The effect of *Lactobacillus acidophilus* on the changes in the acidity of probiotic milk during storage

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Data of the article

First received : 24 November 2021 | Last revision received : 06 Jun 2022
Accepted : 22 August 2022| Published online :20 September 2022
DOI : 10.17170/kobra-202204136019

Keywords

Lactobacillus acidophilus; Probiotic products; *Bifidobacteria*; Fermentation process; Health promotion; Dairy products.

Probiotic products are functional products for human health. To evaluate the acidity changes of milk containing probiotic bacterium *Lactobacillus acidophilus*, three containers containing 3 litre of sterilized low-fat milk with 1.5% fat prepared by Mihan Company were considered in three groups. The results showed changes in milk containing *Lactobacillus acidophilus* bacteria because the acidity index was recorded for two hours to reach the acidity of 42 °C in milk in the incubator at 38 °C and during storage in the refrigerator. An increase in the acidity of probiotic milk compared to the control sample was observed on the first day after production. The acidification rate increased during the fermentation under the influence of L. *acidophilus* bacterium also the final acidity after the end of the fermentation period in the probiotic milk was higher than in the control sample.

1. Introduction

Functional food is a food that contains at least one specific and proven health property in addition to the basic nutritional properties which is functionally recommended by the manufacturer or by nutritionists and consumed by the consumer (Farahmand et al., 2021). One of the important things related to the selection and production of functional foods is their safety. Nowadays, considering the practical proof of the unfavourable results due to imbalance in human societies, the tendency to produce and consume a variety of functional foods has increased significantly (Bull et al., 2013). Nowadays, food consumers pay special attention to the items such as pleasant flavour, low calories and fat, and the beneficial effect of food on health (Widyastuti et al., 2021). Therefore, the food industry tries to manufacture products with better flavour and properties, among which a dairy product processed with probiotics is of great importance in promoting health (Zhang et al., 2019).

Probiotics are one of the most emerging and popular functional products which are of particular importance in this regard (Ye et al., 2017). Probiotic products with valuable nutritional and therapeutic properties are among the most controversial topics in the field of industry, nutrition, and medicine (Raygan et al., 2018). According to FAO/WHO definition, probiotics are "living microorganisms that have beneficial effects on maintaining the health of their host if consumed in sufficient quantities" (Depree & Savage, 2001). Probiotics are used not only as dietary and pharmaceutical supplements but also in dairy products, juices, chocolates, and even meat products (Parker et al., 2018). Two major groups of probiotic microorganisms are *Lactobacillus* pp and Bifidobacterium (Toropov et al., 2020). Probiotics proliferate in the gastrointestinal tract and compete with the production of antagonistic metabolites with saprophytic micro flora. This ability can be observed in lactic acid bacteria such as Lactobacilli and *Bifidobacteria* (Motey et al., 2021). Epidemiological studies showed that regular use of probiotics may reduce the risk of cancer, improve heart health, improve the immune system, improve gastrointestinal health, anti-inflammatory, antimicrobial and antiviral effects, lower blood pressure, prevent weight gain, prevention of urinary tract infections, and treatment of allergic diseases such as eczema (Parker et al., 2018; Barros et al., 2020). Continuity and amount of consumption of probiotics are effective in playing their beneficial therapeutic roles. A daily intake of 106 to 108 CFU/g live bacteria was considered an acceptable minimum. Therefore, daily consumption of 100 g of probiotic product with 1×106 to 5×108 CFU of live bacteria per gram of product can provide the optimal limit (Cruz et al., 2009).

Probiotic bacteria (*Bifidobacteria* and *Lactobacillus acidophilus*) are substantially decreased during refrigerated storage, particularly in acidic conditions, according to extensive studies. As a result, it is critical to keep the quantity of these microorganisms constant throughout storage (Nyanzi et al., 2021). Research has looked at the probiotic properties of this group of bacteria, and strains with specified probiotic characteristics have been introduced (Saarela, 2019).

Besides, consumer-perceived sensory characteristics are important from a commercial and market point of view (Wilkinson, 2018). Fermented dairy products always carry a significant population of bacteria of the starter cultures that are common in terms of the type or even species of well-known and commercially known probiotics and for which many health properties have been mentioned (Sarkar, 2018). Milk is one of the most often eaten items in families' food baskets, and it provides important products to the customer in addition to providing a favourable environment for the development and survival of probiotics due to its unique nutritional characteristics (Vinderola et al., 2019). Probiotic milk is produced in a variety of ways, such as by adding bacteria to fresh milk without fermentation (such as sweet milk) or fermenting milk by probiotic bacteria (such as Yakult) (Patro et al., 2016). Adding probiotics to fermented dairy products leads to milk protection by producing lactic acid and antimicrobial compounds and increasing the nutritional value of food by producing compounds such as free amino acids and vitamins (Turkmen et al., 2019). Of its technological and health characteristics, milk is one of the most popular products in the country. The inclusion of probiotic bacteria in such a high-consumption product can help promote community health (Sotoudegan et al., 2019). Among the most common probiotic dairy products in the global market, we can mention yogurt, buttermilk, kefir, probiotic cheese, and other types of fermented milk (Sah et al., 2016). The increasing prevalence of probiotic products and their widespread advertising has led to the fact that in addition to the common control of these products by regulatory agencies, a part of the scientific study is limited to the quality of biomarkers of probiotic microorganisms in these products (Rezazadeh et al., 2019). This research tried to study the possibility of survival and growth of *Lactobacillus acidophilus* in milk.

2. Materials and Methods

The current research used a fully randomized design. Three containers holding 3 litre of sterilized low-fat milk with 1.5% fat produced by Mihan Company were considered three groups to create milk containing the probiotic bacteria *Lactobacillus acidophilus*-5. The probiotic bacterium of *Lactobacillus acidophilus* (LA-5®) (Chr. Hansen, Hørsholm, Denmark) was in freeze-dried (Direct Vat Set) form at the time of purchase. According to the manufacturer's recommendation, it was kept in freezing conditions (-18°C) until consumption.

2.1. Microorganisms

Single-strain probiotic culture of *Lactobacillus acidophilus* (La-5) was purchased in freeze-dried form and DVS type from the Danish company of Kristin Hansen. The reason to choose *Lactobacillus acidophilus* La-5 as a probiotic strain was between two common probiotic bacteria species (*Lactobacillus acidophilus* and Bifidobacterium) in probiotic dairy products, Bifidobacterium Lactis Bb-12 does not survive well at the pH value of about 5.5. *Lactobacillus acidophilus* survives well at this pH (Taheri et al., 2008). Incompatibility of Bb-12 at 45 °C of aerobic medium (42-45°C) and acidic conditions and at the same time, the optimal growth of *Lactobacillus acidophilus* La-5 in these conditions was shown (Taheri et al., 2008).

ISSN-Internet 2197-411x OLCL 862804632

According to the incubation conditions and the final pH of the product, *Lactobacillus acidophilus* La-5 was selected as the probiotic bacterium used in this study.

Tested starter culture and preparation of culture rennet (1% Tween 80) was added to the one-way probiotic culture of *Lactobacillus acidophilus* La-5 by surface cultured in an MRS agar medium. This culture was then sterilized for 15 minutes at 121°C. The 1 g of probiotic strain was then transferred to 100ml of Lactose Broth and incubated for 48 hours at 42°C. Next, at 40°C, 5ml of the produced medium was inoculated into 1 litre of 1.5% fat sterilized milk, homogenized, and incubated at 37°C until about 80°D acidities. The cells were centrifuged at 2360g for 8min and washed twice in NaCl solution (0.85%). The bacterial mass was dissolved in a normal saline solution to contain approximately 109-1010 CFU/ml of viable bacteria (Pedroso et al., 2012; Florence et al., 2012). Finally, the acidity test was performed; also the last sample of fermented milk was used as a culture starter in various experiments.

2.2. Determination of the optimum growth temperature

To determine the optimum growth temperature, first, one litre of low-fat sterile milk was placed in an Erlenmeyer for 20 minutes at the 80 °C temperature of incubator and then the temperature was reduced to about 40 °C using cold water, then 5 ml of the initial culture medium was inoculated and homogenized under sterile conditions. The resulting milk was evenly distributed in the presence of flame in four 250 ml Erlenmeyer and incubated at 38 and 42 °C, respectively, and the acidity was evaluated at 0, 2, 4, 6, and 8 hours.

2.3. Sampling

To evaluate the survival of *Lactobacillus acidophilus* probiotic bacteria during storage and its effect on the characteristics of probiotic milk and comparison with the control sample, sampling was performed on the control and probiotic samples at 38 and 42°C.

2.4. Evaluation of titratable acidity

In the presence of phenolphthalein reagent, 0.1 N soda, and according to the technique described in Iranian National Standard No. 2852, the titration method was employed. In the presence of phenolphthalein reagent, 10 ml of the sample was mixed with 10 ml of distilled water and titrated with 0.1 N soda. The value of this index was calculated based on the Dornic degree (Mortazavian et al., 2010). The acidity of the milk was controlled and recorded every 2 hours, and the total acidity was calculated from Equation (1). One millilitre of 0.1 N soda is equivalent to 0.009008 g of lactic acid.

Equation (1):

$$\%titratable\ acidity = \frac{ml \times N \times 90 \times 100}{V \times 1000}$$

Where:
ml= ml 0.1 NaOH used
N= Normality of 0.1N NaOH
V= ml sample solution used

2.5. Statistical analysis

In this study, the tests were performed in three periods, and each period with three replications. The comparison among the results was performed by ANOVA statistical tests with a 95% confidence level by SPSS 11.5 software. Excel software was used to draw graphs and figures.

3. Results and Discussion

Acidity rate during storage: The changes in milk containing *Lactobacillus acidophilus* bacteria in terms of acidity index in two hours to achieve acidity of 42 degrees Dornic (milk) in the incubator at 38 °C and during storage in the refrigerator were recorded. Acidity values in terms of degree Dornic were almost constant in the early hours, which were assessed because the activities of the probiotics are not started. Figures (1 to 4) compare the acidification process of probiotic milk with the control sample. The first point in this diagram was the higher acidity of probiotic milk than the control sample on the initial day after production.

In the first milk sample at 38 ° C, the process of increasing the acidity was slow so that in the first 6 hours the acidity was from 19 to 30 Dornic degrees, and then in the next 2 hours with faster growth reached 41 Dornic degrees (Figure1). Also, in the second sample at 42 ° C, the process of increasing the acidity is done more rapidly so that in 4 hours, the acidity has reached 25 to 41 Dornic degrees (Figure2). Then, the third sample was examined with twice the amount of the L. *acidophilus* and a temperature of 42 ° C, and the result was somewhat similar to the second sample, so that in four hours, the acidity increased by 19 degrees, reaching 43 Dornic degrees (Figure3). Finally, for a more accurate comparison, the results of all three samples were placed on a graph (Figure4). Therefore, it can be claimed that the presence of L. *acidophilus* increased the acidification rate during fermentation, and the final acidity after the end of the fermentation period in probiotic milk was higher than in the control sample. It was found that the acidification of the probiotic product was more than the control, during production and incubation. Furthermore, to the rate and speed of acidification during production, which is technologically important, acidification occurs during product storage, which is the high rate and speed of acidification at this stage of the maintenance problems of these products (Parker et al., 2018). In addition to post-acidification, the two-phase phenomenon is also one of the problems in maintaining this type of fermented milk. Research on similar products indicated that sedimentation occurs up to day 14 and then stops. This problem was more common in the samples with higher acidification during storage (Oliveira et al., 2002).

Considering the topic of probiotic products is one of the new scientific subjects in Iran and knowing the production and maintenance capacities of such products will open more windows to its industrialization and improve the health level of society. The present study was designed and conducted following recent years' research to evaluate a type of dairy health product according to Iranian taste. This study's results showed that the probiotic microorganism *Lactobacillus acidophilus* La-5 could be used in milk production. In addition to growth, the acidification of the starter bacteria is important during production and maintenance (Farahmand et al., 2021).

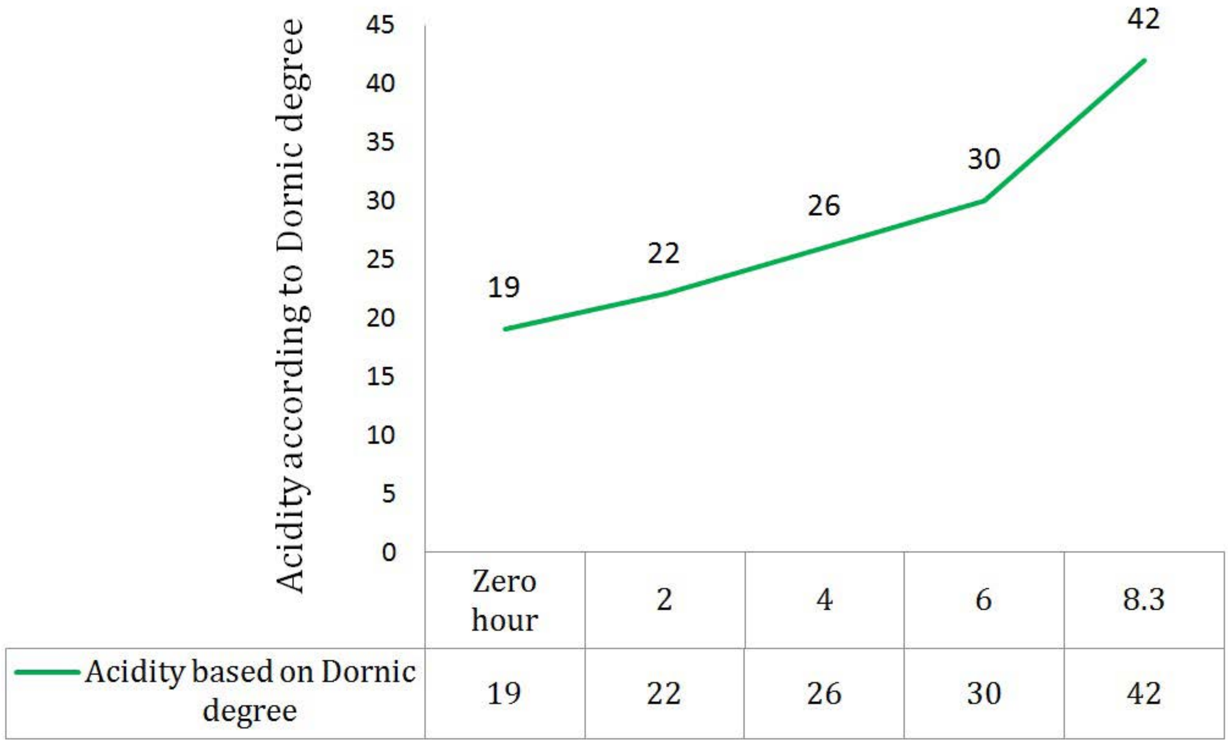


Figure1. The investigation of acidity in the production of *acidophilus* milk at low temperatures (at 38 °C)

ISSN-Internet 2197-411x OLCL 862804632

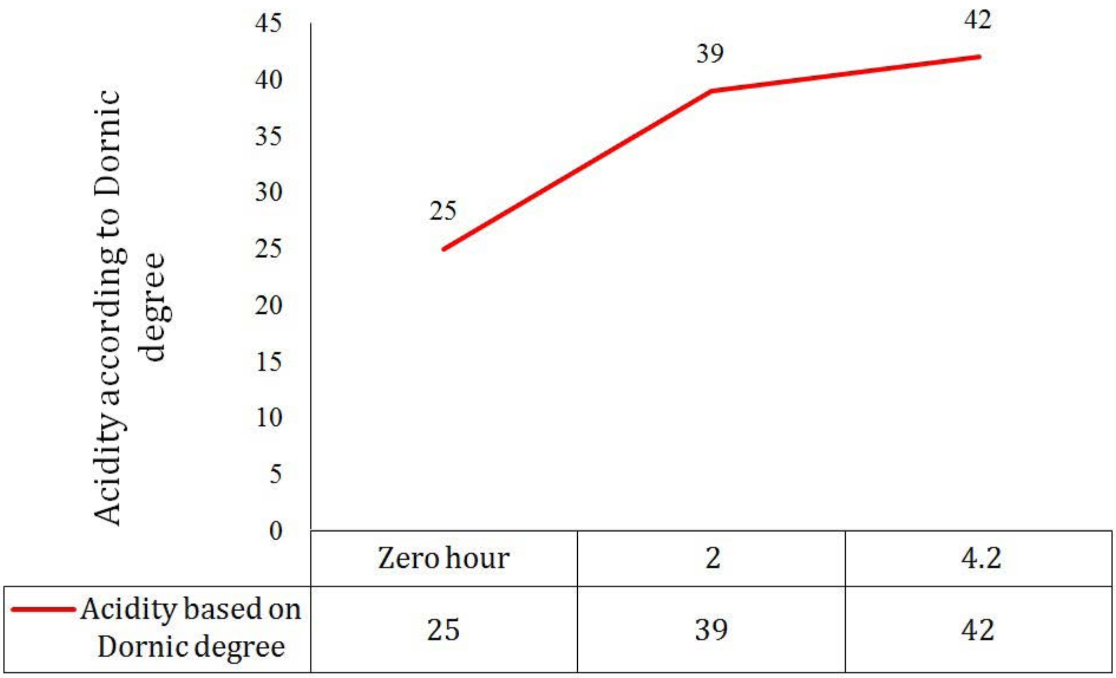


Figure2. The investigation of acidity in the production of *acidophilus* milk (at 42°C)

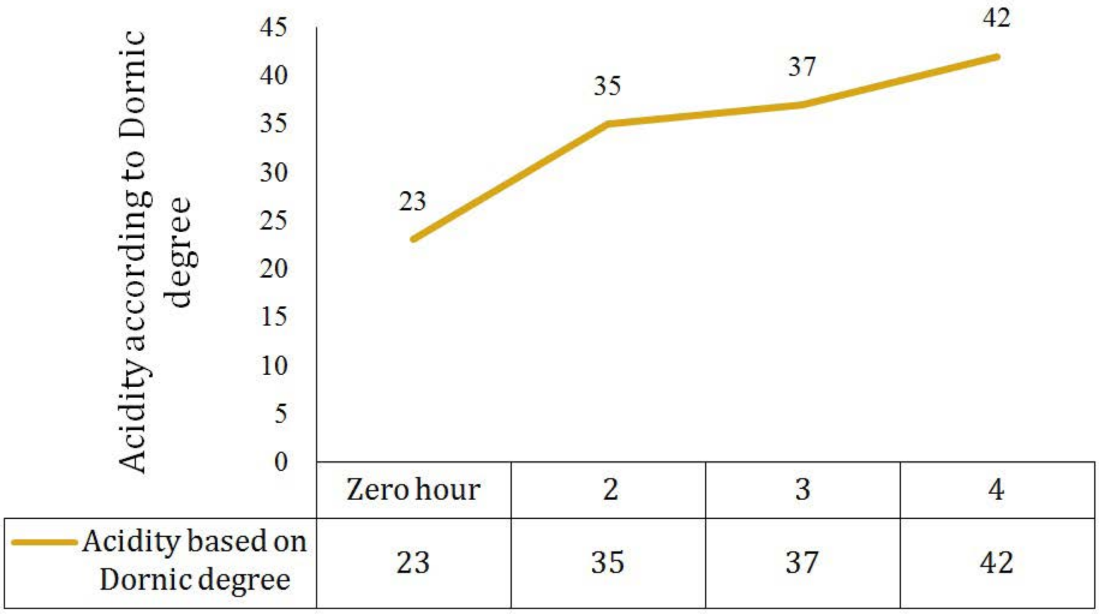
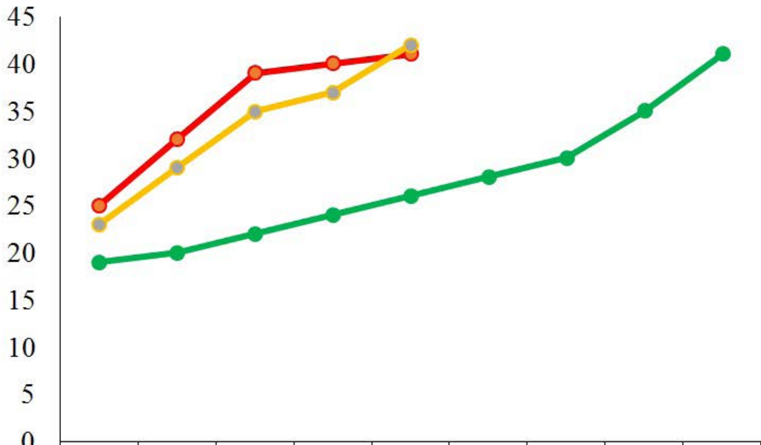


Figure3. The investigation of acidity in the production of the third high-dose *acidophilus* milk (Twice the dose and temperature of 42°C)



	Zero hour	1	2	3	4	5	6	7	8
The first milk (temp 38°C)	19	20	22	24	26	28	30	35	41
The second milk (temp 42°C)	25	32	39	40	41				
The third milk (double dose & temp of 42°C)	23	29	35	37	42				

Figure4. Comparative study of acidity development in the production of all three probiotic milk according to Dornic degree

4. Conclusions

The basis of probiotic products is their medicinal properties (bioavailability). The sensory properties of these products also play a significant role. In other words, the advantage of consuming probiotics via food and not in the form of medicine is their sensory properties. Among probiotic products, fermented products are universally accepted due to their unique sensory properties. Generally, this study's results showed that the acidity rate in probiotic milk during the shelf life is higher compared to the control sample.

Acknowledgment

The authors gratefully appreciate the cooperation of the Veterinary Faculty of Kazerun in carrying out the above research.

Conflict of interest

The authors declare no conflict of interest. Besides, the funders had no role in the design of the study; in the collection, analysis, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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ISSN-Internet 2197-411x OLCL 862804632