10.22059/IJVM.2021.313626.1005141

Iranian Journal of Veterinary Medicine
Volume 16- Issue 1

Original Article
Online ISSN: 2252-0554

Changes in Acidity Parameters and Probiotic Survival of the Kefir using *Lactobacillus acidophilus* and *Lactobacillus paracasei* Complementary Probiotics during Cold Preservation

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Abstract

BACKGROUND: Kefir is a fermented milk product containing some anticarcinogenic organic compounds with nutritional benefits, which made it one of the natural dairy beverages extensively consumed.

OBJECTIVES: The present study was conducted to assess the effect of two selective probiotics on the values of acidic criteria and probiotic survival in the kefir produced in this study.

METHODS: In the first step, the cow milk, preheated at 90°C for 5 min, was inoculated with the commercial starter and divided into two groups. They were complemented with *L. acidophilus* LA-5 and *L. paracasei* 431 and incubated at 30°C for 6 h. They were then preserved at refrigerated temperature up to 14 days and then sampling was carried out to evaluate the changes of values of organic acids (lactic acid and acetic acid), pH, titratable acidity and survival of probiotic complemented bacteria on the 1st, 7th, and 14th days.

RESULTS: The pH values of *L. acidophilus* LA-5 and *L. paracasei* 431 were 4.34 and 4.36 at the beginning of the cold storage and reached 4.27 and 4.31 at day 14. The acidity of *L. acidophilus* LA-5-complemented kefir on the 1st day was 0.80 gr/100 gr higher than *L. paracasei* 431-complemented kefir which showed 0.72 gr/100 gr. Lactic acid was ranging from 1.57 to 2.40 gr/100 mL or 2.17 to 2.42 gr/100 mL (from the 1st to the 14th day) in the kefirs complemented with *L. acidophilus* LA-5 and *L. paracasei* 431, respectively. In the kefirs complemented with *L. acidophilus* LA-5 and *L. paracasei* 431, the acetic acid was stable (from 0.11 to 0.13 gr/100 mL) during 14 days but increased in the later (from 0.11 to 0.23 gr/100 mL) .The survival of both bacteria was higher than 7 logs CFU/gr in the kefir.

CONCLUSIONS: Adding *L. acidophilus* LA-5 and *L. paracacei* 431 can moderate the acidity of the kefir and extend the survival of complementary probiotics at a standard level during two weeks of cold preservation.

KEYWORDS: Acidic parameters, Kefir, Lactobacillus acidophilus, Lactobacillus paracasei, Survival

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Received: 2021-04-22

Accepted: 2021-07-28

How to Cite This Article

Pourbaba, H., Anvar, A A., Pourahmad, R., Ahari, H. (2022). Changes on Acidity parameters and probiotic survival in the kefir using *Lactobacillus acidophilus* and *Lactobacillus paracasei* complementary probiotics during cold preservation. *Iranian Journal of Veterinary Medicine*, *16*(1), 89-98.

Introduction

Kefir is a dairy beverage produced during fermentation process, which includes several beneficial probiotics (Bengoa *et al.*, 2019b). The fermentation process is done with kefir grains, a complex probiotic, which comprises a consortium of microorganisms (Lim *et al.*, 2019; Mitra and Ghosh, 2020) such as yeasts and lactic acid bacteria (LAB) encompassing *Lactococcus* and *Lactobacillus*. Kefir grains have been deployed for the production of Kefir as starter in the Caucasus (Kabak and Dobson, 2011). Kefir is produced through a homofermantative or heterofermentative process of milk (or both of them), which is characterized by a typical sour taste and firmness due to a combination of lactic and acetic acids, ethanol, CO_2, exopolisaccharides and other mixtures produced, as well as pH and titratable acidic changes (Garofalo *et al.*, 2015).

One of the compounds found in milk is lactose. The persons who exhibit lactose intolerance cannot consume milk (Demir, 2020) but approximately 30% of lactose presented in the milk is fermented to lactic acid and acetic acid or other volatile combinations (Zareba *et al.*, 2012). These acids particularly lactic acid reach the normal concentration and give an appropriate sour taste to Kefir (Kök-Tas *et al.*, 2013). While lactose is broken down into glucose and galactose, lactic acid bacteria such as *L. acidophilus* and *L. paracasei* convert the glucose via the main fermentation process (Hikmetoglu *et al.*, 2020).

The biological and Physico-chemical parameters of the produced beverages are necessary for the final properties of Kefir. These attributes are principally related to the milk content, starter, complementary probiotics, and acidic condition (Wang *et al.*, 2017). In addition to lactic acid and acetic acid, titratable acidity and pH are two important indicators to determine the quality of produced Kefir but on the other hand, measuring them is costly and requires more techniques (Magalhães *et al.*, 2011b).

Probiotics are distinct live cells and confer a health advantage on the host when being administered in an adequate volume (da Costa *et al.*, 2020). Their efficiencies include anticarcinogenic properties, protecting gastrointestinal cells against improper situations specifically acidic conditions, eliminating pathogenic microbes (Kim *et al.*, 2019), enhancement of the immune system, and modulation of gut microbiota (Bengoa *et al.*, 2019a). Out of the probiotic microorganisms, *Lactobacillus paracasei* and *L. acidophilus* are of distinct interest on account to their health-enhancing activities (Mantis *et al.*, 2011). Zendeboodi *et al.* (2020) suggested three main categories of probiotics including 'true probiotic' (TP) describing live cells, 'pseudo-probiotic' (PP) describing live but inactive cells, and finally the features of vegetative or spore (PPV or PPS) and 'ghost probiotic' (GP) describing non-live cell, intact or ruptured (GPI or GPR) cells. Each of these categories is divided into two groups according to their site of effectiveness, which could be as in vivo or in vitro.

However, a few Lactic acid bacteria have appropriate potential to regulate the acid production in Kefir, increasing the pH and enhancing bacterial growth up to the standard level. Accordingly, the greater the pH is, the greater the survival rate of bacteria in the Kefir is obtained, which results in increase in the shelf-life of Kefir (Nejati *et al.*, 2020).

This study was aimed to assess the values of different acidic criteria including pH, titratable acidity, lactic acid, acetic acid and also probiotic survival in the Kefir produced in this study.

Materials and Methods

Materials

About 2 liters (L) cow milk containing 2.5% fat (Pak Dairy Co., Iran) was deployed to produce the Kefir in this study. Commercial starter, containing *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*, *Leuconostoc mesenteroides subsp. cremoris* as pack 1 along with the pack 2 including *Kluyveromyces marxianus* subsp. *Marxi-anus* were prepared from Hansen (Denmark). They were arranged in direct vat set (DVS) form, which could be supplemented directly to the milk samples. Commercial probiotics including *L. acidophilus* LA-5 and *L. paracasei* 431 were purchased from Hansen to be used as complementary probiotics.

Study Design

Approximately 0.1 gr of pack 1 and 0.002 gr of pack 2 were added to 1 L cow milk. The *L. acidophilus* LA-5 and *L. paracasei* 431 were added at 0.001 gr/L of milk as groups 1 and 2, respectively, so that a 100 mL of the milk of each group in triplicate was pre-boiled at 90C for 5 min, added into 200-mL Falcone tubes and slightly vibrated for half an hour. The tubes were incubated at 30°C for 6 h. Then, the kefir samples were cooled until 4°C and preserved in a refrigerator for 2 weeks (Figure 1). Sampling days were assigned on the 1st, 7th, and 14th days to evaluate the probiotic survival, acidity value as well as quantities of organic acid of the Kefir.

Microbiological Analysis

On each sampling day, 10.0 mL of the Kefir was mixed with 90.0 mL peptone water (Merck, Darmstadt, Germany) and well homogenized. Based on the methods of Sohrabvandi *et al.*, (2012), it was serially diluted and cultured in Man, Rogosa, and Sharpe (MRS) bile agar (Merck, Darmstadt, Germany) and incubated at 37°C for 2-3 days to observe and count colonies of *L. acidophilus* and *L. paracasei.*

Total Titratable Acidity (TA) and pH Measurement

At the time of kefir sampling, the pH value was measured using a microprocessor pH meter armed with a glass probe (Hanna, USA). On the other hand, the TA value of the Kefir was measured in triplicate by the titration of 10 mL of each Kefir with 0.11 M NaOH. The titration was continued until the pink color disappeared (Affane *et al.*, 2011).

Determination of Organic Acid Concentration

Acetic and lactic acids were determined using HPLC according to the method of Garrote *et al.*, (2000) with minor modification. A total of 18 kefir samples were prepared by mixing 10 mL of each with 50 mL of H_2SO_4 (pH = 2.5, adjusted with NaH_2PO_4) and homogenized for 60 min. It was then centrifuged at 10,000 × g, and the supernatant was filtered through 0.45 µm filter. The mobile phase was performed using an elution (combination of A: acetonitrile (5%) and B: 0.1% orthophosphoric acid (95%)) at 1 mL/min flow rate for 10 min at room temperature. The filtered solution (100 µL) was vortexed with 900 µL of A+B combination and re-centrifuged at 5,000 × g. The supernatant (25 µL) was eventually injected into a HPLC system (Shimadzu Corp., Tokyo, Japan) equipped with C18 columns (250 mm × 4.6 mm; 5µm), and the absorbance was read at 210 nm in triplicate. Acid identification was based on the matching retention times with standards.

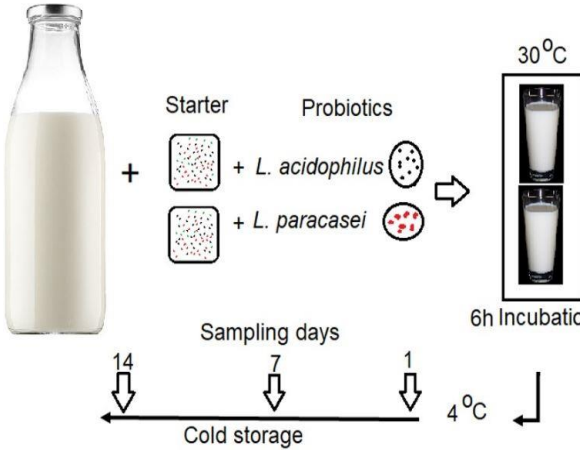

Figure 1. A Schematic of the experiment—there were 2 treatments including *L. acidophilus* LA-5 and *L. paracasei* 431. Initially, the milk was heated at 90°C for 5 min. The treated milks (in triplicate) were then supplemented with the determined probiotics, incubated at 30°C for 6 h and ultimately stored at 4°C. The sampling was carried out on days 1, 7 and 14.

Statistical Analysis

Data are presented as the mean ± standard deviation (SD) in triplicate. The results were statistically analyzed by analysis of variance (ANOVA) followed by Tukey's test. The difference between two kefirs at the same time was assessed using an Independent two-sample t-test. Differences were considered significant at $P<0.05$. The statistical analyses were performed using the IBM SPSS for Windows, V. 26 (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Figure 2 shows the trend of pH in kefir samples supplemented with *L. acidophilus* LA-5 and *L. paracasei* 431 during the sampling days of cold storage. At the same time, the differences between the pH values of both kefirs were negligible ($P>0.05$). The pH values of *L. acidophilus* LA-5 and *L. paracasei* 431 were 4.34 and 4.36, respectively at the beginning of the cold storage and reached 4.27 and 4.31, respectively at day 14. Similar to this study (Figure

2), another finding (Magalhães *et al*., 2011a) exhibited the pH of Kefir reached 4.40 after a day at 25°C. The rise in acidity or drop in pH of the kefir beverage is illuminated by organic acid, which is made by probiotics through the fermentation process (Magalhães *et al*., 2011b). The pH of Kefir decreased significantly by rise in temperature, so that it was 4.3 and 3.9 when the fermentation temperature was 23 and 29ºC, respectively and they used different natural Chinese starters. This result was not in accord with our study results (Figure 2), which showed the pH of both kefirs reached 4.3 at 30ºC of the fermentation period. This difference could be due to the type of starter and complementary probiotic. The results of titratable acidity (based on the lactic acid) of the kefir samples are presented in Figure 3. As such, the TA of both kefirs increased slightly in a time-dependent manner. The acidity of *L. acidophilus* LA-5-complemented Kefir on the 1st day (0.80 gr/100 gr) showed a significant difference (P<0.05) compared to the *L. paracasei* 431 one (0.72 gr/100 gr). This difference was not significant (P>0.05) on the 7th (0.81 and 0.79 gr/100 gr, respectively) and 14th days (0.83 and 0.84 gr/100 gr, respectively). Despite the fact that there is no direct correlation between pH and TA, a general association showed that pH decreases when TA increases in Kefir (Walstra *et al*., 2005). This finding is in the line with our study (Figures 2 and 3), which showed that the pH of *L. paracasei* 431-complemented kefir was slightly decreased from 4.36 to 4.31 when its TA has increased from 0.72 to 0.84 gr/100 gr during two weeks of the experiment. These values were different compared to another study that produced normal kefir from the goat milk. The pH and TA were 4.47 and 0.174 gr/100 gr (Setyawardani and Sumarmono, 2015) but the TA was increased from 0.70 to 0.78 gr/100 gr in the Kefir in which starter was applied and ranging from 0.80 to 0.87 gr/100 gr in Kefir fermented by kefir grains during 14 days of cold preservation. The pH decreased slightly from the beginning to the end of the cold preservation in the Kefir fermented by kefir grains in 10% CO_2 atmosphere (Kök-Tas *et al*., 2013). Özdestan and Üren (2010) exhibited that pH and TA of Kefir varied from 4.11 to 4.53 and from 0.652 gr/100 gr to 1.047 gr/100 gr, respectively.

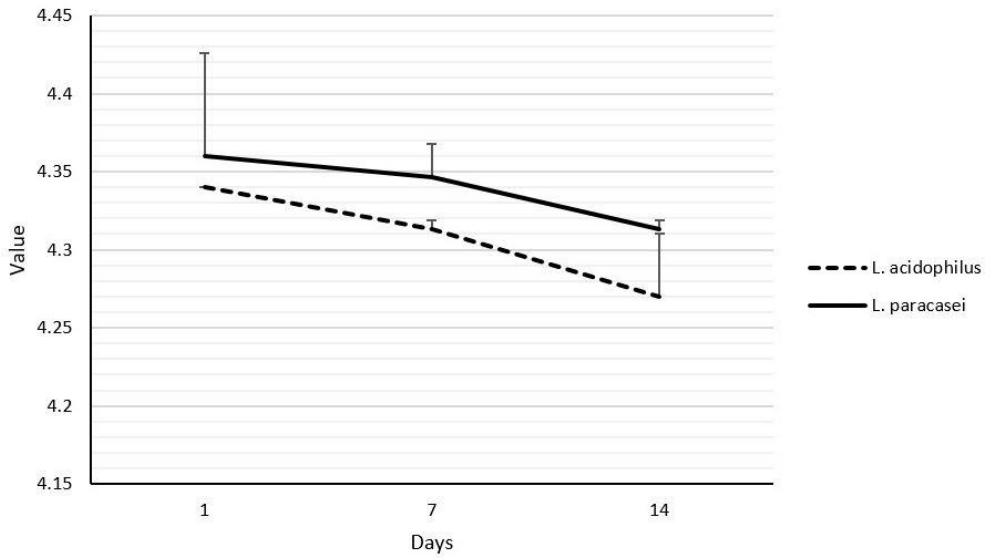

Figure 2. Trend of the pH of two types of kefir groups complemented with probiotics during 2 weeks of cold storage (n=3). Different small scripts in each line indicate a significant difference (P<0.05). Different capital scripts between two lines and the same day indicate a significant difference (P<0.05).

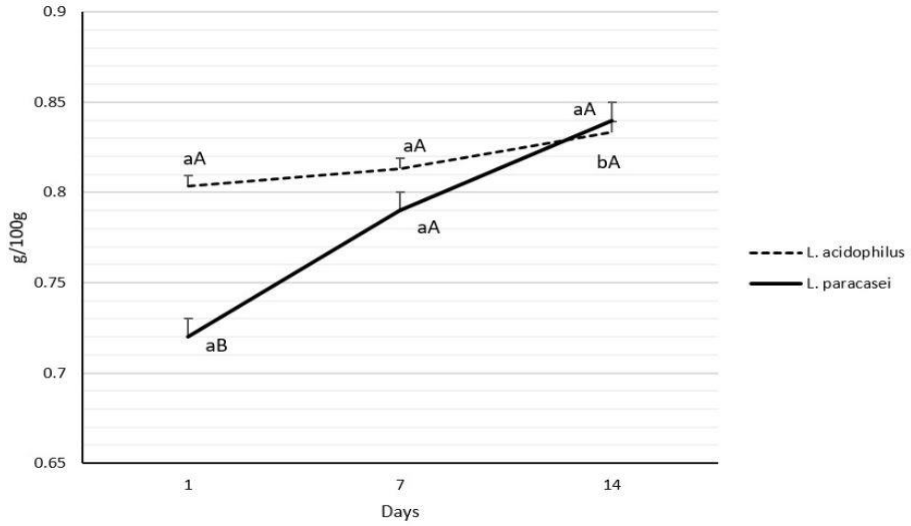

Figure 3. Trend of the titratable acidity (g/100 g) of two types of kefir groups complemented with probiotics measured during 2 weeks of cold storage (n=3). Different small scripts in each line indicate a significant difference (P<0.05). Different capital scripts between two lines and the same day indicate a significant difference (P<0.05).

The titratable acidity could not definitely show lactic acid (LA) or acetic acid (AA) values produced in the Kefir. On the other hand, a study (Affane *et al*., 2011) showed a direct correlation and relatively equal values between TA and LA in the normal Kefir, so that the TA and LA reached 0.8 gr/100 gr and 0.8 gr/100 mL, respectively. But in this study, the LA showed a significant increase (P<0.05) from 1.57 to 2.40 gr/100 mL (from the 1st to the 14th day) or 2.17 to 2.42 gr/100 mL in the kefirs complemented with *L. acidophilus* and *L. paracasei* 431, respectively (Figures 3 and 4). This increase could be due to adding the bacteria to the Kefir, in addition to the starter at incubation time. Lengkey and Balia (2014) showed that LA value increased from 0.82 to 1.26 gr/100 mL after 8 h by dose increase from 10% to 25% of complementary starters, similar to this study (Figure 4). The greater the activity of starter bacteria is, the more the LA value of Kefir is obtained. It indicates that the produced LA would be increased when the fermentative bacteria is available. Another finding showed that the LA of goat kefir was 1.31 gr/100 mL (Setyawardani *et al.*, 2020) less than that of this study (Figure 4). It exhibited that the LA was 1.57 and 2.17 gr/100 mL when the kefirs were complemented with *L. acidophilus* LA-5 and *L. paracasei* 431, respectively. The less production of AA in the *L. acidophilus* LA-5-complemented Kefir compared to *L. paracasei* 431-complemented one (Figure 5) after 14 days of the cold preservation could be due to this fact that *L.* acidophilus, a homofermentative bacterium, cannot produce AA by itself (Fazio *et al.*, 2020). Thus, the produced AA in this type of Kefir was pertinent to the starter at the beginning of the study. The *L. paracasei* can produce either lactic acid or acetic acid (Yamamoto *et al.*, 2019). Additionally, *L. paracasei* proficiently consumes lactose more than *L. acidophilus* (Watson *et al.*, 2013), which was expected that the value of LA in *L. paracasei*-complemented Kefir (2.17 gr/100 mL) was greater than *L. acidophilus* one (1.57 gr/100 mL), at least on the first day (Figure 4). Delgado-Fernández *et al.* (2019) exhibited that LA and AA were 0.63 and 0.038 gr/100 mL, respectively without any changes during the first 7 days.

The Figure 6 presents data on the survival of *L. acidophilus* LA-5 and *L. paracasei* 431 complementary probiotics added to the kefirs. *Lactobacillus paracasei* count is greatly associated with the production of lactate (Bergmann *et al.*, 2010). The count of probiotic in Kefir should be higher than 7.0 log CFU/mL (Rosa *et al.*, 2017). Similarly, the probiotic count of both bacteria-complemented kefirs decreased from 7.6 to 7.2 log CFU/mL during 14 days of the cold preservation (Figure 6), which showed a greater value than the standard. The bacteria count for both decreased slightly from 7.6 to 7.2 log

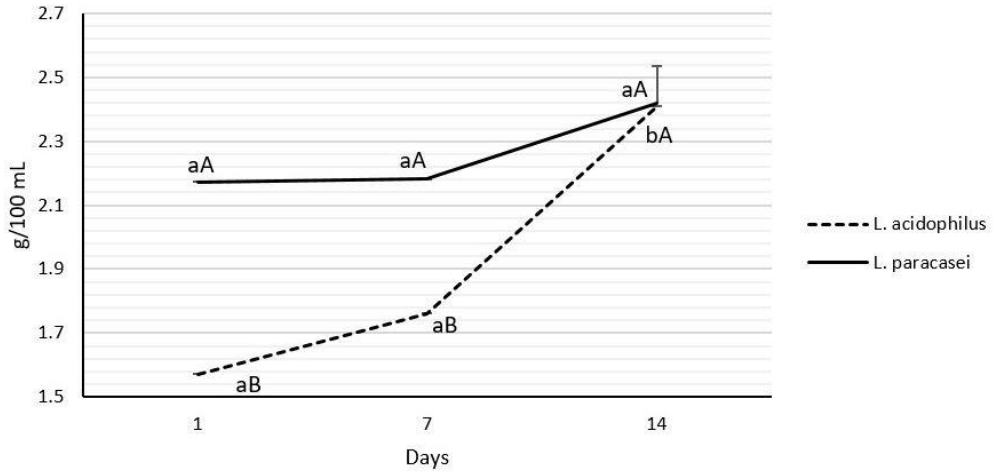

Figure 4. Trend of the lactic acid of two types of kefir groups complemented with probiotics during 2 weeks of cold storage (n=3). Different small scripts in each line indicate a significant difference (P<0.05). Different capital scripts between two lines and the same day indicate a significant difference (P<0.05).

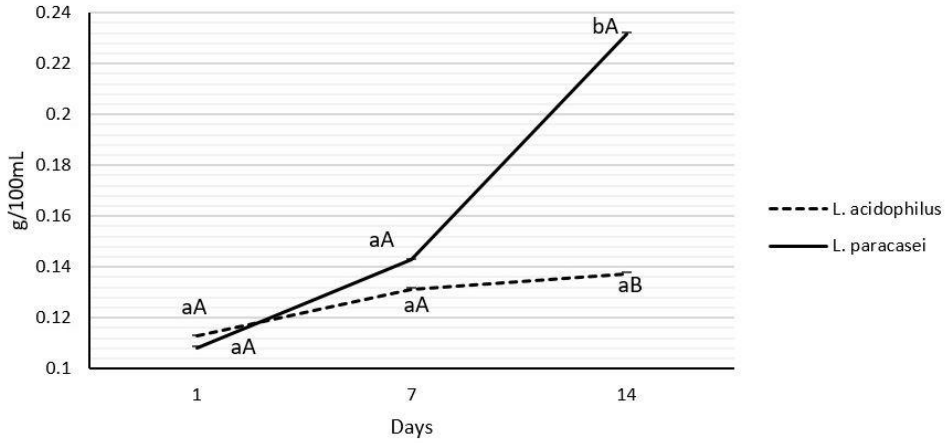

Figure 5. Trend of the acetic acid of two types of kefir groups complemented with probiotics during 2 weeks of cold storage (n=3). Different small scripts in each line indicate a significant difference (P<0.05). Different capital scripts between two lines and the same day indicate a significant difference (P<0.05).

CFU/mL from the beginning to the end of the experiment (2 weeks), but they were not less than the standard value; 7.0 log CFU/mL (Rosa *et al*., 2017). Similarly, in another study (Setyawardani and Sumarmono, 2015), the total count of lactic acid bacteria in the Kefir produced from goat milk ranged 7.6-7.2 log CFU/mL at the same time at refrigerated temperature. The counts of *L. acidophilus* LA-5 in Kefir fermented by kefir grains in 10% CO_2 atmosphere were 7.02, 7.21, and 6.42 log CFU/mL after 1, 4 and 14 days, respectively (Kök-Tas *et al*., 2013). On the other hand, *L. acidophilus* count (Tomar *et al*., 2020) was less than this study (Figure 5), so that, it was decreased from 5.92 to 4.79 log CFU/mL in the Kefir that kefir grains were used and was extremely mitigated to 2.0 log CFU/mL in Kefir fermented by starter during 14 days of cold storage. On the other hand, the total lactic acid bacteria count was 7.20 log CFU/mL in the goat kefir (Setyawardani *et al*., 2020), which was even less than *L. acidophilus* LA-5 (7.64 CFU/mL) or *L. paracacei* 431 (7.63 CFU/mL) in the kefirs examined in this study. In another study (Bengoa *et al*., 2018), *L. paracasei* reached 10^6 log CFU/mL when the Kefir incubated at 30°C, which was less than the standard (Rosa *et al*., 2017).

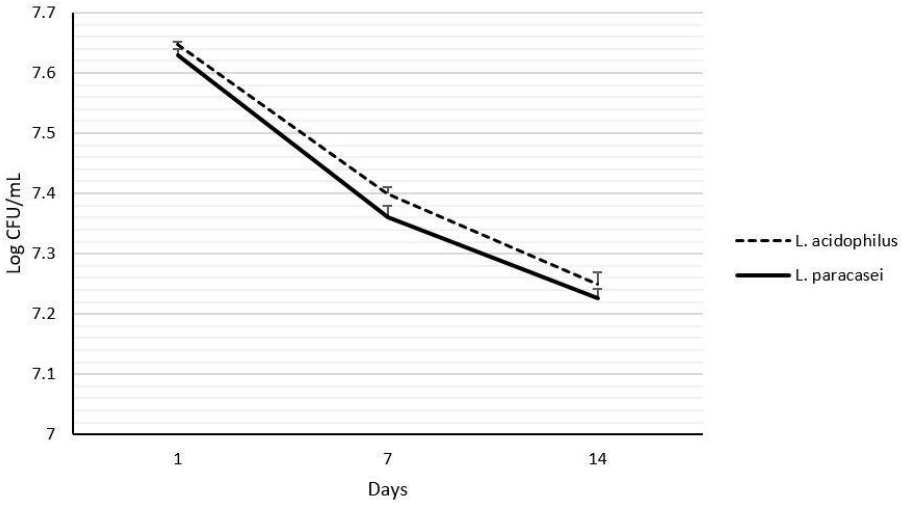

Figure 6. Trend of the probiotic survival in two types of kefirs complemented with these probiotics during 2 weeks of cold storage (n=3). Different small scripts in each line indicate a significant difference (P<0.05). Different capital scripts between two lines and the same day indicate a significant difference (P<0.05).

Conclusion

In conclusion, our study indicated that the addition of the *L. acidophilus* LA-5 and *L. paracacei* 431 can moderate the acidity of the Kefir and prolong survival of complementary probiotics at least up to two weeks of cold storage.

Acknowledgments

We would like to acknowledge the Islamic Azad University, Science and Research Branch of Tehran for the scientific support of this research.

Conflict of Interest

The authors declared no conflict of interest.

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10.22059/IJVM.2020.313626.1005141

Iranian Journal of Veterinary Medicine

Abstracts in Persian Language

ISSN Online 2252-0554

مجله طب دامی ایران، ۱۴۰۰، دوره ۱۶، شماره ۱، ۸۹-۹۸

تغییر شاخص های اسیدی و زنده مانی پروبیوتیک ها در کفیر تولید شده با پروبیوتیکهای کمکی لاکتوباسیلوس اسیدوفیلوس و لاکتوباسیلوس پاراکازئی در طول دوره نگهداری در سرما

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(دریافت مقاله: ۰۲ اردیبهشت ماه ۱۴۰۰، پذیرش نهایی: ۰۵ مرداد ماه ۱۴۰۰)

چکیده

زمینه مطالعه: مطالعه: کفیر محصول شیر تخمیر شده است که حاوی ترکیبات آلی ضد سرطان با مزایای تغذیه‌ای است که باعث شده به عنوان نوشیدنی لبنی طبیعی پر مصرف معرفی گردد.

هدف: مطالعه حاضر برای ارزیابی مقادیر شاخص‌های درگیر در تغییرات اسیدی و زنده‌مانی پروبیوتیک‌های کمکی اضافه شده به کفیر انجام شده است.

روش کار: ابتدا، شیر گاو در دمای ۹۰ درجه سانتی‌گراد جوشیده شد، سپس به آن استارتر اضافه و نهایتا به دو گروه تقسیم‌بندی گردید. شیرها به‌صورت جداگانه به ترتیب با لاکتوباسیلوس اسیدوفیلوس و لاکتوباسیلوس پاراکازیی تلقیح شدند و در دمای ۳۰ درجه سانتی‌گراد به مدت ۶ ساعت گرمخانه‌گذاری شدند. پس از زمان طی‌شده، در داخل یخچال به مدت ۱۴ روز نگهداری و نمونه‌برداری برای تعیین مقادیر اسیدهای آلی از جمله اسید لاکتیک و اسید استیک، اسیدیته، اسید تیتر شده، و زنده‌مانی پروبیوتیک‌های تلقیح‌شده در روزهای ۱، ۷ و ۱۴ صورت پذیرفت.

نتایج: مقدار اسیدیته (pH) در کفیر تلقیح شده با لاکتوباسیلوس اسیدوفیلوس و لاکتوباسیلوس پاراکازئی به ترتیب ۴/۳۴ و ۴/۳۶ در روز اول تحقیق و ۴/۲۷ ۴/۳۱ در روز ۱۴ نگهداری مشخص گردید. مقدار اسیدیته در کفیر اول در روز اول برابر با ۰/۸۰ گرم در ۱۰۰ گرم گردید که از مقدار آن در کفیر دوم (۰/۷۲ گرم در ۱۰۰ گرم) بیشتر گردید. مقدار اسید لاکتیک در کفیر تلقیح‌شده با لاکتوباسیلوس اسیدوفیلوس و نمونه‌های تلقیح‌شده با لاکتوباسیلوس پاراکازئی به ترتیب در محدوده ۱/۵۷ تا ۲/۴۰ و ۲/۱۷ تا ۲/۴۲ گرم در ۱۰۰ گرم اندازه‌گیری گردید. در کفیرهای تکمیل‌شده با لاکتوباسیلوس اسیدوفیلوس و لاکتوباسیلوس پاراکازئی، به ترتیب اسید استیک در طول ۱۴ روز پایدار بود (از ۰/۱۱ به ۰/۱۳ گرم در ۱۰۰ میلی‌لیتر) اما در کفیر بعدی افزایش یافت (از ۰/۱۱ به ۰/۲۳ گرم در ۱۰۰ میلی‌لیتر). مقدار زنده‌مانی در هر دو کفیرهای مورد تحقیق از مقدار log CFU/g ۷ بیشتر ثبت گردید.

نتیجه‌گیری نهایی: اضافه کردن لاکتوباسیلوس اسیدوفیلوس و لاکتوباسیلوس پاراکازئی باعث تعدیل میزان اسید و افزایش زمان زنده‌مانی پروبیوتیک‌های تلقیح شده در حد استاندارد، حداقل تا دو هفته زمان نگهداری در یخچال گردید.

واژه‌های کلیدی: زنده‌مانی، شاخص‌های اسیدی، کفیر، لاکتوباسیلوس اسیدوفیلوس، لاکتوباسیلوس پاراکازئی

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