in kefir (drink) samples

As illustrated by Table 1, after some time as it approached the last day of expiration of the kefir samples, the pH decreased significantly ($p<0.05$) and the product became acidic. The highest and lowest pH levels in each of the two periods were respectively related to samples K2 and K1. In addition, the pH of all samples in both periods was tested using ISIRI measurement, with a maximum limit of 4.50.

In a study conducted on kefir, the results showed that the pH decreased with an increase in storage duration, added concentrations, and flavors (Yilmaz et al., 2006). During the storage time, the pH decreased significantly because lactic acid bacteria were active even at refrigerator temperature. Moreover, they produced lactic acid and reduced pH by fermenting lactose as stated by Kailasapathy et al. in (2008). Similar results have been reported by other researchers, which are in line with the results of this study (Özer et al., 2005; Ramachandran & Shah, 2010).

Table 1. Comparison of pH values of yogurt, cheese, and kefir samples during the storage period.

Samples	pH on production day	pH on expiration day	Standard pH
(Yogurt) Y_1	$4.04 \pm 0.03^{A,c}$	$3.96 \pm 0.01^{B,c}$	Maximum 4.50
Y_2	$4.30 \pm 0.02^{A,a}$	$4.16 \pm 0.01^{B,a}$	
Y_3	$4.12 \pm 0.02^{A,b}$	$4.01 \pm 0.03^{B,b}$	
(Cheese) Ch_1	$4.50 \pm 0.02^{A,b}$	$4.42 \pm 0.03^{B,b}$	Maximum 5.20
Ch_2	$4.52 \pm 0.04^{A,b}$	$4.18 \pm 0.04^{B,c}$	
Ch_3	$4.62 \pm 0.03^{A,a}$	$4.45 \pm 0.04^{B,b}$	
Ch_4	$4.66 \pm 0.03^{A,a}$	$4.55 \pm 0.02^{B,a}$	
(Kefir) K_1	$3.38 \pm 0.02^{B,b}$	$3.65 \pm 0.02^{A,b}$	Maximum 4.50
K_2	$3.52 \pm 0.02^{B,a}$	$3.81 \pm 0.05^{A,a}$	

*Results are reported as mean ± standard deviation.
The presence of at least one similar uppercase Latin letter indicates the absence of a significant difference between the values of each row and the presence of at least one similar lowercase Latin letter indicates the absence of a significant difference between the values of each column at the 5% confidence level.

3.4. The acidity changes in yogurt samples

As revealed in Table 2, an increase in acidity was observed in all the examined samples during the storage period, and this increase was evaluated as a significant change ($p<0.05$). Furthermore, the acidity of all samples in each of the two periods was examined by the ISIRI standards considering at least 0.70.

There are some other studies reported that the acidity of yogurt samples showed a considerable increase by improving the storage time (Foutohi & Manafi Dizaj Yekan, 2021; Jafari-Najafabadi & Fadaei-Noghani, 2021). During storage, the acidity increased significantly because lactic bacteria were active even at refrigerator temperature. They produced lactic acid by fermenting lactose and increasing the acidity (Kailasapathy et al., 2008).

3.5. Acidity changes in cheese samples

Disclosing by Table 2, after some time and approaching the last day of the expiration date of the cheese samples, the acidity increased significantly ($p<0.05$), and the product transformed into an acidic state. The lowest and highest amounts of acidity in each of the two periods were related to Ch_3 and Ch_2 samples, respectively. Additionally, the acidity of Ch_1, Ch_2, and Ch_4 samples was higher than the standard in the last week of the expiration date. According to the national standard, the acidity of cheese samples should be between 0.8-1.40. However, the level of acidity in the mentioned samples exceeded the standard.

In 2014, Farahani et al evaluated some the physicochemical, rheological, and textural characteristics of salted white cheese during the ripening period. The results also indicated an increase in acidity levels during storage. According to the researchers, this was attributed to the activity of bacteria during the ripening period. The findings of this study align with the current research conducted by Farahani et al. (2014).

3.6. Acidity changes in kefir samples

As revealed by Table 2, an increasing trend in acidity level was observed in all the examined samples during the storage period, which was evaluated as significant ($p<0.05$). The lowest and highest acidity levels in both periods were related to samples K_1 and K_2, respectively, while the acidity of all samples in both periods was examined by the national standards of Iran considering at least 0.60.

Table 2. Comparison of acidity values (% lactic acid) of yogurt, cheese, and kefir samples during the storage period.

Samples	Acidity on production day	Acidity on expiation day	Standard acidity
(Yogurt) Y_1	$1.34 \pm 0.05^{A,a}$	$1.10 \pm 0.03^{B,a}$	Minimum 0.70
Y_2	$1.34 \pm 0.04^{A,a}$	$1.19 \pm 0.08^{B,a}$	
Y_3	$1.13 \pm 0.04^{A,b}$	$1.03 \pm 0.02^{B,b}$	
(Cheese) Ch_1	$1.12 \pm 0.18^{B,ab}$	$1.44 \pm 0.12^{A,ab}$	0.8 - 1.40
Ch_2	$1.29 \pm 0.07^{B,a}$	$1.56 \pm 0.11^{A,a}$	
Ch_3	$0.94 \pm 0.04^{B,b}$	$1.24 \pm 0.12^{A,b}$	
Ch_4	$1.24 \pm 0.11^{B,a}$	$1.46 \pm 0.08^{A,a}$	
(Kefir) K_1	$0.84 \pm 0.02^{B,a}$	$0.97 \pm 0.02^{A,a}$	Minimum 0.60
K_2	$0.88 \pm 0.05^{B,a}$	$1.11 \pm 0.02^{A,a}$	

3.7. The viability of bacteria (Lactobacillus) in kefir samples

Considering Figure 1, the average number of live *lactobacillus* in sample K_1 was 5.84 log CFU/mL in the first week of production, while this figure in sample K_2 reached 6.75 log CFU/mL for live bacteria in the first week of production. The number was consistent with the standard rate of probiotic dairy products, which is at least 6 log CFU/mL. There was a significant difference between separate samples of kefir in terms of the number of probiotics in the first week of production ($p<0.05$). During storage, the average number of bacteria decreased sharply and reached zero.

The reason for the decrease in the bacterial population is related to the effect of the resulting acid on the bacterial cell wall and their digestion. As a result, the resistance of the bacteria decreases (Mohammadi et al., 2012). It is also related to the secretion of alkaline substances by the bacteria to neutralize and adapt to the acidic culture medium outside. However, with the excessive secretion of these substances and the rise of the internal pH, the conditions for the continued growth of bacteria become worse leading to cell death (Jayamanne & Adams, 2006; Shah, 2000).

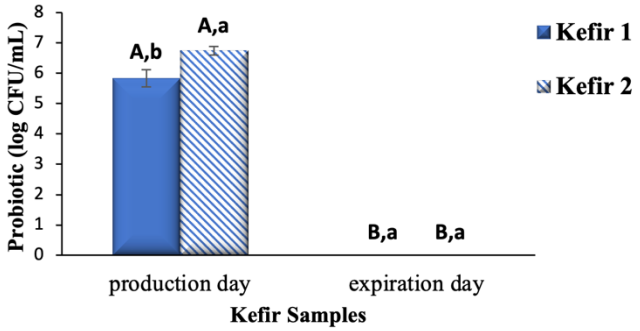

Fig. 1. The average number of living *Lactobacillus* in kefir samples. Columns labeled with different letters are significantly different, lowercase Latin letter and uppercase Latin letter show significant statistical differences between samples and storage time respectively.

3.8. The survival of Lactobacillus in cheese samples

According to the Iranian National Standard No. 6629, in the case of the production of probiotic and synbiotic cheese, the viability (live count) of each of the probiotic strains used in the cheese should not be less than 10^6 (CFU/g) until the end of the expiration date. (ISIRI, 2015)

As illustrated by Figure 2, the average number of live *Lactobacillus* in Ch_1, Ch_2, Ch_3, and Ch_4 samples was 4.15, 3.66, 4.35, and 5.67 log CFU/mL, respectively. The highest number of probiotics was observed in sample Ch_4. There was a significant difference between several samples of cheese in terms of the number of probiotics in the first week of production ($p<0.05$). During storage time the average number of bacteria decreased significantly so that in Ch_4 a number of 4.14 log CFU/mL live probiotic bacteria was observed in the last week of expiration, although no live probiotic bacteria were observed in the other cheese samples.

Dabour et al (2006) observed a decrease in the number of probiotic bacteria during 6 months of storage in probiotic cheddar cheese containing exopolysaccharides. The high salt content and relatively low pH of white cheese may cause the decrease in the number of probiotic bacteria during the storage time limiting the growth of probiotics (sensitive to salt and acid) used freely (Rolim et al., 2015). The results of Ehsani et al.'s study in 2013 indicated that the simultaneous use of starter and probiotic bacteria in the production of Iranian white cheese reduces the ability of probiotics to survive. It can stem from the unfavorable environmental conditions such as low pH and nutritional competition between the starter bacteria (Ehsani et al., 2011).

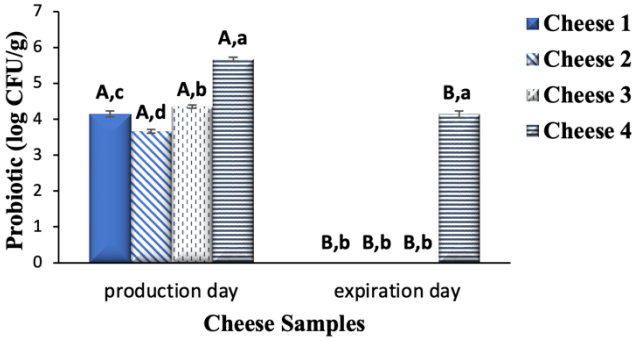

Fig. 2. The average number of living *Lactobacillus* in cheese samples.

3.9. The survival of Lactobacillus in yogurt

According to the Iranian National Standard No. 11325, the viability (live count) of each probiotic strain used in probiotic yogurt should not be less than 10^6 (CFU/g) until the end of the expiration date (ISIRI, 2008).

The average count of the probiotic yogurt plate showed that none of the samples had the minimum number of live *Lactobacillus* according to ISIRI measurements. The average number of bacteria in both periods was zero when the samples contained only traditional yogurt bacteria (lactic acid bacteria) and no probiotic bacteria were observed. Moreover, the minimum number of live and active probiotic cells in each gram of yogurt was claimed to be 10^6 (CFU/g). On the other hand, the test results were contrary to the manufacturer's claim.

Various factors such as pH reduction, lactic acid production due to fermentation, peroxide production, hydrogen, microbial

composition of the starter used, and improper storage temperature may lead to a decrease in the viability of probiotics during yogurt storage (Lourens-Hattingh & Viljoen, 2001). Donkor et al reported that the decrease in environmental pH and the accumulation of organic acids during storage are among the factors affecting the viability of probiotic bacteria in probiotic yogurt. Of course, the survival of probiotics in addition to pH and acidity is also under the effect of factors such as oxidation and reduction potential (Donkor et al., 2006). Yeast contamination of yogurt samples is one of the main reasons for a reduction in the number of probiotic bacteria. The high growth of dairy yeasts in kefir and cheese products is very common because yeasts provide part of the starter culture of these products. However, in the case of yogurt and buttermilk, yeast contamination (due to its ability to produce and tolerate high acidity) is associated with the creation of over acidity or post-acidity phenomenon in yogurt. It not only reduces the sensory properties of yogurt but also provides the basis for reducing the growth of live bacteria, including probiotic strains (Rezaei et al., 2017). In 2011, Heidarpor and Mazdarani examined 35 probiotic yogurt products from the domestic market of Iran. They did not refer to the commercial brand of the products. They concluded that the number of existing bacteria was less than the required amount in 8 samples. Therefore, these samples lacked the initial condition to be introduced as a probiotic product. The processing and storage conditions of the product were the main factors necessary for the survival of the bacteria in question (Heydarpour & Mazdarani, 2011). In 2010, Jafarpor et al conducted research at various points of the production line to investigate the number of live bacteria in probiotic yogurt. They concluded that part of the reduction of the number of probiotic bacteria occurs during the production procedure and even before product distribution (Jafarpor-Sadegh et al., 2010).

3.10. Gram staining of the samples

During the experiment conducted under the microscope, it was determined that the colonies grown on the culture medium of the samples were gram-positive, please refer to Figure 3.

Probiotic bacteria were seen with purple color (Lee et al., 2000).

3.11. The catalase test

Among the set of colonies grown on a culture medium, one sample of each colony with different shapes was selected for testing. During the experiment, it was observed that all the colonies were catalase-negative and the reason was lack of gas production, which indicated that the test was negative (Halt et al., 1985).

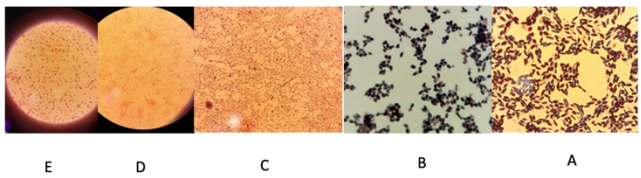

Fig. 3. A: The results of Gram staining of kefir sample (K_1). B: The results of Gram staining of kefir sample (K_2). C: The results of Gram staining of yogurt sample (Y_1). D: The results of Gram staining of cheese sample (Ch_2). E: The results of Gram staining of cheese sample (Ch_4).

4. Conclusion

Among the numerous studies conducted in the country in the field of production, optimization, and introduction of various probiotic products, the number of quality control studies on industrial probiotic products available in the market is very small. Although the production or maintenance of many probiotic products has been successfully carried out in a controlled laboratory workshop and ideal conditions, which is an important step in spreading healthy products in society, there are still several uncertainties in maintaining their quality and understanding the effective factors that reduce the number of live bacteria and probiotic strains at the commercial and sales level.

Production and maintenance of industrial probiotic products in real conditions are very complicated and challenging. In the present study, it was observed that out of 18 samples of dairy products labeled as probiotics, only one sample (5.5%) was probiotic and included the minimum number of live bacteria according to the standards. This issue, unlike the formulation of the standard of probiotic products in Iran, requires greater monitoring of the implementation of this standard. However, it seems that the lack of live bacterial growth in the products (which is investigated in the current research) is because of the insufficient number of them to maintain the sensory properties of the product among consumers. Also, factors such as inappropriate supply conditions and non-uniformity of the quality of the starter cultures used in the factories have affected the results of the present study.

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Conflict of interest

The authors declare that there is no conflict of interest.

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