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## Summary

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# Evaluation of manufacturing conditions of a probiotic product using culture of *Lactobacillus acidophilus*

*Bewertung der Herstellungsbedingungen eines probiotischen Produkts unter Verwendung einer Kultur von *Lactobacillus acidophilus**

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Probiotic bacteria are among the dietary supplements with beneficial effects on human health. To produce a probiotic product from milk using auxiliary culture of *Lactobacillus acidophilus*, the possibility of using an initial culture step was first studied. For this purpose, the first 0.33 g of Lyophilized bacteria was added to each litre of low-fat sterilized milk of Mihan brand (in total 1 gram to 3 litres of milk) and was incubated at 42 °C until reaching the acidity of 42° Dornic and 15 grams of this product with 30 grams of low-fat yoghurt of Pegah brand was added to 3 litres of low-fat sterilized milk of Mihan brand to produce acidophilus yoghurt and was incubated at 42 °C to reach the acidity of 96° Dornic. Two other types of acidophilus yoghurt, one with a temperature of 38 °C and the other with a temperature of 42 °C and a double bacterial dose were prepared and their flavour was compared by 150 people with control yoghurt (low-fat Pegah brand). In general, the shelf life of acidophilus yoghurt with twice the bacterial dose was the lowest but the consistency was better and its flavour was sourer, the other two acidophilus yoghurts were evaluated with longer shelf life and sweeter than the control yoghurt. Also, with increasing the shelf life, the acidity indicated a significant increase.

**Keywords:** Probiotic yoghurt, titration acidity, *Lactobacillus acidophilus*, *Bifidobacterium*, fermentation process, health promotion, dairy products

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## Introduction

Dairy products are products that are commonly manufactured in probiotic form; so various forms of these probiotic products have an important part of the market of such products (Kamel et al., 2021): The results of studies have shown that dairy products are appropriate carriers in terms of maintaining the number of probiotic microorganisms. Refrigerated and cold storage conditions applied to maintain the shelf life of dairy products help maintain the number of live bacteria (Hamdy et al., 2021): In addition, the consumption of dairy products, especially fermented types such as fermented milk and yoghurt, due to the history of consumption and their role in health has always been faced with a positive attitude from society and the addition of beneficial probiotic strains to these products is well justifiable (ACU, M et al., 2021).

The study of the physicochemical and sensory properties of food is of great importance and it is impossible to produce a desirable high-quality food product without considering these properties (Vasconcelos et al., 2019): Examining the trend of these changes in a food product also allows us to estimate the shelf life of the product (Ranadheera et al., 2018): In recent years, the desire to use beneficial bacteria called probiotics as an adjunct diet in the food industry has been growing. Probiotic bacteria used in common products today are mostly different species of *Lactobacillus* and *Bifidobacterium* (Keshavarzi et al., 2021): Recently, various probiotic products such as yoghurt, ice cream, and probiotic cheese have been produced and many valuable types of research have been done in this regard (Pandey et al., 2015): The word probiotic in Greek means life-giving and by definition is a living microorganism that has beneficial effects on consumer health if consumed in sufficient quantities (Hill et al., 2014): Probiotics are live microbial supplements that are added to the diet and have beneficial effects on the host by improving the balance of intestinal microbial flora (Markowiak et al., 2017): The effect of probiotics on the microbial flora of the gastrointestinal tract and their therapeutic role was related to the daily intake of these bacteria and, the most commonly defined range for the presence of live probiotic bacteria is  $10^6$ – $10^7$  CFU per gram (Maleki et al., 2021): The main mechanism by which probiotics promote host health is unclear. Certain strains of probiotics appear to have a positive effect on the host's health and this effect cannot be generalized to other strains (Afzaal et al., 2019): *Lactobacillus* and *Bifidobacterium* are among the common probiotics that inhibit the growth of pathogenic bacteria by producing lactic acid, acetic acid, and propionic acid and lowering the intestinal pH, and maintaining a balance in the number of bacteria in the normal intestinal flora (Ghaleh Mosiyani et al., 2017; Ghasempour et al., 2020): *Lactobacillus* is one of the probiotic bacteria. *Lactobacillus* is bacilli and coccobacilli-shaped bacteria (Champagne et al., 2018): This gram-positive spore-free, catalyse, oxidise and indole-negative bacteria are not able to reduce nitrate and belong to the *Lactobacillaceae* family and, approximately 56 species of this bacterium have been identified (Ayar et al., 2018): *Lactobacillus* has numerous benefits including decreasing blood cholesterol and blood fibrinogen, an antipathogenic effect due to the production of bacteriocin called plantaricin, reducing bloating in irritable bowel syndrome in humans (Kalicka et al., 2019; Guimarães et al., 2020).

Properties such as acidity, free fatty acid content, and sensory and nutritional properties are influenced by the

chemical composition of milk, process conditions, the addition of flavourings, and the activity of starter bacteria during milk fermentation (Nyanzi et al., 2021): In order to obtain probiotic yoghurt, in addition to the yoghurt starter bacteria, probiotic bacteria must be present during the fermentation process and in the final product. Yoghurt starter culture can enhance the growth of probiotics by producing the materials needed for their growth (Prasanna Pradeep & Charalampopoulos, 2019): The characteristics of probiotic bacteria and the culture medium they require vary. Therefore, by changing the fermentation conditions of milk or optimizing this process, we can try to increase the number of probiotic bacteria or increase their lifespan in yoghurt (Yao et al., 2020).

## Materials and methods

The present study was conducted in a completely randomized design. In order to produce milk containing the probiotic bacterium of *Lactobacillus acidophilus* (*Lactobacillus acidophilus*-5), three containers containing 3 litres of sterilized low-fat milk of 1.5% fat prepared by Mihan Company were considered as three groups. The probiotic bacterium of *Lactobacillus acidophilus* (LA-5®) (Chr. Hansen, Hørsholm, Denmark) was freeze-dried (Direct Vat Set) at the time of purchase. According to the manufacturer's recommendation, it was kept in freezing conditions ( $-18$  °C) until consumption.

### Isolation of *Lactobacillus* from yoghurt using specific culture medium

The culture medium for probiotic bacteria (de Man, Rogosa, and Sharpe (MRS)) is a specific environment for the growth and isolation of the desired bacteria and is able to meet their complex nutritional needs. This medium was prepared by the German company Merck and was prepared in solid form (MRS agar) according to the instructions of this company. In the next step, 2 grams of each sample was poured into a sterile forge and reached 100 CC (w/volume) with MRS broth medium and incubated for 24 hours at 37 °C in a CO<sub>2</sub> incubator.

### Microorganisms

One-way probiotic culture of *Lactobacillus acidophilus* (La-5) was purchased in freeze-dried and DVS type from the Danish Kristin Hansen Company. The reason for selecting *Lactobacillus acidophilus* La-5 as a probiotic strain was that between two common species of probiotic bacteria (*Lactobacillus acidophilus* and *Bifidobacteria*) in probiotic dairy products, *Bifidobacterium lactis* Bb-12 does not have good survival of *Lactobacillus* at a pH of about 5.5, but *Lactobacillus acidophilus* survives well at this pH (Taheri et al., 2008): Incompatibility of Bb-12 at the temperature of 45 °C in aerobic medium (42–45 °C) and acidic conditions and at the same time, the optimal growth of *Lactobacillus acidophilus* La-5 in these conditions has been shown (Taheri et al., 2008): 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 containing *Lactobacillus acidophilus* La-5

In this study, commercial bacterial culture including the one-way probiotic culture of *Lactobacillus acidophilus*

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La-5 by surface culture in MRS agar medium (Merck Germany) was used. In a way that, after preparing the MRS broth culture medium according to the manufacturer's instructions, 1% Tween 80 was added to it, then it was sterilized for 15 minutes at 121 °C. According to the recommendation of the Danish Cher-Hansen Company, under sterile conditions, 1 g of probiotic strain was transferred to Erlenmeyer containing 100 ml of broth lactose and incubated for 48 hours at 42 °C. Then 5 ml was taken from the prepared medium and inoculated into 1 litre of 1.5% sterilized milk at 40 °C and the resulting milk sample was incubated at 37 °C after homogenization until the milk acidity reached the acidity of about 80D°in. The cells were also centrifuged at 2360 g for 8 minutes and washed twice in NaCl solution (0.85%): The bacterial mass was redissolved in a normal saline solution to contain approximately 10<sup>9</sup>–10<sup>10</sup> CFU/ml of viable bacteria (de Lara Pedroso et al., 2012; Florence et al., 2012): Finally, an acidity test was performed every 2 hours at 38 and 42 °C. This operation was repeated 3 times to find favourable conditions for the probiotic strain to grow and the last sample of fermented milk was used as the primary culture medium in various experiments.

30 grams of low-fat Pegah yoghurt along with 15 grams of acidophilus milk from the first group was added to the first 3-litre group (low-fat sterilized milk) and placed in a 42 °C incubator and an acidity test was performed every 2 hours. 30 grams of low-fat Pegah yoghurt along with 15 grams of acidophilus milk from the first group was added to the second 3-litre group and placed in a 38 °C incubator and acidity tests were performed as in the above method. 45 g of low-fat Pegah yoghurt with 30 g of acidophilus milk from the first group was added to the third 3-litre group and placed in an incubator at 42 °C. Acidity tests were performed as in the previous method. The criterion for removing from the incubator was to reach an acidity of about 96°Dornic. In this method, the incubation duration and the acidity improvement of the three groups of yoghurt produced were compared.

#### Determination of the optimum growth temperature

To determine the optimum growth temperature, first put one litre of low-fat sterile milk was placed in an Erlenmeyer oven for 20 minutes at 80 °C and then the temperature was reduced to about 40 °C using cold water, then 5 ml of the initial culture rennet was inoculated and homogenized under sterile conditions. The resulting milk was evenly distributed in the presence of flame in four Erlenmeyer 250 ml and incubated at 38 and 42 °C, respectively, and the acidity was assessed at 0, 2, 4, 6, 8, and 9 hours.

#### Sampling

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

#### Evaluation of titratable acidity

The titration method was used in the presence of phenolphthalein reagent with 0.1 N soda and according to the method mentioned in Iranian National Standard No. 2852.10 ml of the sample was mixed with 10 ml of distilled water and titrated with 0.1 N normal in the presence of phenolphthalein reagent. The value of this index was determined by 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): It should be noted that one millilitre of 0.1 normal soda is equivalent to 0.009008 g of lactic acid:

$$\% \text{titratable acidity} = \frac{\text{ml} \times N \times 90 \times 100}{V \times 1000} \quad (1)$$

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

#### Sensory evaluation

After producing all 3 acidophilus yoghurts, the flavour of all three products with low-fat Pegah yoghurt (as a control) was tested by 150 people. This evaluation was performed by tables provided to individuals, in whom the parameters of smell, colour, consistency, flavour, fat status, and sourness and sweetness of the product were examined and compared with control yoghurt. Also, from each group of yoghurt produced, some were placed in the refrigerator to compare their shelf life.

#### Statistical analysis

In this study, the tests were performed in 3 periods, and each period with three replications. Comparison between the results was performed by ANOVA statistical tests with a 95% confidence level by SPSS 11.5 software. Excel software was used to plot graphs and figures.

## Results and discussion

The results of the present study indicated that the acidity of all three types of yoghurt increased significantly from the beginning to the end of the study (Fig. 1 to 4): The factor of time also had a significant effect on acidity changes. The third treatment, the third high-dose acidophilus yoghurt (Double dose and 42 °C), has the shortest growth time (6.30 hours) among all the treatments and shows that the medium has quickly become unfavourable for starter bacteria.

In the first yoghurt sample at 38 °C, the process of increasing the acidity was dramatic so that in the first 7 hours the acidity rose from 23 to 85 Dornic degrees, and then in the next 2 hours with slow growth reached 90 Dornic degrees (Fig. 1): In the second sample at 42 °C, we witnessed a slight increase in acidity in the first 2 hours, but during the next 5 hours, a significant increase of about 60 degrees was recorded. Then, in the last 2 hours, the acidity reached 90 degrees Dornic with a gentle slope (Fig. 2): The third yoghurt was tested with a double dose at 42 degrees Celsius, which reached 92 degrees Dornic acid within 6.5 hours with an upward slope, minor fluctuations, and an increase of 66 degrees. In this sample, compared to the other two yoghurts, the speed of the process increased by about 28 % (Fig. 3): Also, for a more accurate comparison, the results of all three samples were placed on a graph (Fig. 4): According to this graph, the third sample showed the highest growth of acidity in the shortest time, while the first sample had a more uniform growth of acidity. Then a questionnaire based on 5 factors of aroma, colour, consistency, taste, and fat was prepared and 3 samples produced were compared with control yoghurt by 150 students the second sample had the best score in taste (Fig. 5).

The addition of probiotic bacteria may interfere with yoghurt bacteria and alter the physicochemical and sensory properties of the product. Therefore, probiotic yoghurt may

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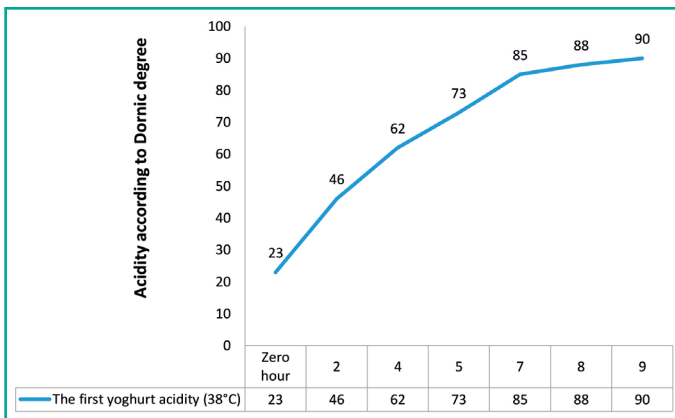


FIGURE 1: Investigation of acidity in the production of the first acidophilus yoghurt at low temperature (38 °C).

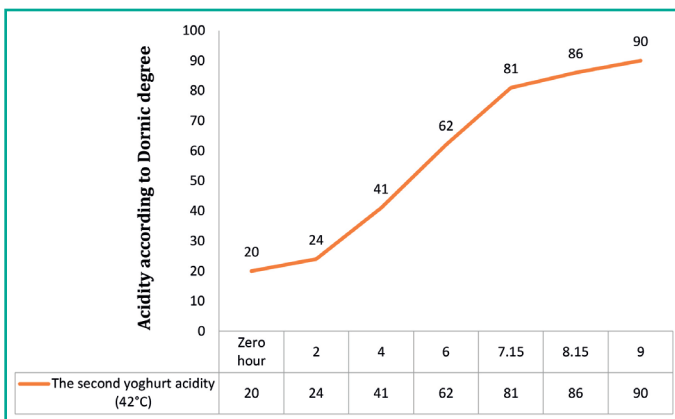


FIGURE 2: Investigation of acidity throughout the manufacture of the second acidophilus yoghurt (42 °C).

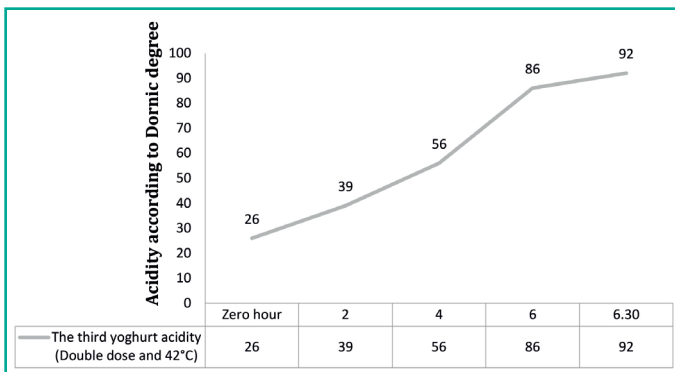


FIGURE 3: Investigation of acidity in the production of third high-dose acidophilus yoghurt (Twice dose and 42 °C).

be different from regular yoghurt in terms of these properties (Homayouni Rad et al., 2019): According to the obtained results, among acidophilus yoghurts, the second yoghurt achieved the highest score. Meanwhile, the third yoghurt had the highest consistency, the sourest flavour, and the shortest shelf life among probiotic yoghurts, but its manufacture duration was shorter and it ripened faster (Fig. 6): Therefore, the second yoghurt is the best alternative to regular yoghurt, because it will be more beneficial for the health of the digestive system due to the presence of probiotics.

Today, many studies have been done on probiotic bacteria and their beneficial effects on human health. Among probiotic products, fermented products, especially yoghurt, are globally accepted (Nikbakhet et al., 2014): Normally, the storage capacity of yoghurt is 21 days. Given

the high acidity of yoghurt, moulds are among the most important microbial agents that can be proliferated in this product and cause its spoilage. In this regard, the standard limit of 100 CFU/gr has been set (Ghaffari et al., 2018): During the storage period, the acidity rate increased due to the post-acidification resulting from the activity of the starters. In addition to the acidification rate throughout manufacturing, which is technologically important, acidification occurs during product storage; the high acidification rate

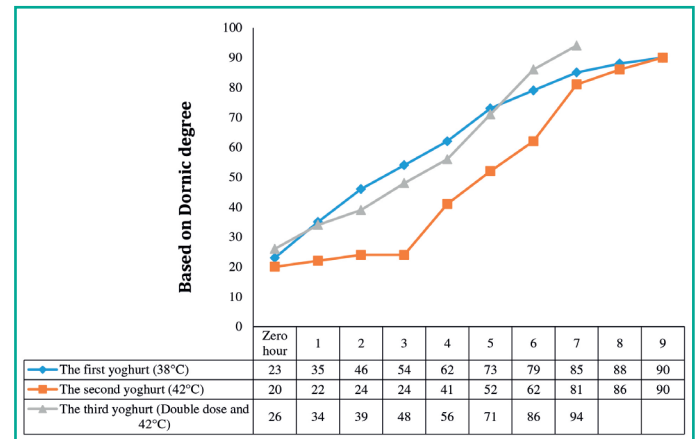


FIGURE 4: Comparative assessment of acidity growth in the production of all three probiotic yoghurts.

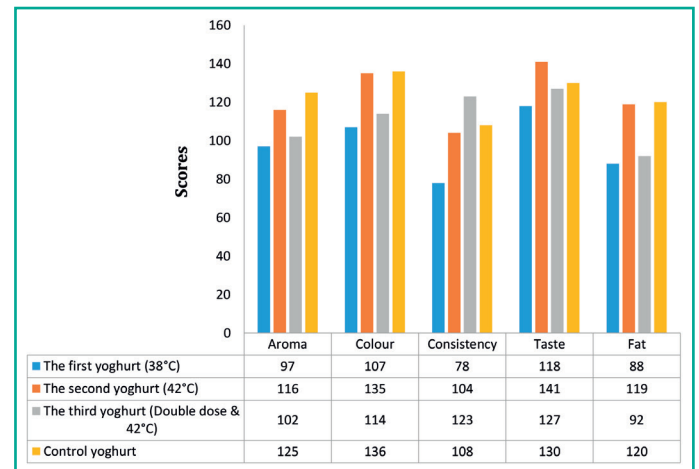


FIGURE 5: Comparative results of flavour test questionnaire by 150 people.

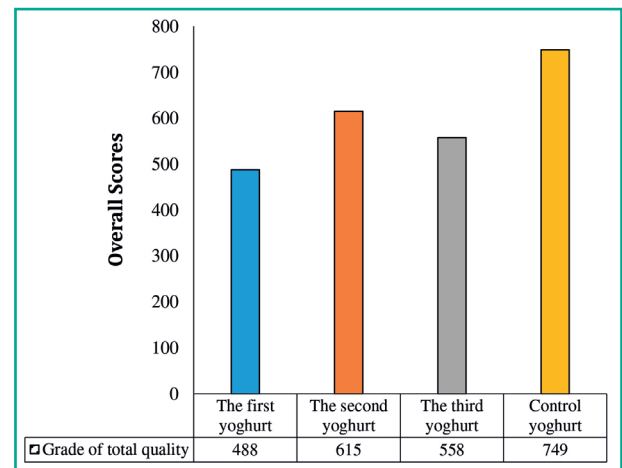


FIGURE 6: Comparison of all 3 acidophilus yoghurts in different parameters.

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at this stage is one of the problems for maintaining these products (Mani Lopez et al., 2014): Also, the increase in acidity during the storage period of the product is due to post-acidification, because of the activity of the Beta-galactosidase enzyme at refrigerated temperatures. The results of the present study are similar to the study of Ozcan and Horne, (2015) who reported that yoghurt acidity increases during storage. The researchers studied the acidity variation trend in yoghurt produced with milk heated at various temperatures. Also, Ali Mohammadi and Koohdar (2019) in the study of the effect of storage time on the acidity of yoghurt stated that in all samples, the number of probiotic bacteria and the acidity rate decreased and increased significantly, respectively.

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.

## Conclusions

In general, probiotic yoghurts have a longer shelf life than normal yoghurts and become sour later. Also, yoghurt No. 2 had a better flavour than the control yoghurt, but in other parameters was somewhat similar. Yoghurt No. 1 was weaker than the control yoghurt in the measured parameters. Yoghurt No. 3 scored higher in terms of consistency, almost equal in terms of flavour, and in other parameters scored less than the control yoghurt. The interesting point we encountered during the yoghurt acidity test was that whenever we sampled yoghurt (liquid between the clots that were forming yoghurt), its Dornic degrees differed by about 9 degrees from the Dornic degrees of the semi-solid part of the yoghurt (It was lower by about 9° Dornic).

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## 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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