



Determination of Probiotic Viability in Yoghurts Produced with Acid Adapted *Lactobacillus acidophilus* ATCC 4356 and *Bifidobacterium bifidum* ATCC 11863 During Refrigerated Storage

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ABSTRACT

Microorganisms have various stress response systems to maintain their viability when exposed to different stress conditions. In this study, *Lactobacillus acidophilus* ATCC 4356 and *Bifidobacterium bifidum* ATCC 11863 strains, used in probiotic yoghurt production, were subjected to acid (lactic and hydrochloric acid) stress to induce acid tolerance response (ATR). Yoghurt samples produced with both acid-adapted and non-adapted strains were stored at +4°C for 21 days. During the storage period, the pH and titratable acidity values of the yoghurts were measured, and the viability levels of the probiotic strains in the yoghurts were determined. In all yoghurt groups, a decrease in pH values and an increase in titratable acidity were observed during storage. The highest viability levels of the probiotic strains were detected on the first day of storage. Lactic acid-adapted *Lb. acidophilus* ATCC 4356 and *B. bifidum* ATCC 11863 in yoghurt showed growth at a level of 8.08 ± 0.12 and 8.08 ± 0.09 log₁₀ Cf_u/g at the first day of storage, respectively. Additionally, hydrochloric acid-adapted *Lb. acidophilus* ATCC 4356 and *B. bifidum* ATCC 11863 in yoghurt exhibited growth at levels of 7.90 ± 0.08 and 5.99 ± 0.03 log₁₀ Cf_u/g, respectively. The viability of acid-adapted *Lb. acidophilus* ATCC 4356 and *B. bifidum* ATCC 11863 showed a decrease similar way to that of the control group (non-acid adapted) during the storage period.

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Introduction

Starting from the late 20th century and encompassing the first quarter of the 21st century, there has been an increase in the quality of life for individuals due to various factors such as technological advancements that have impacted their lives and the rise in healthcare expenses. Therefore, the need for individuals to consume healthy food has arisen, and in this context, the demand for foods with beneficial properties such as “functional foods” has increased (Evren et al., 2017).

The term “functional food” was initially defined as “Foods for Specified Health Uses (FOSHU)” in Japan in the early 1980s (Granato et al., 2010). Functional foods are natural or processed foods that contain biologically active compounds with proven health benefits when consumed in adequate amounts (Martirosyan and Singh, 2015). Foods enriched with probiotics, on the other hand, are known as functional food products containing a sufficient number of live microorganisms capable of altering the microbiota in

the host to create beneficial effects on health (Chávarri et al., 2010; Tufarelli and Laudadio, 2016).

The term “probiotic” is derived from the Latin word “bio-tikos”, meaning “for life”, and it was first used by researchers Lily and Stillwell in 1965 to refer to substances that promote the growth of other microorganisms (Parracho et al., 2007). Probiotic bacteria, which have been defined in various ways to date, are broadly described as living microbial food supplements that can survive in the host’s intestinal microbiota and exert beneficial effects there to maintain the microflora (Saarela et al., 2000; Fuller, 2004). The first experimental study related to probiotic microorganisms was conducted by the Russian scientist Metchnikoff in 1907, who investigated the intestinal microflora and reported that fermented dairy products prevented the effects of toxic substances in the body (Vasiljevic and Shah, 2008; Galdeano et al., 2010).

In the first quarter of the 21st century, approximately 500 probiotic food products have been introduced to the global market, indicating a continuous expansion of the applications of probiotic bacteria (Dinkçi et al., 2019). Probiotic products can contain one or more types of microorganisms. Among the various types of microorganisms, species belonging to the *Lactobacillus* and *Bifidobacterium* genera are the most commonly used (Timmerman et al., 2004; Yaşar and Kurdaş, 2009). These bacteria are commonly used in the production of fermented foods, where they can remain highly viable. They exhibit their therapeutic effects only when consumed in specific quantities (10^6 - 10^7 Cfu/g or Cfu/ml) in the body. Therefore, while various foods are being researched as probiotic carriers, fermented foods are recommended as the best probiotic carriers. Yoghurt, fermented milk, and other fresh fermented products, or non-fermented products with an equivalent number of live probiotic bacteria added, are preferred food carriers for probiotic bacteria that have been used until today (Lourens-Hatting and Viljoen, 2001; Afzaal et al., 2019).

Milk and dairy products have become the primary product group in the probiotic market due to their buffering capacity, diverse product varieties, and the presence of nutrient elements that support the viability of probiotic microorganisms during fermentation and storage. These products are also known as functional dairy products and/or probiotic dairy products. Among them, yoghurt is considered the best carrier food (Gürsoy and Kınık, 2004; Meybodi et al., 2020; Gao et al., 2021). However, traditional yoghurt is not a probiotic product. The bacteria used in yoghurt production, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, are not resistant to stomach acid, bile salts, and digestive enzymes. Consequently, they lose their viability in the gastrointestinal system. Additionally, since they are not part of the intestinal microbiota, they lack the ability to adhere to and colonize the intestines (Çelikel et al., 2018).

Traditional yoghurt, while not carrying probiotic microorganisms, is still an important functional product with many properties, such as containing health-beneficial components like organic acids and providing lactase enzyme to the body through yoghurt starters. The use of probiotic cultures in yoghurt production has become a common practice with the aim of enhancing the positive effects and functional properties of yoghurt on health. This helps transform yoghurt, which holds a significant place in the Turkish diet, into a carrier for probiotics, contributing to the intake of these beneficial microorganisms. This application allows the production of a high-nutrient functional yoghurt, also known as “bio-yoghurt”. The use of *Bifidobacterium* species and *Lb. acidophilus* in yoghurt production is increasingly popular, and the resulting product is sold under the name “probiotic yoghurt” (Lourens-Hatting and Viljoen, 2001; Güler-Akın et al., 2007).

The pH level of the environment is crucial for maintaining the viability of probiotic bacteria. Probiotic bacteria belonging to the *Lactobacillus* genus have higher acid tolerance (pH 3.70 - 4.30). However, for *Bifidobacterium* species, it becomes more challenging to maintain their viability below pH 5.00. The pH value of yoghurt, which is commonly used as a probiotic carrier, typically ranges from 4.00 to 4.50. As a result, the number of probiotic bacteria tends to decrease

during storage, which can also reduce their viability during passage through the digestive system (Boylston et al., 2004; Tripathi and Giri, 2014).

Exposing microorganisms to adverse conditions for a short period can lead to these microorganisms' developing tolerance or adaptation to these adverse conditions (Hill et al., 1995; Uğuz and Andıç, 2016). A similar situation exists for probiotic microorganisms. Short-term adaptation of probiotic bacteria to an acidic environment can enhance their viability (Shah, 2000).

In this study, probiotic bacteria commonly used in yoghurt production were exposed to moderately high acidic conditions (pH 4.5) before fermentation to determine whether the bacteria developed an acid tolerance response. Therefore, two probiotic bacteria frequently used in probiotic yoghurt production, *Lb. acidophilus* ATCC 4356 and *B. bifidum* ATCC 11863, were adapted to both organic (lactic acid (LA)) and inorganic (hydrochloric acid (HCl)) acids, and the bacteria's acid tolerance responses were examined. Additionally, the viability of probiotic bacteria in yoghurt samples was monitored during storage.

Materials and Methods

Material

The UHT milk used in the research was obtained from Torku - Panagro Tarım Hayvancılık Gıda Sanayi ve Ticaret A.Ş. (Konya, Türkiye) company. The thermophilic yoghurt culture used for probiotic yoghurt production was obtained from Chr. Hansen/İstanbul company. The probiotic cultures (*Lb. acidophilus* ATCC 4356 and *B. bifidum* ATCC 11863) were provided from the distributor Oxoid/Türkiye.

Method

Activation of probiotic culture, preparation and preservation of stock culture

In the study, *Lb. acidophilus* ATCC 4356 was activated and prepared as a stock culture using MRS Broth (Merck, Germany). *B. bifidum* ATCC 11863 was activated and prepared as a stock culture using MRS Broth supplemented with 0.05% L-cysteine HCl (for anaerobic medium) (Dave and Shah, 1996; Tharmaraj and Shah, 2003). The probiotic cultures were inoculated into MRS Broth and incubated at 42°C for 24-48 hours. The activation of cultures was repeated for two passages. The active cultures obtained from the second passage were transferred to sterile tubes containing glycerol (1:1) and stored at -20°C (VELP Scientifica, Italy) for long-term preservation.

To determine the microbial loads of the activated cultures, the spread plate method was used for *Lb. acidophilus* ATCC 4356 on MRS-Sorbitol Agar (Merck, Germany), and the Petri dishes were incubated at 42°C for 72 hours in a carbon dioxide incubator containing 10% CO₂ (under microaerophilic condition) (Nüve EC 160, Türkiye). For *B. bifidum* ATCC 11863, MRS-NNLP Agar (Merck, Germany) was used, and the plates were placed in an anaerobic jar (containing Gas pack) and incubated at 42°C for 72 hours.

The solutions of L-cysteine HCl, sorbitol, and NNLP (nalidixic acid, neomycin sulfate, lithium chloride, and paromomycin sulfate from Sigma-Aldrich, Germany) used in the growth media were sterilized using a 0.45 µm pore-sized sterile syringe filter (Millipore, Ireland).

Adaptation of probiotic cultures to acid environment

In order to adapt the probiotic cultures to an acidic environment, sterilized MRS growth media were prepared by heating at 121°C for 15 minutes and then cooled to 45-50°C. Sterilized 1 N HCl and 1 N LA solutions were added to the MRS media using a 0.45 µm pore-sized sterile syringe filter to adjust the pH of the media to 4.5 (Shah and Lankaputhra, 1997; Matsumoto et al., 2004). For *B. bifidum* ATCC 11863, sterilized L-cysteine HCl solution was also added to the media at a concentration of 0.05% (Tharmaraj and Shah, 2003).

A 1 ml of inoculum was taken from active cultures and inoculated into centrifuge tubes containing 9 ml of acidic broth. The tubes were then incubated at 42°C for 3 hours. After incubation, the tubes were centrifuged using a cooling centrifuge (Hettich Universal 32R, Germany) at 4°C, 4500 rpm for 10 minutes due to cell pellets. The cell pellets were washed three times with sterile peptone water to remove the remaining acidic media.

Probiotic yoghurt production

For the production of probiotic yoghurt, UHT (Ultra-High Temperature) cow's milk was used. The milk was subjected to a heat treatment at approximately 85-90°C for about 10 minutes and rapidly cooled to 44-45°C. The yoghurt culture + non-adapted probiotic culture pellets and yoghurt culture + acid-adapted probiotic culture pellets were added into the milk at the same time for production of yoghurt groups. The yoghurt culture was inoculated at a 2% ratio and probiotic bacteria were added at a level of 10⁷ Cfug (7.30 log Cfug, which is pellets's microbial load). The cultured milk was quickly distributed into sterile sample containers of 100 ml each. A total of three groups of yoghurt have been produced in this research. The yoghurt samples were coded *LO+B0* (non-acid adapted *Lb. acidophilus* ATCC 4356 (L0) + non-acid adapted *B. bifidum* ATCC 11863 (B0) – control group), *LL+LB* (lactic acid-adapted *Lb. acidophilus* ATCC 4356 (LL) + lactic acid-adapted *B. bifidum* ATCC 11863 (LB)), *HL+HB* (HCl-adapted *Lb. acidophilus* ATCC 4356 (HL) + HCl-adapted *B. bifidum* ATCC 11863 (HB)).

The containers were immediately sealed, and the yoghurt samples were placed in an incubator set at 44-45°C. Fermentation was stopped when the pH of the yoghurt reached approximately 4.6. The probiotic yoghurt samples were then stored at 4 ± 1°C for 21 days. During the storage period, pH measurements, titratable acidity analysis, and cultural count of probiotic bacteria were performed on the yoghurt samples on the 1st, 7th, 14th, and 21st days.

pH and titration acidity analysis

The pH values of yoghurt samples were determined using a benchtop pH meter (Orion™ Star A215, Thermo Fisher Scientific Inc, the USA). Before each analysis, the pH meter was calibrated using pH 4.0 and pH 7.0 buffer solutions at 20°C (Bradley et al., 1992).

For the titration acidity analysis, 10 g of the yoghurt sample was taken and mixed with 10 ml of distilled water. Then, 4-5 drops of phenolphthalein indicator were added to the mixture, and it was titrated with 0.1 N NaOH solution (Sigma, Germany) until a permanent light pink color was formed. The result was expressed as the acidity percentage in terms of lactic acid (AOAC, 1995).

Cultural counts of probiotic bacteria

MRS-Sorbitol Agar was used for *Lb. acidophilus* ATCC 4356, and MRS-NNLP Agar was used for *B. bifidum* ATCC 11863 in order to the count of probiotic bacteria (Dave and Shah, 1996; 1997). To prepare the samples, 1 gram of yoghurt was weighed and added to a test tube containing 9 ml of 0.1% sterile peptone water. Serial dilutions were then prepared from the initial dilution (10⁻¹) by taking appropriate dilution volumes. A 0.1 ml sample was taken from the suitable dilutions and spread onto Petri dishes using the spread plate method. For the count of *Lb. acidophilus* ATCC 4356, the Petri dishes were incubated in a carbon dioxide incubator containing 10% CO₂ at 42°C for 72 hours. For the count of *B. bifidum* ATCC 11863, the Petri dishes were placed in an anaerobic jar (containing a Gas pack) and incubated at 42°C for 72 hours. After incubation, the Petri dishes were examined, and those containing 30-300 colonies were selected for counting. The results were then calculated as colony-forming units per gram (Cfu/g). To present the results in a table, the obtained bacterial counts were subjected to logarithmic transformation (log₁₀) for better representation and comparison.

Statistical analysis

In this study, the obtained results were analyzed using the SPSS software package (version 20.0 for Windows, SPSS Inc., Chicago, Illinois). One-Way ANOVA (Analysis of Variance) was used to determine whether there is a statistically significant difference between the group means of the yoghurt samples. To assess the significance of differences, the Duncan multiple comparison test was employed.

Results and Discussion

After activation of probiotic culture, the colonies were counted, and the microbial loads were determined to be 1.36x10⁸ Cfug/ml for *Lb. acidophilus* ATCC 4356 and 1.6x10⁸ Cfug/ml for *B. bifidum* ATCC 11863. In our study, we monitored both the viability of *Lb. acidophilus* ATCC 4356 and *B. bifidum* ATCC 11863 after 3 hours acid adaptation and the viability of these bacteria during storage in yoghurt.

Adaptation of probiotic cultures to acid environment

Acid stress exhibits lethal or sublethal effects on numerous microorganisms (Beales, 2004, Uğuz and Andiç, 2016). Although this effect primarily affects the viability of various microorganisms, short-term acid stress (acid adaptation) is one of the strategies to improve the survival of probiotic bacteria. Bifidobacteria are more sensitive to acids than lactobacilli (Upadrasta et al., 2011; Tripathi and Giri, 2014).

Following 3 hours exposure to an acidic environment at pH 4.5, the viability of *Lb. acidophilus* ATCC 4356 and *B. bifidum* ATCC 11863 was found to be 9 log₁₀ Cfug. The microbial loads before and after acid adaptation of probiotic cultures are given in Table 1. Considering the bacterial counts after 3 hours incubation, it is seen that ATR was formed and acid adaptation was successful for both types of acids (De Angelis and Gobbetti, 2011; Guan and Liu, 2020).

Table 1. Microbial loads of probiotic bacteria before and after 3 hours acid adaptation

Acid type	Bacteria group	Adaptation Time (hours)	Counts (log 10 Cfu/g)
LA	LL	0	8.13 ± 0.02
		3	9.02 ± 0.03
	LB	0	8.20 ± 0.01
		3	9.16 ± 0.02
HCl	HL	0	8.13 ± 0.01
		3	9.22 ± 0.02
	HB	0	8.20 ± 0.01
		3	9.04 ± 0.02

LA: Lactic acid, HCl: Hydrochloric acid, LL: Lactic acid-adapted *Lb. acidophilus* ATCC 4356; LB: Lactic acid-adapted *B. bifidum* ATCC 11863, HL: Hydrochloric acid-adapted *Lb. acidophilus* ATCC 4356; HB: Hydrochloric acid-adapted *B. bifidum* ATCC 11863

Table 2. Changes in pH and acidity values of yoghurts produced with yoghurt culture + non-adapted and acid-adapted probiotic cultures

pH	Storage time (Days)	L0 + B0	LL + LB	HL + HB
pH	1	4.70 ± 0.04 ^{Aa}	4.32 ± 0.01 ^{Ab}	4.20 ± 0.01 ^{Ac}
	7	4.59 ± 0.01 ^{Ba}	4.23 ± 0.01 ^{Bb}	4.14 ± 0.02 ^{Bc}
	14	4.59 ± 0.02 ^{Ba}	4.19 ± 0.01 ^{Cb}	4.11 ± 0.00 ^{Cc}
	21	4.56 ± 0.02 ^{Ba}	4.01 ± 0.01 ^{Dc}	4.07 ± 0.01 ^{Db}
Acidity (%)		L0 + B0	LL + LB	HL + HB
	1	0.86 ± 0.02 ^{Bc}	0.97 ± 0.01 ^{Ba}	0.92 ± 0.01 ^{Cb}
	7	0.90 ± 0.01 ^{Ac}	0.97 ± 0.01 ^{Bb}	1.00 ± 0.01 ^{Ba}
	14	0.90 ± 0.02 ^{Ac}	0.99 ± 0.02 ^{Bb}	1.05 ± 0.01 ^{Aa}
	21	0.91 ± 0.02 ^{Ac}	1.01 ± 0.01 ^{Ab}	1.06 ± 0.01 ^{Aa}

a-c: Different letters on the same line indicate a statistically significant difference between samples (P<0.05); A-D: Different letters in the same column indicate a statistically significant difference between days (P<0.05); L0 + B0: Non-lactic acid-adapted *Lb. acidophilus* ATCC 4356 + non-lactic acid-adapted *B. bifidum* ATCC 11863; LL + LB: Lactic acid-adapted *Lb. acidophilus* ATCC 4356 + lactic acid-adapted *B. bifidum* ATCC 11863, HL + HB: Hydrochloric acid-adapted *Lb. acidophilus* ATCC 4356 + hydrochloric acid-adapted *B. bifidum* ATCC 11863

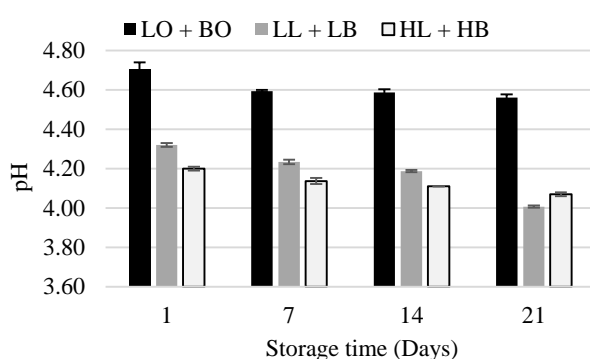


Figure 1. Changes of pH values during storage time

L0 + B0: Non-lactic acid-adapted *Lb. acidophilus* ATCC 4356 + non-lactic acid-adapted *B. bifidum* ATCC 11863; LL + LB: Lactic acid-adapted *Lb. acidophilus* ATCC 4356 + lactic acid-adapted *B. bifidum* ATCC 11863, HL + HB: Hydrochloric acid-adapted *Lb. acidophilus* ATCC 4356 + hydrochloric acid-adapted *B. bifidum* ATCC 11863

pH and titration acidity values of yoghurts

The pH changes of probiotic yoghurt samples during the 21-day storage period are presented in Table 2 and Figure 1. Throughout the storage period, it was observed that the pH values of probiotic yoghurts ranged from 4.20 - 4.70 on day 1, 4.14 - 4.59 on day 7, 4.11 - 4.59 on day 14, and 4.07 - 4.56 on day 21. In all probiotic yoghurts produced with the non-adapted culture (L0+B0) and with the acid-adapted culture (LL+LB and HL+HB), a decrease in pH values was noted during storage, and this decrease was statistically significant (P<0.05).

It was observed that yoghurt produced with acid-adapted cultures had significantly lower pH values compared to the control samples throughout all storage

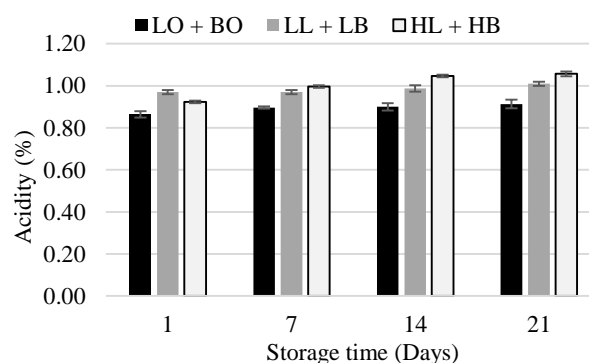


Figure 2. Changes of Acidity (%) during storage time

L0 + B0: Non-lactic acid-adapted *Lb. acidophilus* ATCC 4356 + non-lactic acid-adapted *B. bifidum* ATCC 11863; LL + LB: Lactic acid-adapted *Lb. acidophilus* ATCC 4356 + lactic acid-adapted *B. bifidum* ATCC 11863, HL + HB: Hydrochloric acid-adapted *Lb. acidophilus* ATCC 4356 + hydrochloric acid-adapted *B. bifidum* ATCC 11863

periods. Yoghurts produced with HCl-adapted cultures generally had lower pH values than those produced with LA-adapted cultures. The effect of acid adaptation and the type of acid on the pH of yoghurts was found to be statistically significant (P<0.05).

Shah et al. (1995) found that the pH of commercially produced probiotic yoghurts (containing *Lb. acidophilus* and *B. bifidum*) decreased during storage by 0.07 to 0.42 units, compared to the pH values observed 2-3 days after production. Yerlikaya et al. (2013) reported a regular decrease in pH values of probiotic fermented beverages produced using *Lb. acidophilus* (0.75%), *Bifidobacterium animalis* subsp. lactis (1.0%), and *Lactobacillus casei* (1.0%) cultures during the storage period.

Settachaimongkon et al. (2015) produced set-type probiotic yoghurts using *Lactobacillus rhamnosus* GG and *B. animalis* subsp. *lactis* BB12 cultures exposed to sublethal levels of NaCl and acid stress, along with yoghurt starter culture. During the storage period, the pH values of probiotic yoghurt samples showed a decrease; however, this decrease was not statistically significant ($P>0.05$). In the study conducted by Çomak-Göçer et al. (2016), probiotic yoghurts were produced using *Lb. acidophilus* ATCC 4356 in combination with yoghurt starter culture. The yoghurts were subjected to different incubation temperatures and terminated at different pH values. After storage, all yoghurt samples showed a decrease in pH values due to the storage period. In our study, it was determined that the decrease in pH values observed in yoghurt samples during storage is consistent with the findings of other studies on probiotic yoghurt.

The changes in titration acidity of probiotic yoghurt samples are shown in Table 2 and Figure 2. Throughout the storage period, the titration acidity values increased, while the pH decreased during storage. The titratable acidity of yoghurt samples varies between 0.86% and 1.06%. This increase was found to be statistically significant ($P<0.05$).

The lowest titratable acidity values were determined on the 1st day of storage. The yoghurt produced using HCl-adapted cultures showed the highest titration acidity values, whereas the yoghurts produced with non-adapted culture exhibited the lowest titratable acidity values during the end of storage period. According to the Turkish Food Codex Regulation on Fermented Dairy Products, the titration acidity in yoghurt should be between 0.6% and 1.5% (Anonymous, 2022). All of the titration acidity values determined in this research are within the limits specified in the regulation. Overall increase from first day to end of the storage period is compatible with the results reported by Shah et al. (1997), Çakmakçı et al. (2012), Shoji et al. (2013), Başyigit-Kılıç and Akpınar Kankaya (2016), Demirci et al (2017), and Ghaderi-Ghahfarokhi et al. (2021).

Counts of probiotic bacteria in yoghurts

In the probiotic yoghurts produced using yoghurt starter and probiotic cultures (*Lb. acidophilus* ATCC 4356 and *B. bifidum* ATCC 11863), it was determined that the non-adapted cultures showed a decrease in counts during storage; however, their viability was maintained throughout all analysis periods. *Lb. acidophilus* ATCC 4356 and *B. bifidum* ATCC 11863 strains managed to maintain their viability during storage. The viable cell count of non-adapted and acid-adapted probiotics in yoghurt was given Table 3, and changes in count were shown Figure 3 and Figure 4.

On the first day of storage, *Lb. acidophilus* ATCC 4356 exhibited a viability of 7.71 ± 0.04 log₁₀ Cf/g, while *B. bifidum* ATCC 11863 showed a viability of 7.12 ± 0.04 log₁₀ Cf/g. Throughout storage, except for a slight fluctuation observed on the 14th day for *Lb. acidophilus* ATCC 4356, the counts of both bacteria decreased during the storage period. This reduction in probiotic bacteria counts was found to be statistically significant ($P<0.05$).

In the research conducted on 5 commercial probiotic yoghurts containing *Lb. acidophilus* and *B. bifidum*, it was observed that during the storage period, only 3 yoghurt

samples were able to maintain *Lb. acidophilus* counts at approximately 7-8 log₁₀ Cf/g. However, for *B. bifidum* counts in the same yoghurt samples, only one sample was able to maintain a count of 6 log₁₀ Cf/g until the 9th day of storage, while none of the samples could sustain their viability by the end of the storage (Shah et al., 1995). Ng et al. (2011) investigated the relative viability rates of 5 different *Lb. acidophilus* strains (NCFM, ATCC 700396, PIM703, SBT2062, and LA-5) in combination with yoghurt culture. They found that the SBT2062 strain, which was used at levels of 7-8 log₁₀ Cf/g along with yoghurt culture, exhibited the highest viability rate. However, they observed that the relative viability rates of the other *Lb. acidophilus* strains rapidly decreased throughout the storage period, reaching levels between 4.11 to 5.04 log₁₀ Cf/g by the end of storage period. In the search by Çakmakçı et al. (2012), they observed a general decrease in the counts of *Lb. acidophilus* DSMZ 20079 and *B. bifidum* DSMZ 20456 in probiotic yoghurt produced using banana marmalade during the storage period. Due to a significant reduction in the counts of *Lb. acidophilus* and *B. bifidum* in these yoghurts, they reported that the products lost their probiotic properties after the 7th day of storage. The viability of *Lb. rhamnosus* GG and *B. animalis* subsp. *lactis* BB12 cultures used in yoghurt production without sublethal stress, it was found that after 28 days of storage, there was a decrease of 0.5 log₁₀ Cf/g in *Lb. rhamnosus* GG culture count and 1.2 log₁₀ Cf/g in *B. animalis* subsp. *lactis* BB12 culture count (Settachaimongkon et al., 2015). Similarly, the probiotic bacterial counts in our study also showed a decrease during storage. As the acidity of the yoghurt increased, the viability of the probiotic bacteria decreased. The possible reason for this decrease could be attributed to the increased acidity in the yoghurt. In yoghurt production, the number of organic acids such as lactic acid and acetic acid increases due to the metabolism of lactose by yoghurt cultures and probiotic cultures. Due to the organic acids, the increased ionized hydrogen in the environment interferes with microbial cell membrane integrity, disrupts the cell's internal pH balance, and fundamental biochemical processes. As a result, it can hinder the growth and survival of probiotic bacteria. On the other hand, the impact of metabolites produced by yoghurt cultures and probiotic cultures, along with antagonistic interactions between cultures, may have reduced the viability of probiotic bacteria (Shah, 2000; Tripathi and Giri, 2014; Sendra et al., 2016; Bisson et al., 2023).

In the yoghurt samples produced using *Lb. acidophilus* ATCC 4356 and *B. bifidum* ATCC 11863 adapted to lactic acid (LL and LB groups), both bacteria exhibited a growth level of 8.08 log₁₀ Cf/g on the first day of storage. It is known that bacteria can maintain their viability in acidic environments through certain mechanisms present in their structures. The activation of stress response systems and the development of acid tolerance response (ATR) in bacteria exposed to moderately low pH are common phenomena, inducing the synthesis of proteins that help bacteria survive in low pH conditions (Ventura et al., 2011; Uğuz and Andiç, 2016). Probiotic cultures can survive in the low pH environment resulting from milk fermentation by developing ATR during adaptation to the acidic conditions (De Angelis and Gobbetti, 2011).

Table 3. The viable cell counts of non-adapted and acid-adapted probiotics in yoghurt (Log CFU/g)

Probiotics	Storage time (Days)	L0	LL	HL
<i>Lb. acidophilus</i> ATCC 4356	1	7.71 ± 0.04 ^{Ac}	8.08 ± 0.12 ^{Aa}	7.90 ± 0.08 ^{Ab}
	7	6.96 ± 0.05 ^{Ca}	5.47 ± 0.06 ^{Bb}	7.00 ± 0.01 ^{Ba}
	14	7.06 ± 0.04 ^{Ba}	ND	5.63 ± 0.03 ^{Cb}
	21	5.94 ± 0.03 ^{Da}	ND	ND
<i>B. bifidum</i> ATCC 11863		B0	LB	HB
	1	7.12 ± 0.04 ^{Ab}	8.08 ± 0.09 ^{Aa}	5.99 ± 0.03 ^{Ac}
	7	7.07 ± 0.01 ^{Aa}	5.92 ± 0.07 ^{Bb}	5.61 ± 0.05 ^{Bc}
	14	6.00 ± 0.02 ^{Ba}	4.22 ± 0.02 ^{Cb}	ND
	21	5.61 ± 0.05 ^{Ca}	ND	ND

a-c: Different letters on the same line indicate a statistically significant difference between samples (P<0.05); A-D: Different letters in the same column indicate a statistically significant difference between days (P<0.05); L0: Non-lactic acid-adapted *Lb. acidophilus* ATCC 4356, LL: Lactic acid-adapted *Lb. acidophilus* ATCC 4356, HL: Hydrochloric acid-adapted *Lb. acidophilus* ATCC 4356, B0: Non-lactic acid-adapted *B. bifidum* ATCC 11863; LB: Lactic acid-adapted *B. bifidum* ATCC 11863, HB: Hydrochloric acid-adapted *B. bifidum* ATCC 11863, ND: not detected

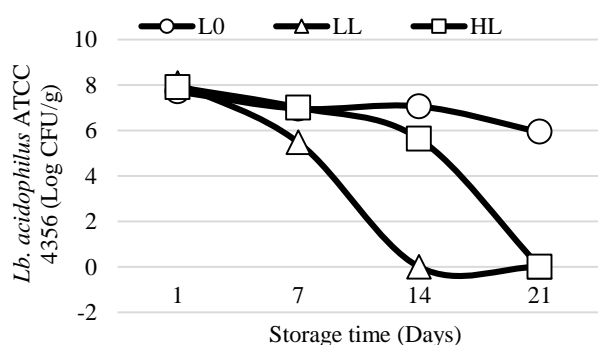


Figure 3. Changes in viable counts of *Lb. acidophilus* ATCC 4356 during storage time

L0: Non-lactic acid-adapted *Lb. acidophilus* ATCC 4356, LL: Lactic acid-adapted *Lb. acidophilus* ATCC 4356, HL: Hydrochloric acid-adapted *Lb. acidophilus* ATCC 4356

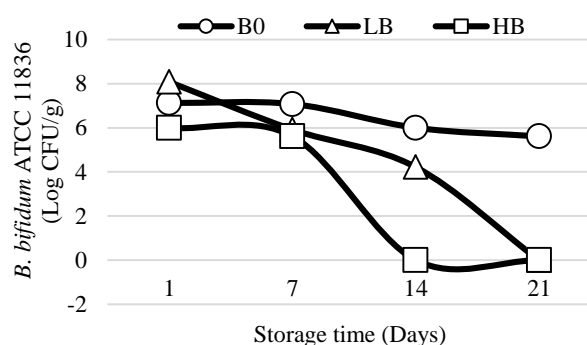


Figure 4. Changes in viable counts of *B. bifidum* ATCC 11863 during storage time

B0: Non-lactic acid-adapted *B. bifidum* ATCC 11863; LB: Lactic acid-adapted *B. bifidum* ATCC 11863, HB: Hydrochloric acid-adapted *B. bifidum* ATCC 11863

In our study, the probiotic bacterial counts on the first day of storage being above 7.30 log₁₀ Cfu/g, as inoculated in yoghurt production, can be explained by this mechanism. According to this result, both probiotic bacteria have developed ATR in the acidic environment. In the yoghurt group containing *B. bifidum* ATCC 11863, viability was observed until the 14th day of storage, while in the yoghurt containing *Lb. acidophilus* ATCC 4356, viability was observed until the 7th day.

In yoghurt samples produced with hydrochloric acid-adapted probiotic bacteria (HL and HB groups), on the first day of storage, *Lb. acidophilus* ATCC 4356 showed growth at a level of 7.90 ± 0.08 log₁₀ Cfu/g, while *B. bifidum* ATCC 11863 strain showed growth at a level of 5.99 ± 0.03 log₁₀ Cfu/g. Considering the initial microbial load in the pellet inoculated into this yoghurt group (7.30 log₁₀ Cfu/g), it can be said that *Lb. acidophilus* ATCC 4356 was positively affected by HCl adaptation, whereas *B. bifidum* ATCC 11863 was negatively affected. In this yoghurt group, *Lb. acidophilus* ATCC 4356 could maintain its viability until the 14th day of storage, while *B. bifidum* ATCC 11863 could only maintain its viability until the 7th day of storage. Probiotic cultures belonging to the *Bifidobacterium* genus have less tolerance to HCl in the stomach environment compared to species belonging to the *Lactobacillus* genus (Ventura et al., 2011, Tripathi and Giri, 2014; Soni et al., 2020). It is predicted that the decrease in the number of *B. bifidum* ATCC 11863 observed in this yoghurt group is due to this reason.

The counts of both acid-adapted and non-adapted *Lb. acidophilus* ATCC 4356 and *B. bifidum* ATCC 11863 exhibited a decrease in all yoghurt groups during the storage period. Bifidobacteria generally show optimal growth within a pH range of 6.0 to 7.0. When the pH drops below pH 5.0, the growth of bifidobacteria decrease significantly (Boylston et al., 2004; Dinkçi et al., 2019). The decrease in the counts of probiotic bacteria can be attributed to organic acids (lactic acid, acetic acid), bacteriocins (bifidin and bifidosin), and antibiotic-like substances produced by both yoghurt cultures and probiotic bacteria (Hill et al., 1995; Çelikyurt and Arıcı, 2008; Fraise et al., 2013, Güngör and Özçelik, 2014, Alipour and Mofarrah, 2022). Additionally, the induced acid tolerance responses in probiotic bacteria can vary based on the bacterial species, growth phase of the bacteria (log phase, stationary phase, etc.), and the type of acid used for adaptation (organic or inorganic acid) (Saarela et al., 2004).

In the study investigating the viability of *Bifidobacterium longum* biotype *longum* NCIMB 8809 and its mutant strain adapted to HCl (pH 4.0 - *B. longum* biotype *longum* 8809dpH), a significant decrease in viability was observed in the non-adapted strain, where it decreased by 5 log after a 90-minute incubation in the simulated gastric environment. However, there was no significant decrease in the viability of the mutant strain (Sánchez et al., 2007). Saarela et al. (2009) aimed to determine the stability of lyophilized *Lb. rhamnosus* cells, which they developed at pH 5.0 and pH 5.8, in their

research. For this purpose, they inoculated cultures into environments containing malic acid and HCl. They observed that the bacteria developed at pH 5.0 showed higher viability than those developed at pH 5.8. Additionally, in acidic environments, higher viability was achieved in the HCl medium. Jiang et al. (2016) investigated the impact of acid stress on *B. longum* BBMN68 (wild type) and found that *B. longum* BBMN68m (mutant strain - acid-adapted), incubated for 2 hours in a medium adjusted to pH 2.5 with HCl, exhibited 4.4 log₁₀ Cf/g higher viability compared to the wild-type strain. In the research evaluating the viability of different probiotic bacteria and their binary combinations at different pH levels (pH 1.0-4.0), the yoghurt group containing a combination of *Lb. acidophilus* and *B. bifidum* showed the highest probiotic viability in all pH environments, with a survival rate of 66.1% (Soni et al., 2020). In a similar manner, studies conducted by Çakmakçı et al. (2012), Söküt et al. (2021), and Akan (2022) in yoghurt research have also shown a decrease in probiotic counts during storage. Throughout these studies, some products maintained therapeutic levels of probiotic viability by the end of the storage, while others exhibited viability levels below the therapeutic range. In our own study, similar to these previous works, a reduction in probiotic counts was observed in both the control group and yoghurt produced with acid-adapted cultures. However, it was found that the *therapeutic level* was generally maintained within the first 7 days.

In some yoghurt studies produced with probiotic cultures, the detection of high viability during storage might be depend on the use of commercial lyophilized cultures (10¹¹ to 10¹² Cf/g; e.g., *Lb. acidophilus* LA5® and *B. animalis* subsp. *lactis* BB-12®). However, in our study, despite the low initial levels of acid-adapted probiotic cultures (10⁷ Cf/g), therapeutic level of viability was observed at the end of storage period.

Conclusion

In this research, it was observed that *Lb. acidophilus* ATCC 4356 and *B. bifidum* ATCC 11863 cultures adapted to both organic (lactic acid) and inorganic acid (hydrochloric acid) environments and they developed an acid tolerance response of bacteria. Following a 3-hours acid adaptation, both probiotic cultures exhibited a viability level of 9 logs, indicating the successful adaptation process. *Lb. acidophilus* ATCC 4356 showed better adaptation to the HCl (9.22 log 10 Cf/g), whereas *B. bifidum* ATCC 11863 adapted more effectively to the lactic acid (9.16 log 10 Cf/g).

In yoghurt samples, pH decreased and titratable acidity increased over the storage period. The viability of probiotic bacteria adapted to lactic acid and HCl environments decreased during storage, similar to the control group. HCl-adapted *Lb. acidophilus* ATCC 4356 and lactic acid-adapted *B. bifidum* ATCC 11863 could maintain their viability until the 14th day. However, by the end of the 21-day storage period, viability of probiotic culture was only observed in yoghurts produced with non-adapted strains. It has been observed that the majority of probiotic viability levels maintained the therapeutic dosage range that should be present in probiotic yoghurts.

To maintain the survival of these probiotic bacteria in the product, some measures can be taken, such as adding prebiotic substances to the product and selecting appropriate packaging material. Additionally, applications such as using components that reduce oxidation-reduction potential, incorporating antioxidant compounds, or using food products containing these substances can be used to preserve and enhance the viability of acid-adapted probiotic cultures in the product. Moreover, in potential products where probiotic cultures are used, such as cheese, ice cream, bakery products, and meat products, the cultures can be exposed to stress conditions like high salt, low temperature, high temperature, and acidic environments to induce the development of stress response systems. This approach would enable the use of probiotic cultures in products with high salt content or products subjected to low or high temperatures.

Conflict of Interest

No conflict of interest was declared by the authors.

Ethics Committee Approval

This research does not require ethics committee permission.

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