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Effects of Adding Non-viable *Lacticaseibacillus casei* and *Lactobacillus acidophilus* on Physicochemical, Microbial, Chemical and Sensory Attributes of Probiotic Doogh

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Abstract

Background and Objective: Inactivated probiotics provide various health and technological benefits, making them appropriate for the production of functional dairy products. The aim of this study was to investigate effects of adding nonviable probiotics (*Lactobacillus acidophilus* LA-5 and *Lacticaseibacillus casei* 431) to doogh (a typical Iranian fermented milk drink).

Material and Methods: Probiotics were inactivated by heat or sonication and added to the samples before or after fermentation. Various parameters such as pH, titratable acidity, redox potential, antioxidant capacity, color, viscosity, and phase separation, viability of traditional starter bacteria and probiotics and sensory characteristics were assessed during fermentation and refrigerated storage at 5 °C.

Results and Conclusion: Sonicated probiotic-containing treatments included the highest pH decrease rate (0.011 pH min^{-1}) during fermentation, as well as the highest antioxidant capacity (16.45%) and viscosity (35.15 mPa.s), while heat-inactivated probiotic- containing treatments included the lowest viscosity (17.60 mPa.s). Treatments with viable probiotics reasonably included the highest post-acidification rate during storage (4.14 °D d^{-1}), compared to those containing nonviable cells, as well as the minimum phase separation rate. The b* and L* values of color did not differ significantly within treatments, but the highest a* value was observed in the treatments with sonication. The highest populations of *Lactobacillus delbrueckii* ssp. *bulgaricus* (log 11,891 CFU ml^{-1}) and *Streptococcus thermophilus* (log 14,977 CFU ml^{-1}) at the end of the storage were observed in treatments with heated probiotics (compared to viable probiotics) and treatments with sonicated probiotics, respectively. In addition, *Lactobacillus acidophilus* was more susceptible than *Lacticaseibacillus case* and included lower viability. Taste, mouth feeling and total acceptance of all samples did not differ significantly within treatments. The present study suggests that inactivated probiotics can successfully be used for the production of fermented milk beverages with appropriate sensory characteristics and higher antioxidant capacity, compared to the control group.

Conflict of interest: The authors declare no conflict of interest.

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1. Introduction

Recently, it has been suggested that probiotics, viable or non-viable, are bacterial cells that include positive effects on human health. By this general definition, probiotics are divided into two categories of viable and non-viable probiotics [1, 2]. The idea of using non-viable probiotics in food industries is originated from the fact that probiotic bacteria are susceptible to environmental conditions during passage through the gastrointestinal tract (GIT), include limited stability over a wide range of pH and temperature, include a shorter shelf-life and need refrigerated storage. Therefore, their use in various industries is further technologically and economically feasible [3-6]. Additionally, it has been verified that non-viable probiotics include beneficial effects for humans such as immune-stimulating activity [7], cholesterol decrease [8], anticancer characteristics [9], healing gastrointestinal disorders [10] and suppression of pro-inflammatory cytokine production [11]. There are several available methods to inactivate probiotics, including heat treatment, ultraviolet (UV) irradiation, irradiation, sonication (ultrasound), high pressure, ionizing radiation, pulsed electric field (PEF), supercritical CO_2, drying and changes in pH [8,12]. Sonication and heating are the most commonly used methods for inactivating probiotics, majorly because they are cost-effective and time-efficient. Ultrasound at frequencies of 20–40 kHz can be lethal to microorganisms by creating acoustic cavities on their cell membrane (CM), leading to the release of their contents [13]. In contrast, during heating, intracellular contents are not released.

Doogh is a fermented beverage whose major ingredients include yogurt, water, salt and flavoring agents [14]. However, studies on adding non-viable probiotics to fermented foods are limited. Parvayi et al. studied effects of inactivated *Lactobacillus acidophilus* ATCC SD 5221 and *Bifidobacterium lactis* BB-12 on yogurt characteristics and reported that incorporation of heat-inactivated probiotics to yogurts included less technological challenges and could be deliberated as an appropriate alternative for probiotics in functional yogurts [15]. Overall, there is still a research gap in the development and commercialization of inactivated probiotic dairy products in food industries. While interests in probiotics and prebiotics are increasing, inactivated probiotics have not received much attention for product development and market availability. In addition, knowledge on specific inactivated probiotic compounds in dairy products and their potential effects on human health is limited. Further research are needed to identify and characterize these compounds and assess their potential health benefits and uses in functional foods [16,17]. Moreover, there is a lack of standardized methods for the production and quality control of inactivated probiotic dairy products, which limits their widespread commercialization. Research in this area is essential to establish industry standards and guidelines for the production and commercialization of inactivated probiotic dairy products. The aim of this study was to assess effects of adding non-viable forms of *Lacticaseibacillus casei* 431 and *lactobacillus acidophilus* LA-5 probiotics inactivated by heating or sonication on the quality characteristics of doogh, a traditional fermented milk beverage from Iran. Probiotics were added before or after the milk fermentation processes.

2. Materials and Methods

2.1 Materials

Skim milk powder was purchased from Pegah, Tehran, Iran. Starter culture included *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* (YF-3331) and the probiotics (*Lacticaseibacillus casei* 431 and *Lactobacillus acidophilus* LA-5) were provided by Chr. Hansen, Copenhagen, Denmark. De Man-Rogosa-Sharpe (MRS) agar and M17 agar were purchased from Quelab, Montreal, Canada, and salt from a local market.

2.2 Preparation of nonviable probiotics

Probiotic suspension was subjected to thermal inactive-ation by heating at 121 °C for 15 min [18]. To achieve ultrasound inactivation, probiotic suspension was exposed to ultrasound waves at a frequency of 250 kHz for 25 min [19].

2.3 Preparation of doogh

To prepare doogh, skim milk powder was reconstituted and diluted to a total solid content of 3.5%. Mixture was heated to 90 °C and set for 15 min before cooling down to 45 °C. Probiotics in viable or nonviable form were added before heat treatment (B) or after fermentation (A). Mixture was incubated at 42 °C until the pH reached 4.5, cooled down to 5 °C and stored in refrigerator for 28 d, as presented in Fig. 1.

2.4 Assessment of pH, redox potential and titratable acidity

The pH, RP (redox potential) and titratable acidity of the doogh samples were checked every 30 min during fermentation. After fermentation, doogh samples were cooled and stored in refrigerator for 28 d, during which, pH, RP (redox potential) and titratable acidity were assessed every 7 d to monitor the shelf life. The pH and RP were assessed using pH meter at room temperature (RM). Titratable acidity was assessed by titrating with 0.1 M NaOH solution and 0.5% phenolphthalein indicator [20]. Increase in acidity, decrease in pH value (pH value min^{-1}) and increase in redox potential (mV min^{-1}) were calculated using Eqs. 1, 2 and 3:

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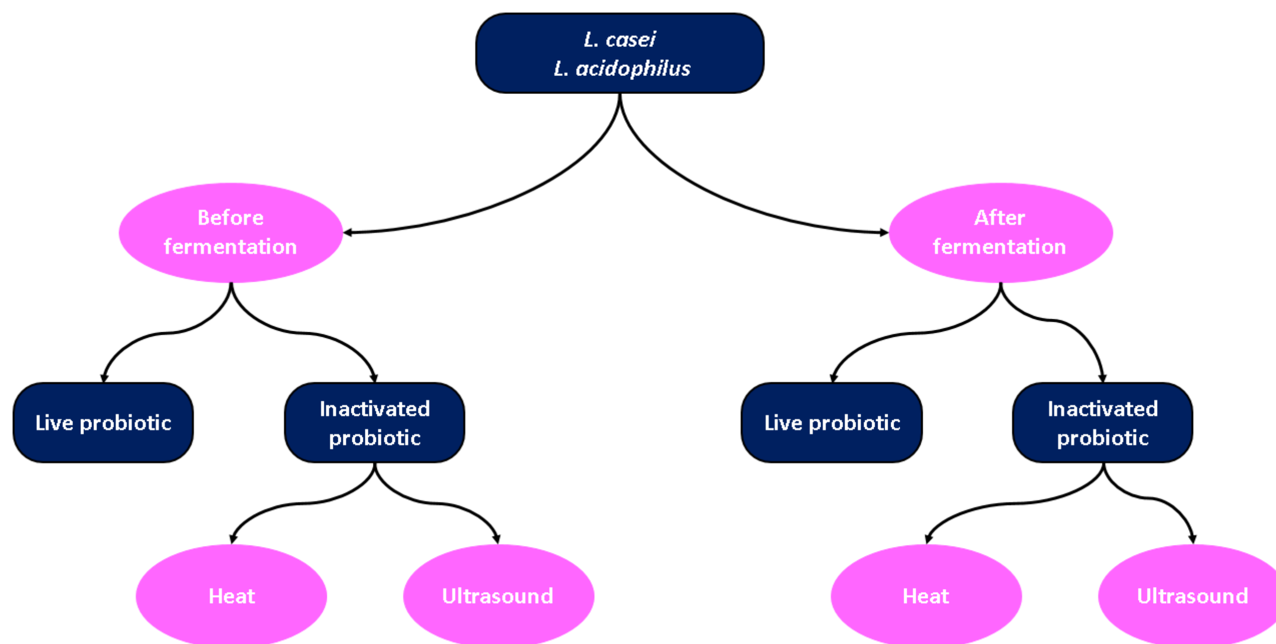

Figure 1. Study design of the present study.

$$\text{pH drop rate} = \frac{\text{Final pH value} - \text{Initial pH value}}{\text{Incubation time}} \quad \text{Eq. 1}$$

$$\text{Acidity increase rate} = \frac{\text{Final acidity value} - \text{Initial acidity value}}{\text{Incubation time}} \quad \text{Eq. 2}$$

$$\text{RP increase rate} = \frac{\text{Final Eh value} - \text{Initial Eh value}}{\text{Incubation time}} \quad \text{Eq. 3}$$

2.5 Serum separation analysis

After cooling down, samples were stored in 10 ml vials and incubated at 5 °C to assess serum separation. During the shelf-life period, height of the supernatant was assessed every 7 d to assess degrees of serum separation that were expressed as proportions using the following Eq. 4 [21]:

$$\text{Separation rate} = \frac{\text{Tallness of supernatant} * 100}{\text{Tallness of sample}} \quad \text{Eq. 4}$$

2.6 Rheological assessment

Rheological assessments were carried out using Brookfield viscometer at refrigerator temperature, one day after the samples were prepared [22]. Briefly, no. 2 cylindrical spindle and spindle speeds of 0.3, 0.6, 1.5, 3, 6, 12, 30 and 60 rpm were used during 90 s if the torque to rotate the spindle in the samples was between the 15.0 and 85.0% of the maximum torque.

2.7 Assessment of antioxidant capacity

To assess antioxidant capacity of the samples, a method was used based on the ability of antioxidants to scavenge the stable radical DPPH (1,1-diphenyl2-picrylhydrazyl). This method was described by Farahmandfar et al. essentially, sample ability to reduce the concentration of DPPH was assessed by measuring absorbance of the solution before and after exposure to the samples [23]. Antioxidant capacity of all samples and inactivated bacterial suspension were assessed on two occasions. The first assessment was carried out on the day of production, while the second assessment was carried out on Day 28 of the shelf-life.

$$\text{Absorbance (\%)} = (\text{blank absorbance} - \text{sample absorbance}) \div \text{blank absorbance} \times 100 \quad \text{Eq. 5}$$

2.8 Color assessment

Color characteristics of doogh were assessed using Hunter Lab Color Flex EZ explained by Milovanovic et al. [24]. Color parameters were L* (brightness, white = 100, black = 0), a* (+, red; -, green) and b* (+, yellow; -, blue).

2.9 Bacterial enumeration

Pour plate method was used to count numbers of *L. delbrueckii subsp. bulgaricus, S. thermophilus* and *L. casei* [25]. The *L. bulgaricus*, starter bacteria of doogh, was cultured in MRS-bile agar at 42 °C for 72 h under anaerobic conditions using Gas Pac system. Enumeration of *S. thermophilus* was carried out using M17 agar at 37 °C for 24 h under aerobic conditions [26]. *Lactobacillus acidophilus* LA-5 and *L casei* were cultured in MRS agar with added bile (0.15% w w^{-1}) to prepare selective media of probiotic enumeration at 37 °C for 72 h under aerobic conditions [27, 28]. The initial counts of *L. acidophilus* and *L. casei* were 10^7 CFU ml^{-1}. To calculate the viability proportion index, final cell population of the microorganisms was divided into the initial cell population based on the Eq. 6 [25].

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$$\text{Viability proportion index} = \frac{\text{Final cell population}}{\text{Initial cell population}} \quad \text{Eq. 6}$$

2.10 Sensory evaluation

Taste, mouth feel and overall acceptance of doogh were assessed using 5-point hedonic scale rating test (with 5 excellent, 4 good, 3 acceptable, 2 bad and 1 very bad) [29]. Twenty consumers assessed the sensory attributes of doogh samples after the first day of preparation.

2.11 Statistical analysis

All experiments were carried out in triplicate and expressed as mean ±SD (standard deviation) ($n = 3$). Data were analyzed using univariate analysis of variance (Tukey test) AND SPSS statistical software v.26 (SPSS, Chicago, USA). Generally, $p < 0.05$ was addressed as the significance threshold.

3. Results and Discussion

3.1 Assessments of pH, redox potential and titratable acidity

During milk fermentation, growth of starter bacteria leads to the conversion of lactose into various compounds such as lactic acid, acetate, formate, acetaldehyde and ethanol. This process results in lactic acid production, causing decreases in pH and increases in redox potential and titratable acidity [30]. Figure 2 illustrates changes in pH, redox potential and titratable acidity during the fermentation process. The initial pH of milk at the beginning of fermentation was 6.8, dropping to 4.5 by the end of fermentation. As shown in Fig. 2, and Table 1 fermentation process included three distinct phases of lag, log and constant phases. During the first 30 min, the lag phase, no significant changes were seen, possibly due to the adaptation of the starter bacteria and buffering characteristics of milk [31]. The fastest decrease in pH and increase in redox potential were observed in sample with ultrasound-inactived *L. casei*. This might be attributed to the ultrasound treatment, which caused puncturing of the membrane of the probiotics, resulting in the release of their cell contents into doogh [5,13]. Feeding the starter bacteria resulted in decreases in the rate of pH and pH of BUC reached 4.5 as the fastest rate (after 210 min). However, BUA included the highest titratable acidity, indicating that the type of probiotic bacteria included major effects on the rate of pH drop and acidity increase. Similarly, Tian and colleagues (2017) reported that the type of bacteria included effects on the quantification of organic acids [32]. In addition, postbiotics produced from *L. acidophilus* LA-5, *L. casei 431* and *L. salivarius* included 62 vrious components, including alcohols, terpenes, norisoprenoids, acids, ketones and esters [33]. Hence, these compounds were available in the environment and might improve the fermentation stage.

Based on Fig. 2, BHC included similar rates of pH decrease and increase in redox potential through the fermentation process as the sample without probiotics. However, BHA showed the lowest rate of acid increase at the end of the fermentation, indicating that the starter bacteria alone were responsible for lactic acid production and the intact cells of the probiotic bacteria included no significant effects on acid production. Furthermore, heat-inactivated *L acidophilus* demonstrated the antibacterial activity [34]. Samples containing live probiotics needed longer times (240 min) to reach pH 4.5. This finding was similar to the finding of Parvayi (2021), who reported that live probiotic samples needed longer times to reach pH 4.5, compared to paraprobiotic samples [15]. Based on a study by Vinderola et al. (2002), adding *L. casei* and *L. acidophilus* to the media with the starter bacteria included negative effects on the growth of the starter bacteria, resulting in decreases in lactic acid production [35].

Statistical analysis showed no significant differences in redox potential between various types of bacteria ($p>0.05$). However, *L. acidophilus* resulted in further decreases in pH and increases in titratable acidity during the storage, compared to that *L. casei* did ($p<0.05$) as represented in Table 2. These results suggested that the selection of probiotic bacteria should carefully be considered based on the specific goals of the fermentation process [36]. Throughout the storage, the highest level of titratable acidity was seen in sample containing live probiotics of *L. acidophilus* (181AD°), which could be attributed to the ongoing acid production by the live probiotics at the refrigerated storage. In contrast, BUC sample included the lowest acidity (117A°), suggesting that the addition of probiotics after the fermentation process could lead to uncontrolled increases in acidity and continued fermentation during cold storage [15]. Moreover, samples containing sonicated and live probiotics included the maximum and the minimum RP increasing rates because of producing the minimum and the maximum lactic acid quantities during storage ($p<0.05$).

3.2 Serum separation

The study detected that the activity of starter bacteria and their ability to generate acids included significant effects on the separation of serum in the samples [37]. Data of Table 3 show increases in serum separation values for all samples during the storage. The initial and the final separation rates of BUC were the highest (32.4%), suggesting that the released intracellular contents were heavier than the whole bacterial cells, causing further sedimentations. In addition, Samples containing live probiotics included smaller serum separation ratios at the end of storage, indicating that they frequently produced lactic acid and their pH was further different from the isoelectric pH [38].

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Figure 2. The pH, reduction potential and titratable acidity of changes in the treatmets during fermentation.

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Table 1. The pH, redux potential and titratable acidity decrease/increase rates in various treatments

	During fermentation			During storage		
Sample	pH-rate (pHmin^{-1})	RP-rate (mVmin^{-1})	Acidity-rate (°Dmin^{-1})	pH-rate (pHday^{-1})	RP-rate (mVday^{-1})	Acidity-rate (°Dday^{-1})
BUC	0.011±0.00 ^{A}	0.733 ±0.00 ^{A}	0.252 ±0.00 ^{A}	0.020±0.00 ^{C}	0.500 ±0.00 ^{B}	1.78±0.00 ^{E}
BUA	0.010±0.00 ^{A}	0.714 ±0.00 ^{A}	0.322 ±0.00 ^{A}	0.023±0.00 ^{B}	0.500 ±0.00 ^{B}	3.07±0.01^{B}
BHA	0.009±0.00 ^{C}	0.627±0.00 ^{C}	0.193±0.00 ^{D}	0.020±0.00 ^{C}	0.398±0.00 ^{D}	2.57±0.00 ^{C}
BHC	0.010±0.00 ^{B}	0.704±0.00 ^{B}	0.250±0.00 ^{B}	0.022±0.00 ^{B}	0.460 ±0.00 ^{C}	1.89±0.00 ^{E}
BC	0.009±0.00 ^{C}	0.645 ±0.00 ^{C}	0.212±0.01 ^{C}	0.025 ±0.00 ^{B}	0.570±0.00 ^{A}	2.89±0.00 ^{C}
BA	0.009±0.00 ^{C}	0.645±0.00 ^{C}	0.241±0.02 ^{C}	0.042±0.00 ^{A}	0.607±0.00 ^{A}	2.42±0.00 ^{D}
AUC	0.010±0.00 ^{B}	0.704±0.00 ^{B}	0. 257±0.02 ^{B}	0.023±0.00 ^{B}	0.500±0.00 ^{B}	2.60 ±0.00 ^{C}
AUA	0.010±0.00 ^{B}	0.704±0.00 ^{B}	0. 257±0.01 ^{B}	0.022±0.00 ^{B}	0.500 ±0.00 ^{B}	2.42±0.00 ^{D}
AHC	0.010±0.00 ^{B}	0.704±0.00 ^{B}	0. 257±0.01 ^{B}	0.024±0.00 ^{B}	0.600±0.00 ^{A}	3.17±0.00 ^{B}
AHA	0.010 ±0.00 ^{B}	0.704 ±0.00 ^{B}	0. 257±0.02 ^{B}	0.022±0.00 ^{B}	0.390± 0.00 ^{D}	2.35±0.00 ^{D}
AC	0.010±0.00 ^{B}	0.704 ±0.00 ^{B}	0. 257±0.00 ^{B}	0.022 ±0.00^{B}	0.460 ±0.00 ^{C}	2.96±0.00 ^{C}
AA	0.010 ±0.00 ^{B}	0.704 ±0.00 ^{B}	0. 257±0.01 ^{B}	0.020±0.00 ^{C}	0.420±0.00 ^{D}	4.14±0.00 ^{A}

Relatively, Amani et al. reported effects of the activity of starters during storage due to their protein hydrolyzing characteristics on phase separation [37]. In addition, *L. casei* was reported to include lower serum separation ratios than that *L. acidophilus* did (p<0.05). This suggested that the type of bacteria in the samples played important roles in the serum separation rate because various strains of probiotic bacteria included various abilities to ferment and break down organic compounds and producing exo-endo polysaccharides as discussed in viscosity section [39]. However, np statistically significant differences were detected between the sequences of probiotic additions (p> 0.05).

3.3 Viscosity

Naturally, acidification and lowering of pH during fermentation cause milk casein proteins to clump, affecting viscosity of the final products. Figure 3 shows assessed viscosity of the samples. Sonicated probiotic-containing treatments (BUC and BUA) included the highest viscosity (3.083 ±0.6 and 3.515 ±0.5, respectively). Additionally, addition of live probiotics during fermentation led to increased viscosity, compared to samples without probiotics. It was reported that the release of exopolysaccharides and intracellular polysaccharides from the probiotics significantly increased viscosity [40, 41]. Exopolysaccharides secreted by *Lactobacillus* spp. during their growth affect viscosity of dairy products [42]. Moreover, "intracellular polysaccharides" are polysaccharides that accumulate within cells. The intracellular biosynthetic process involves transferring sugar residues into the cell, converting them into various monomeric units, partially polymerizing them and attaching them to isoprenoid lipid carriers [43]. Viscosity of heat-inactivated treatments was similar to that of control treatment, possibly because intact cells of probiotic bacteria did not release biopolysaccharides into doogh samples. Furthermore, type of bacteria significantly affected the viscosity (p<0.05). It was previously reported that variations in the viscosity values could be affected by characteristics of the probiotics cultures as well as adaptability of the bacteria [44].

3.4 Antioxidant activity assessment

The DPPH radical scavenging method, widely used to assess antioxidant activities, is simple, rapid, sensitive and reproducible compared to other methods [45]. Figure 4 shows antioxidant capacities of the samples on Days 1 and 28. Antioxidant capacity of the samples decreased significantly during the storage due to inappropriate sealing, oxygen entry into the samples and uncontrolled bacterial activity. Sonicated probiotic-containing treatments increased the antioxidant capacity, as the intracellular content of lactic acid bacteria (LAB) demonstrated greater antioxidant characteristics than that the whole cell or the extracellular metabolites did [46,47]. Antioxidant activity of the intra-cellular contents of LAB was linked to the activity of superoxide dismutase (SOD), glutathione peroxidase (GPx), nicotinamide adeninedinucleotide (NADH)-oxidase, NADH peroxide and glutathione (GSH) enzymes [48]. In addition, use of live or heat-inactivated probiotics did not result in significant differences (p>0.05). This was due to the cell contents that were not released. Relatively, lyophilized cells of *Lactococcus lactis* subsp. *cremorishave* included the highest antioxidant capacity, compared to those the heat-killed and intact cells did [49].

In contrast, use of *L. acidophilus* rather than *L. casei* significantly increased the antioxidant capacity (p<0.01). Amdekar et al. assessed antioxidant and anti-inflammatory potentials of *L. casei* and *L. acidophilus* in *in-vitro* models of arthritis. Results indicated that arthritic rats treated with *L. acidophilus* included higher glutathione peroxidase and decreased glutathione concentration, compared to that arthritic rats treated with *L. casei* did [50]. Additionally, adding probiotics before fermentation improved the antioxidant capacity of doogh samples (p>0.05).

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Table 2. The pH, oxidation-reduction potential and titratable acidity changes during storage

Sample	pH					Reduction potential					Acidity				
	Day 1	Day 7	Day 14	Day 21	Day 28	Day 1	Day 7	Day 14	Day 21	Day 28	Day 1	Day 7	Day 14	Day 21	Day 28
BUC	4.5±0.00 Aa	4.02±0.00 Db	3.96±0.00 Ec	3.94±0.00 Fd	3.92±0.00 Fe	130±0.00 Ia	135±0.00 Gb	139±0.00 Ec	140±0.00 Dd	141±0.00 De	67±0.00 Ma	91±0.00 Jb	108±0.80 Ic	116±0.10 Hd	117±2.00 He
AUC	4.5±0.00 Aa	4.01±0.00 Db	3.94±0.00 Fc	3.88±0.00 Gd	3.84±0.00 Fe	130±0.00 Ia	134±0.10 Gb	141±0.00 Dc	143±0.00 Cd	144±0.00 Be	70±0.00 La	83±1.00 Kb	110±0.70 Hc	138±0.00 Fd	143±0.30 Ee
BUA	4.5±0.00 Aa	4.01±0.02 Db	3.94±0.01 Fc	3.87±0.00 Gd	3.85±0.00 He	130±0.10 Ia	135±0.20 Gb	141±0.00 Dc	143±0.20 Cd	144±0.10 Be	84±2.00 Ka	105±1.00 Ib	127±0.70 Gc	130±0.30 Fd	170±0.20 Be
AUA	4.5±0.00 Aa	4.06±0.01 Cb	3.94±0.00 Fc	3.88±0.01 GD	3.86±0.01 Ge	130±0.00 Ia	133±0.10 Hb	139±0.00 Ec	141±0.10 Dd	144±0.00 Be	71±0.00 La	90±0.60 Jb	127±0.00 Gc	130±1.00 Fd	139±0.40 Ee
BHC	4.5±0.00 Aa	4.02±0.01 Db	3.97±0.00 Ec	3.92±0.02 Fd	3.88±0.00 Ge	130±0.00 Ia	137±0.00 Fb	138±0.10 Ec	139±0.00 Ed	143±0.20 Ce	70±0.90 La	97±1.00 Jb	119±0.30 Hc	121±0.80 Gd	123±2.00 Ge
AHC	4.5±0.01 Aa	3.94±0.00 Fb	3.87±0.01 Gc	3.85±0.01 Hd	3.82±0.01 He	130±0.00 Ia	140±0.00 Db	143±0.00 Cc	144±0.10 Bd	146±0.20 Ae	71±100 La	110±0.40 Hb	145±2.00 Ec	157±0.70 Cd	160±0.60 Ce
BHA	4.5±0.00 Aa	4.12±0.00 B b	4.06±0.00 Cc	3.95±0.00 Fd	3.93±0.00 Fe	130±0.10 Ia	132±0.10 Hb	135±0.00 Gc	139±0.20 Ed	141±0.10 De	57±0.00 Na	87±0.00 KB	119±2.00 Hc	123±0.90 Gd	130±1.00 Fe
AHA	4.5±0.01 Aa	4. 00 ±0.00 D b	3.93±0.00 Fc	3.9±0.00 Gd	3.88±0.01 Ge	130±0.00 Ia	137±0.00 Fb	138±0.20 Ec	139±0.00 Ed	141±0.00 De	71±1.00 La	100±0.30 Ib	106±2.00 Ic	121±0.10 Gd	135±0.60 Fe
BC	4.5±0.01 Aa	4.07±0.03 Cb	3.88±0.01 Gc	3.82±0.01 Hd	3.8±0.00 Ie	130±0.00 Ia	135±0.00 Gb	144±0.00 Bc	145±0.00 Bd	146±0.00 Ae	64±1.00 Ma	109±0.60 Ib	115±1.00 Hc	141±0.10 Ed	145±2.00 Ee
AC	4.5±0.00 Aa	3.98±0.00 Eb	3.94±0.01 Fc	3.89±0.00 Gd	3.87±0.02 Ge	130±0.00 Ia	139±0.10 Eb	140±0.00 Dc	142±0.00 Cd	143±0.00 Ce	71±2.00 La	110±0.80 Hb	124±1.00 Gc	146±1.00 Ed	154±2.00 De
BA	4.5±0.00 Aa	4.01±0.01 Db	3.91±0.00 Fc	3.88±0.01 Gd	3.82±0.00 He	130±0.00 Ia	136±0.00 Fb	140±0.10 Dc	143±0.00 Cd	147±0.00 Ae	72±1.00 La	93±0.60 Jb	120±0.00 Gc	134±0.90 Fd	140±0.30 Ee
AA	4.5±0.00 Aa	4.02±0.01 Db	3.98±0.00 Ec	3.95±0.00 Ed	3.93±0.01 Fe	130±0.00 Ia	136±0.20 Fb	138±0.00 Ec	140±0.00 Dd	142±0.00 Ce	71±0.30 La	91±1.00 Jb	121±200 Gc	156±0.70 Dd	187±0.60 Ae

Continue *Means show with different English letters represent significant differences ($p < 0.05$) in the same columns (among treatments).
BUC= Sample contained inactivated *Lacticaseibacillus casei* by ultrasound before added fermentation, AUC= Sample contained inactivated *Lacticaseibacillus casei* by ultrasound after added fermentation
BUA= Sample contained inactivated *Lactobacillus acidophilus* by ultrasound added before fermentation,, BHA: Sample contained inactivated *Lactobacillus acidophilus* by heat added before fermentation
AUA= Sample contained inactivated *Lactobacillus acidophilus* by ultrasound added after fermentation,,, BHC= Sample contained inactivated *Lacticaseibacillus casei* by heat added before fermentation,,
AHC= Sample contained inactivated *Lacticaseibacillus casei* by heat added after fermentation,, AHA= Sample contained inactivated *Lactobacillus acidophilus* by heat added after fermentation,,
AC: Sample contained live *Lacticaseibacillus casei* added after fermentation,,, BA= Sample contained live *Lactobacillus acidophilus* added before fermentation,, AA= Sample contained live *Lactobacillus acidophilus* added after fermentation,,

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Table 3. Phase separation proportions in treatments during refrigerared storage

Sample	Storage day				
	Day 1	Day 7	Day 14	Day 21	Day 28
BUA	47 ±0.36 ^{Ba}	45±0.90 ^{Bb}	42±2.30 ^{Bc}	40±0.21 ^{Bd}	28±0.00 ^{Ce}
BHC	57±0.14 ^{Aa}	52±0.0 ^{Bb}	48.5±0.30 ^{Bc}	44.4±0.40 ^{Bd}	25±0.40 ^{De}
AA	55±0.10 ^{Aa}	50±0.70 ^{Bb}	37±0.80 ^{Cc}	35±0.08 ^{Cd}	30±0.30 ^{De}
AHA	60±0.27 ^{Aa}	55±0.40 ^{Ab}	49±0.50 ^{Bc}	44±0.77 ^{Bd}	31±2.00 ^{Ce}
BHA	50± 0.25 ^{Ba}	46±0.84 ^{Bb}	43±0.30 ^{Bc}	38±0.00 ^{Cd}	30±1.00 ^{Ce}
BA	55± 0.50 ^{Aa}	52±0.20 ^{Bb}	49±1.30 ^{Bc}	41±0.66 ^{Bd}	38±0.54 ^{Ee}
BC	50±0.90 ^{Aa}	46±0.03 ^{Cb}	40±0.90 ^{Cc}	37±0.02 ^{Dd}	33±0.80 ^{De}
AHC	65±0.60 ^{Aa}	52±0. 80 ^{Bb}	39±0.30 ^{Bc}	30±0.40 ^{Cd}	23±0.60 ^{De}
AUC	40±3.00 ^{Ba}	37±0.40 ^{Cb}	34±0.10 ^{Cc}	30±0.50 ^{Cd}	28±0.00 ^{Ce}
AC	65±0.30 ^{Aa}	57±0.80 ^{Bb}	48±1.00 ^{Cc}	40±0.00 ^{Dd}	32±0.07 ^{De}
AUA	54±0.40 ^{Aa}	46±1.00 ^{Bb}	39±0.35 ^{Bc}	33±0.32 ^{Cd}	27±0.09 ^{Ce}
BUC	32 ±0.05 ^{Ca}	28±0.00 ^{Cb}	24±0.09 ^{Dc}	22±0.06 ^{Dd}	21±0.30 ^{De}

*Means show with different English letters represent significant differences ($p< 0.05$) in the same columns (among treatments).

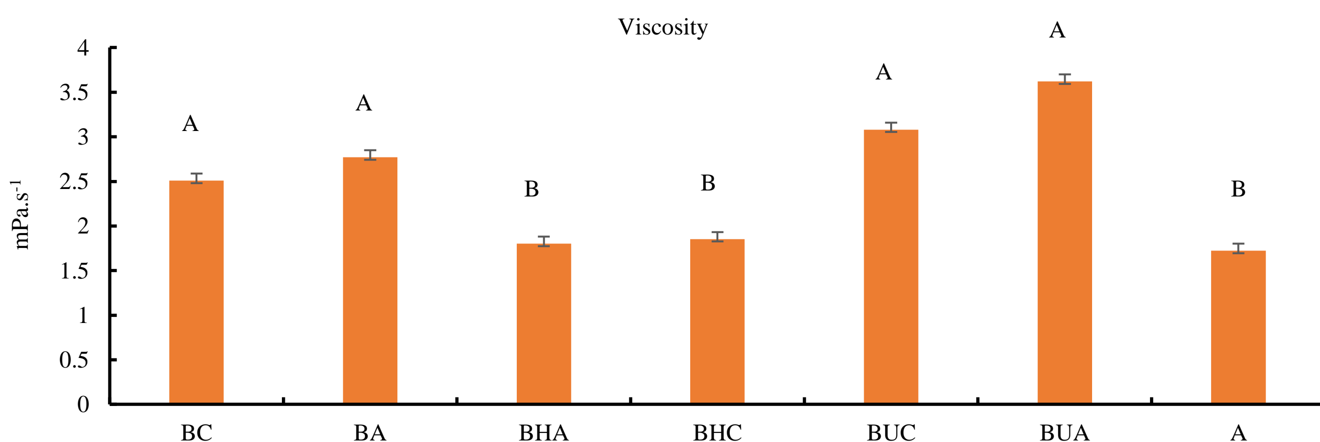

Figure 3. Viscosity of treatments at the end of fermentation. *Means in a raw shown with different capital letters are significantly different ($p < 0.05$).

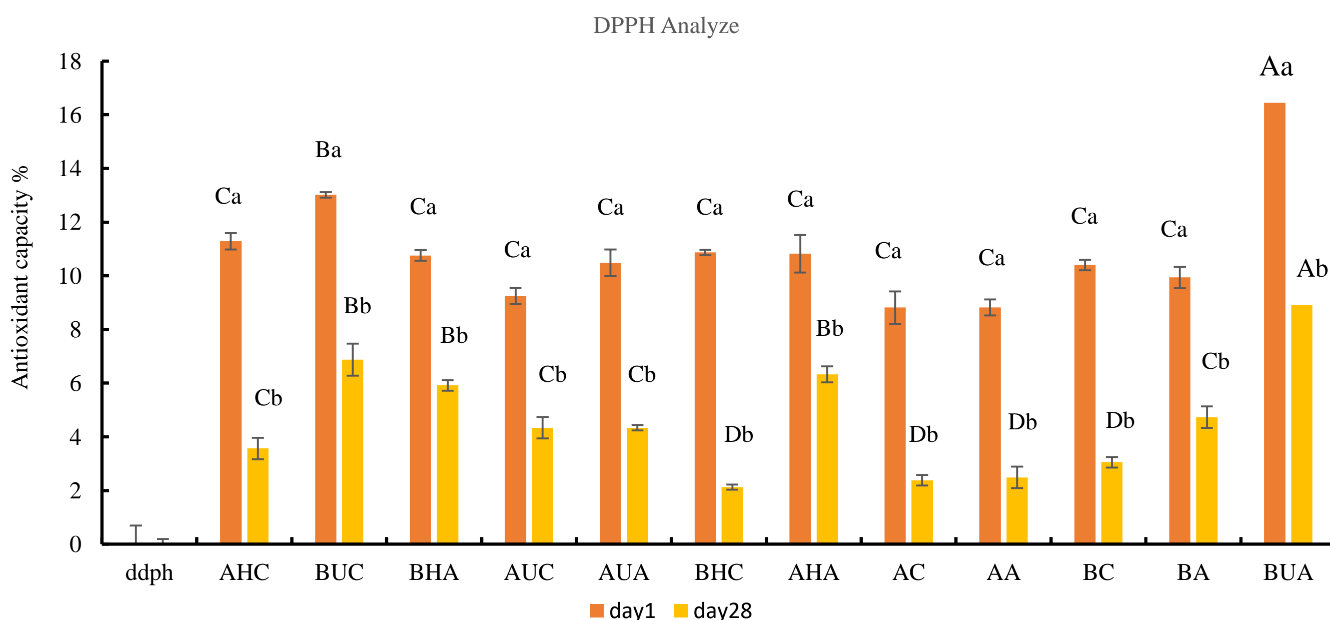

Figure 4. Antioxidant capacities of the samples at the end of fermentation and at the end of storage period.

*Means in the same column shown with diverse small letters are significantly different ($p<0.05$). Means in a raw shown with different capital letters are significantly different ($p<0.05$).

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3.5 Color analysis

Color is a critical characteristic in assessing quality of products such as yogurts and doughs. The L* parameter indicates lightness or darkness of the color, the a* parameter shows redness or greenness of the color and the b* parameter represents yellow or blueness of the color [51]. The color values are shown in Fig. 5. Integration of probiotics inactivated using ultrasound resulted in increases in a* value, indicating release of probiotic contents into the doogh sample ($p<0.05$) and showing that green pigment substances such as thiamine were present in intracellular probiotics [52]. However, no significant differences were reported between the paraprobiotics and probiotics in a* value ($p>0.05$). Additionally, no significant differences were demonstrated between the sequential additions of probiotics in a* value ($p > 0.05$). Type of the probiotics in doogh samples included significant effects on a* value ($p < 0.05$). It has previously been suggested that various types of bacteria with special characteristics can affect color of the products [53]. In L* and b* values, no differences were observed within the addition of active/inactivated probiotics into doogh samples ($p>0.05$). Furthermore, types of probiotic bacteria (*L. casei* or *L. acidophilus*) and probiotic adding sequences did not include significant effects on L* and b* values ($p > 0.05$).

3.6 Viable counts of the starter bacteria and probiotics

The *L. bulgaricus* and *S. thermophilus* are critical for acidification and production of doogh [54]. Survival of the starter and probiotic bacteria in yogurts depends on various factors such as the specific strains, interactions between the species, chemical compositions of the yogurts, the culture conditions, production of hydrogen peroxide during bacterial metabolism, final acidity of the yogurts, rates of lactic and acetic acids, nutrient availability and the storage temperature [46]. Table 4 shows the number of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* for all samples during the storage. The BUA included the highest number of *L. delbrueckii* subsp. *bulgaricus* on the first and the last days of enumeration (logs 11.89 and 10.91 CFU ml^{-1}, respectively), while BC included the lowest number (logs 11.35 and 9.68 CFU ml^{-1}, respectively). Additionally, BA and BUA included the lowest and the highest *L. delbrueckii* subsp. *bulgaricus* counts at the end of storage (logs 7.84 and 10.91 CFU ml^{-1}, respectively). Type of probiotics (viable or non-viable) in doogh samples included significant effects on the viability of *L. delbrueckii* subsp. *bulgaricus* ($p<0.05$) through competitive interactions, metabolic activities, cell-cell interactions and protective effects. However, a study by Parvayi et. al (2021) showed that addition of viable or non-viable probiotics did not affect *L. delbrueckii* subsp. *bulgaricus* when used as the starter bacteria [15].

As a statistical result, significant differences were observed between using *L. casei* and *L. acidophilus* probiotic bacteria ($p<0.05$). Selection of probiotic strains such as *L. casei* and *L. acidophilus* in doogh could affect viability of *L. delbrueckii* subsp. *bulgaricus* through species-specific interactions, metabolic compatibility, competition for resources, synergistic or antagonistic effects and stability of the microbial community. In addition, inhibition of *L. delbrueckii* subsp. *bulgaricus* growth by *L. acidophilus* has previously been reported. Vinderola reported *L. casei* strains did not include effects on the growth of *L. delbrueckii* subsp. *bulgaricus* [35]. Furthermore, no statistical differences were reported between the sequences of adding probiotics (before or after fermentation) into doogh samples ($p>0.05$). In contrast, viability of *S. thermophilus* decreased significantly ($p<0.05$) during the storage. On the first day of storage, BUC included the highest number of *S. thermophilus* (log 14.67 CFU ml^{-1}), whereas BA included the lowest number of *S. thermophilus* (log 13.54 CFU ml^{-1}). In addition, AA and AUC included the lowest and the highest *S. thermophilus* counts at the end of storage (logs 10.11 and 12.85 CFU ml^{-1}, respectively). The highest rate of decrease in *S. thermophilus* viability was associated to AA (0.73), while AUA included the lowest rate of decrease in *S. thermophilus* viability (0.88). Addition of non-viable probiotics caused significant differences in the bacterial population, compared with that addition of live probiotics did ($p<0.05$). This could be due to the indirect antagonistic effects of live probiotics [4, 35].

Doogh samples with inactivated probiotic cells showed significantly higher starter proliferation, compared to those treated with probiotic bacteria due to the cell wall structure of *L. acidophilus* and *L. casei* in their ruptured cells ($p<0.05$). Naturally, cell wall majorly consists of teichoic acids, cell structural protein (S-layer), peptidoglycan and polysaccharides [10]. Additionally, LAB intracellular contents include GABA, B-vitamin complex, poly-saccharides, biopeptides, polysaccharides and lipoteichoic acids [4,47,55]. Therefore, fermentation in the environment is strengthened when the intracellular contents are released into doogh. It is possible that choosing the right time for inoculation can significantly affect growth of starter bacteria. Results showed significant differences ($p < 0.01$) between adding probiotics before or after the fermentation process, affecting viability and activity of the starter bacteria due to its adaptation to the culture medium during fermentation and storage. Moreover, it was reported that *L. acidophilus* included stronger growth inhibitory effects on *S. thermophilus* than that *L. casei* did ($p <0.01$). In a study by Vinderola et. al (2002), *L. casei* strains inhibited growth of *S. thermophilus* while *L. acidophilus* did not affect the growth of *S. thermophilus* [35]. Sample inoculated with live probiotics before fermentation included the lowest count of *S. thermophilus* due to the potential antagonism effects of the probiotics ($p<0.01$).

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Table 4. Viability (log CFU ml^{-1}) of the probiotics and traditional yogurt bacteria at the end of fermentation and during the storage*

Sample	*S. thermophilus log count*						*L. bulgaricus log count*					
	Storage day					Viability	Storage day					Viability
	Day 1	Day 7	Day14	Day21	Day28		Day 1	Day 7	Day14	Day21	Day28	
AA	13.78±0.00^{Ca}	13.04±0.00^{Db}	12.77±0.00^{Ec}	11.80±0.00^{Gd}	10.11±0.04^{Je}	0.73±0.01^{B}	11.88±0.03^{Aa}	11.71±0.01^{Ab}	11.04±0.01^{Ac}	10.77±0.00^{Bd}	8.72±0.02^{Ce}	0.73±0.01^{B}
BA	13.54±0.00^{Ca}	13.14±0.00^{Da}	12.60±0.06^{Eb}	12.34±0.02^{Fc}	10.28±0.07^{Id}	0.75±0.03^{B}	11.55±0.02^{Aa}	10.60±0.01^{Bb}	9.84±0.01^{Cc}	9.20±0.02^{Cd}	7.84±0.01^{Be}	0.73±0.01^{B}
BC	13.57±0.00^{Ca}	13.20±0.00^{Da}	12.76±0.00^{Eb}	12.49±0.06^{Fc}	10.22±0.07^{Gd}	0.75±0.01^{B}	11.35±0.01^{Aa}	11.49±0.02^{Ab}	10.47±0.00^{Bc}	10.14±0.01$^{B d}$	8.68±0.01^{Ce}	0.76±0.00^{B}
AC	13.77±0.01^{Ca}	13.27±0.00^{Db}	12.87±0.00$^{E c}$	12.60±0.00^{Fd}	10.80±0.00^{He}	0.78±0.00^{B}	11.68±0.02^{Aa}	10.67±0.02^{Bb}	9.90±0.01^{Cc}	9.61±0.01^{cd}	8.47±0.02^{De}	0.68±0.02^{B}
AUC	14.77±0.01^{Aa}	14.04±0.02^{Bb}	13.69±0.01^{Cc}	13.30±0.00^{Dd}	12.85±0.01^{Ee}	0.88±0.00^{A}	11.71±0.02^{Aa}	10.81±0.01^{Bb}	9.95±0.03^{Cc}	9.70±0.00^{cd}	9.64±0.01^{Ce}	0.82±0.02^{A}
AUA	14.67±0.00^{Aa}	13.98±0.01^{Cb}	13.49±0.00^{Dc}	13.20±0.00^{Dd}	12.68±0.00^{Ee}	0.88±0.03^{A}	11.74±0.02^{Aa}	10.93±0.01^{Bb}	10.07±0.01^{Bc}	9.63±0.00^{cd}	9.23±0.01^{De}	0.78±0.00^{B}
BUC	14.67±0.01^{Aa}	14.27±0.00^{Bb}	13.89±0.01^{Cc}	13.77±0.00^{Cd}	12.91±0.02^{Ee}	0.86±0.02^{A}	11.72±0.01^{Aa}	10.91±0.02^{Bb}	10.23±0.02^{Bc}	9.62±0.03^{cd}	9.11±0.00^{Ce}	0.85±0.01^{A}
BUA	14.97±0.00^{Aa}	14.79±0.00^{Ab}	13.99±0.00^{Cc}	13.93±0.01^{Cd}	12.83±0.00^{Ee}	0.88±0.01^{A}	11.77±0.02^{Aa}	11.39±0.01^{Ab}	10.50±0.03^{Bc}	10.07±0.02^{Bd}	9.83±0.00^{Ce}	0.92±0.02^{A}
BHA	14.77±0.01^{Aa}	13.94±0.01^{Cb}	13.54±0.00^{Cc}	13.46±0.00^{Dd}	12.79±0.00^{Ee}	0.79±0.01^{B}	11.72±0.01^{Aa}	10.77±0.03$^{B b}$	10.49±0.01^{Bc}	10.36±0.02^{Bd}	9.69±0.01^{Ce}	0.82±0.01^{A}
BHC	14.89±0.01^{Aa}	14.30±0.00^{Bb}	14.07±0.01^{Bc}	12.47±0.02^{Fd}	11.76±0.00^{Ge}	0.86±0.00^{A}	11.77±0.01^{Aa}	11.03±0.02^{Ab}	9.77±0.01^{Cc}	9.00±0.00^{cd}	9.36±0.01^{De}	0.80±0.01^{A}
AHC	14.57±0.00^{Aa}	14.04±0.02^{Bb}	13.49±0.00^{Dc}	13.17±0.01^{Dd}	12.88±0.00^{Ee}	0.87±0.01^{A}	11.74±0.00^{Aa}	11.29±0.01^{Ab}	10.171±0.02^{Bc}	9.740±0.03^{cd}	8.98±0.00^{De}	0.78±0.02^{B}
AHA	14.54±0.01^{Aa}	14.38±0.00^{Bb}	13.81±0.01^{Cc}	13.69±0.00^{Cd}	12.85±0.00^{Ee}	0.87±0.03^{A}	11.89±0.00^{Aa}	11.69±0.02^{Ab}	11.44±0.01^{Ac}	11.14 ±0.02^{Ad}	10.91±0.03^{Be}	0.83±0.0^{A}

Viability (log CFU ml^{-1}) of probiotics

Sample	Probiotic	Storage day					Viability
		Day 1	Day 7	Day14	Day21	Day28	
AA	*L. acidophilus*	9.23±0.01^{Ba}	8.60±0.00^{cb}	7.69±0.00^{Ec}	7.61±0.01^{Ed}	7.31±0.03^{Ge}	0.79±0.01^{A}
BA	*L. acidophilus*	9.84±0.01^{Aa}	9.66±0.00^{Ab}	8.77±0.00^{Dc}	8.08+0.00^{Ed}	7.50±0.00^{Ee}	0.79±0.01^{A}
AC	*L.casei*	9.59±0.01^{Aa}	9.07±0.02^{Bb}	8.76±0.01^{Cc}	8.20±0.00^{Dd}	7.61±0.01^{Ee}	0.80±0.00^{A}
BC	*L.casei*	9.77±0.01^{Aa}	9.43±0.00^{Bb}	8.98±0.04^{Bc}	8.57±0.01^{cd}	7.90±0.00^{Ee}	0.76±0.02^{A}

S. thermophilus log count= Streptococcus thermophilus log count

L. bulgaricus log count= Lactobacillus bulgaricus log count

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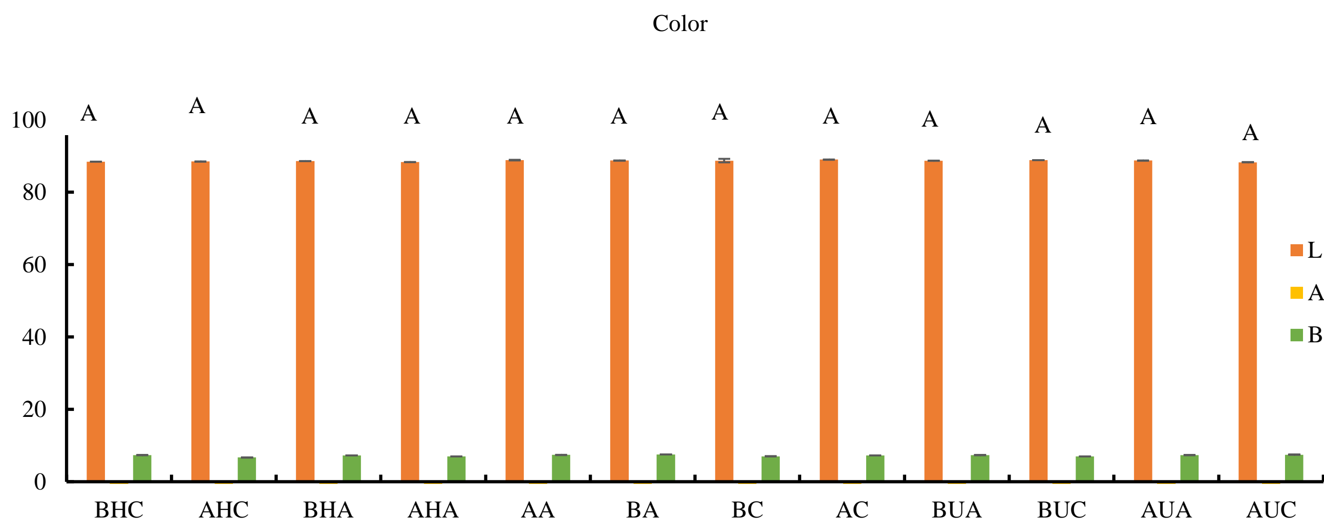

Figure 5. Color parameters in the treatments at the end of fermentation. *Means in a raw shown with different capital letters are significantly different ($p<0.05$).

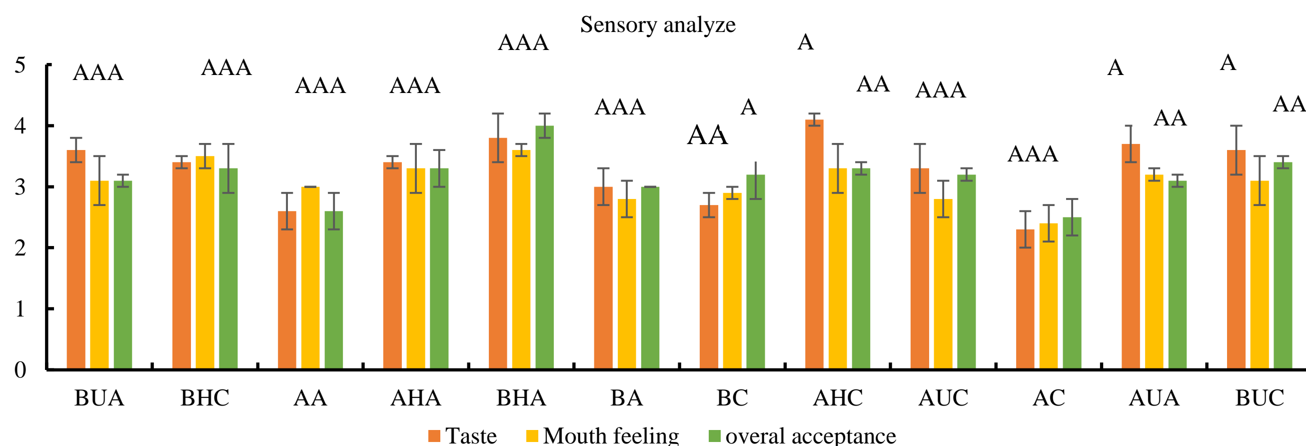

Figure 6. Sensory attributes in the treatments at the end of fermentation. *Means in a raw shown with different capital letters are significantly different ($p < 0.05$).

Generally, live probiotics included side effects on the growth and viability of the starter bacteria. For nonviable probiotic cells, these inhibitory effects are rarely observed and can surprisingly promote starter bacterial activity by providing various nutritious (e.g., amino acids, minerals, B vitamins and saccharides) and growth stimulatory elements [56]. Moreover, live probiotic bacteria include side effects on the starter bacterial growth because of their antimicrobial secretion and competition. Probiotic bacteria included more inhibitory effects on LAB than that LAB did when probiotics were not present [35]. For inactivated probiotics, there are no severe competitions for nutrition between the starter bacteria that may nourish them and enhance their growth due to the release of cytoplasmic contents. It is noteworthy that addition of live probiotics to media before fermentation increased the number of probiotic cells during storage due to better adaptation ($p < 0.05$). Furthermore, *L. acidophil*us was more susceptible than *L. casei* and statistically significant differences were recorded in viability of the probiotic bacteria ($p < 0.05$). Therefore, it can be concluded that selection of an appropriately adaptable strain may play critical roles in preserving viability of probiotics during the shelf life of the products [36].

3.7 Sensory evaluation

Sensory attributes play key roles in attracting consumers. Daily probiotic products may include distinct tastes that are not be accepted by the consumers [57]. Therefore, studies have focused to improve consumer acceptance of probiotic beverages. In this study, taste, mouthfeel and overall acceptability of sensory aspects were assessed on the first day of fermentation (Figure 6). The AHC included the highest score (4.1/5) for taste, while AC included the lowest score (2.6/5) due to uncontrolled lactic acid formation. Nevertheless, no significant differences were seen for taste, mouthfeel and total acceptance of doogh samples ($p > 0.05$). A study demonstrated that probiotic beverages containing *L. casei* included high acceptance, compared with beverages containing *L. acidophilus* due to desirable flavors [58].

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4. Conclusion

These non-viable probiotics have been shown to eleminate technological limitations by enhancing rates of titratable acidity and fermentation, texture, viability of starter bacteria including *S. thermophilus* and *L. bulgaricus* and decreasing post-acidification rate as well as potentially improving gut health and immunity by increasing antioxidant capacity of doogh samples. Further studies are needed to fully understand mechanisms of these effects and optimize formulation of non-viable probiotics in fermented milk products. Overall, findings suggest that incorporating non-viable probiotics into fermented milks can be a valuable strategy for enhancing functional characteristics of dairy products.

5. Acknowledgements

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6. Conflict of Interest

The authors report no conflicts of interest.

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بررسی اثرات افزودن زیستیارهای غیرزنده *لاکتوباسیلوس کازئی* و *لاکتوباسیلوس اسیدوفیلوس* بر خواص فیزیکوشیمیایی، میکروبی و حسی دوغ

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چکیده

سابقه و هدف: زیستیارهای[1] غیرفعال فواید سلامتی و تکنولوژیکی گوناگونی دارند که آنها را برای تولید محصولات لبنی فراسودمند مناسب می‌سازند. هدف از این مطالعه بررسی اثرات افزودن زیست-یارهای غیرقابل زنده (*لاکتوباسیلوس کازئی* ۴۳۱ و *لاکتوباسیلوس اسیدوفیلوس* LA-5) به دوغ (یک نوشیدنی شیری تخمیری معمول در ایرانی) بود.

مواد و روش ها: زیستیارها با حرارت یا فراصوت غیرفعال شده و به نمونه‌ها قبل یا بعد از تخمیر اضافه شدند. پارامترهای گوناگونی مانند pH، اسیدیته قابل تیتراسیون، پتانسیل ردوکس، ظرفیت ضداکسیدانی، رنگ، گرانروی، جداسازی فاز، زنده‌مانی باکتری‌های آغازگر سنتی و زیستیارها و همچنین ویژگی‌های حسی در طی تخمیر و نگهداری در یخچال در دمای ۵ درجه سلسیوس ارزیابی شدند.

یافته‌ها و نتیجه‌گیری: تیمارهای حاوی زیستیار غیرفعال‌شده با فراصوت شامل بالاترین میزان کاهش pH(۰/۰۱۱ pH در دقیقه) در طول تخمیر و همچنین دارای بالاترین ظرفیت ضداکسیدانی (۱۶/۴۵%) و گرانروی (۳۵/۱۵ mPa.s) بودند. این در حالی بود که تیمارهای حاوی زیستیارهای غیر فعال‌شده با حرارت شامل کمترین گرانروی (۱۷/۶۰ mPa.s). تیمارهای با زیستیارهای زنده به‌طور منطقی شامل بالاترین نرخ پس از اسیدی شدن در طول ذخیره‌سازی (۴/۱۴°D d-1)، در مقایسه با تیمارهای حاوی سلول‌های غیرزنده بوده و همچنین حداقل میزان جداسازی فاز را داشتند. مقادیر *b و *L رنگ در تیمارها تفاوت معنی‌داری نداشت، اما بالاترین مقدار *a در تیمارهای غیرفعال شده با فراصوت مشاهده شد. بیشترین جمعیت باکتریایی در مدت نگهداری برای *لاکتوباسیلوس دلبروکی* زیرگونه *بولگاریکوس* (log ۱۱/۸۹۱ CFU ml-1) و *استرپتوکوکوس ترموفیلوس* (log ۱۴/۹۷۷ CFU ml-1) به ترتیب در تیمارهای زیستیارغیرفعال شده با حرارت (در مقایسه با زیستیارهای زنده) و تیمارهای زیستیارهای غیرفعال شده با فراصوت مشاهده شدند. علاوه بر این، *لاکتوباسیلوس اسیدوفیلوس* نسبت به *لاکتی‌کازئی باسیلوس* حساس‌تر بود و زنده مانی کمتری داشت. طعم، احساس دهانی و پذیرش کلی همه نمونه‌ها در تیمارها تفاوت معنی‌داری نداشت. مطالعه حاضر نشان می‌دهد که زیستیارهای غیرفعال را می‌توان با موفقیت در تولید نوشیدنی‌های شیر تخمیر شده با ویژگی‌های حسی مناسب و ظرفیت آنتی‌اکسیدانی بالاتر در مقایسه با گروه شاهد استفاده کرد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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[1] Probiotics غذاها یا مکمل‌هایی که به دلیل داشتن ریزاندامگان‌ها قادر به بازسازی یا تغییر گیاگان میکربی روده هستند و در حفظ سلامت انسان مؤثرند

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