



Biochemical Characteristics and Viability of Probiotic and Yogurt Bacteria in Yogurt during the Fermentation and Refrigerated Storage

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Abstract

This research aimed to investigate the viability of probiotic bacteria (*Lactobacillus acidophilus* LA-5 and *Bifidobacterium lactis* BB-12) and yogurt bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) in yogurt during the fermentation, immediately after fermentation and during refrigerated storage (21 d, 4°C). Also the biochemical characteristics of milk as affected by the commercial 4-strain mixed starter culture were investigated. Storage time affected the viability of all bacterial species. The concentration of lactic acid during the fermentation increased in parallel with the titrable acidity, and the concentration of acetic acid was proportional to the viability of *Bifidobacterium lactis*. The acetaldehyde level was decreased in the yogurt from day 0 up to the end of the storage. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* were multiplied considerably during the fermentation. *Streptococcus thermophilus* could maintain its viability to the highest level, but *Lactobacillus delbrueckii* ssp. *bulgaricus* lost its viability rapidly during the cold storage compared to *Streptococcus thermophilus*. The multiplication and viability of probiotic bacteria were also influenced by the associative strains and species of yogurt organisms. Bifidobacteria counts were satisfactory. The loss of viability for bifidobacteria was gradual and steady during the storage, and they showed good stability during the storage as compared to *Lactobacillus acidophilus*.

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1. Introduction

Probiotics are referred to as 'live micro-organisms, which when administered in adequate amounts confer a health benefit on the host [1]. The popularity of probiotics has continuously been growing and various food products have been marketed. The majority of commercial probiotics are *Lactobacillus* and bifidobacteria species used in products such as yogurt, milk powder and frozen desserts [2, 3]. It has been known that probiotics have many health benefits such as antimicrobial activity, alleviating diarrhea, anticarcinogenic properties, and improving lactose intolerance and immune

system [1, 2, 4]. However, those health benefits are strain-specific, and no single strain has all of the proposed health benefits [2]. Although there is no specific standard on the required concentration of probiotics, giving maximum health benefits for different species and strains, the minimum dose of 10^6 cfu.g⁻¹ of food product at the time of consumption is accepted in general [3, 5, 6]. This concentration could be strain-specific, and the number of survived cells along the gastrointestinal tract the GI tract is more important. However, many probiotics containing food products fail to maintain the recommended

probiotic concentrations due to instability of probiotics in food matrices [7, 8].

Several factors affect the viability of probiotic bacteria. The low pH of fermented foods is one of the most important factors causing pronounced viability loss of probiotics [9-11]. Not only the low pH values but also the rate of drop in pH may affect the viability of probiotic bacteria. Higher acidification rates until a certain final pH of fermentation would lead to pH drop (fast pH decline) in the probiotic cells, and would significantly lower these cells' viability [10]. Hydrogen peroxide, produced by some lactobacilli, is known for its antimicrobial effects [12-14].

Bifidobacteria are anaerobic in nature; therefore, higher oxygen content may affect their growth and viability [15-16]. Antagonism among the bacteria used in the starter culture (caused by the production of antimicrobial substances such as bacteriocins) may decrease the number of sensitive organisms and probiotics in the products [17-18].

Although the biochemical characteristics and viability of probiotic bacteria in fermented milks have been investigated [5, 13], but there is no research on the biochemical characteristics and viability of yogurt bacteria along with probiotics during the fermentation and refrigerated storage in the commercial 4-strain mixed starter culture, which is frequently used in Iran dairy industry. Therefore, the objective of this study was to carefully examine and monitor the biochemical characteristics and viability of yogurt and probiotic bacteria during the fermentation and refrigerated storage (4°C).

2. Materials and Methods

2.1 Starter culture

The ABY culture (containing *Lactobacillus* (*L.*) *acidophilus* LA-5, *Bifidobacterium* (*B.*) *lactis* BB-12, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus* (*S.*) *thermophilus*), known as FD-DVS ABY-3, was supplied by Chr-Hansen (Horsholm, Denmark). This culture is currently used by the dairy industries worldwide for producing probiotic yogurt. The cultures were maintained according to the manufacturer's instructions at -18°C until used.

2.2 Study design and sample preparation

The yogurt treatments were produced using reconstituted skim milk powder and sterilized potable water. Reconstituted milk samples containing 12.0% milk solid non-fat (MSNF) were heat treated at 90°C for 15min. After inoculation, they were distributed in 100 ml glass

cups. Fermentation was carried out at 44°C until pH reached 4.5 ± 0.02 . Three replicates of each treatment were conducted. Biochemical parameters including pH drop, acidity increase, and redox potential increase were monitored during the fermentation period. These parameters were recorded every 30min. Parameters of incubation time (min), final titrable acidity, pH drop rate, acidity increase rate and redox potential increase rate, as well as the concentration of lactic acid, acetic acid and acetaldehyde were determined immediately after fermentation and at the end of the storage time. The probiotic organisms and yogurt bacteria were counted just at the end of fermentation (d 0) and also during the refrigerated storage (d 7, 14, and 21; 4°C).

2.3 Microbiological analysis

Viable counts of bacteria were enumerated using the pour plate and surface culture techniques. Acidified MRS Agar medium (MRS Agar from Merck, Darmstadt, Germany) was used for the selective count of *L. delbrueckii* ssp. *bulgaricus* [19]. The plates were incubated anaerobically at 37°C for at least 72h. Anaerobic conditions were produced using the GasPac system (Merck, Darmstadt, Germany).

M₁₇ Agar medium was used for the selective count of *S. thermophilus* [19]. The plates were incubated aerobically at 37°C for at least 48h. The selective count of *B. lactis* was performed using TOS-propionate Agar medium supplemented with mupirocin lithium salt and sodium propionate [15]. The plates were incubated anaerobically at 37°C for at least 72h. MRS/CL/CIP Agar medium containing clindamycin and ciprofloxacin was used for the selective count of *Lactobacillus acidophilus* [20]. The plates were incubated anaerobically at 37°C for at least 72h, and viable numbers were enumerated using the surface culture technique.

Viability proportion index (VPI) of the microorganisms at the end of fermentation and during the storage period was calculated as follows [9-10, 14]:

$$\text{VPI} = \frac{\text{final cell population (cfu.ml}^{-1}\text{)}}{\text{initial cell population (cfu.ml}^{-1}\text{)}} \quad (\text{Eq. 1})$$

2.4 Chemical analysis

The pH values and the redox potential of the yogurt samples were measured at room temperature using a pH meter. Titrable acidity was determined after mixing 10ml of the sample with 10ml of distilled water and titrating with 0.1N NaOH using 0.5% phenolphthalein [5, 13].

The pH drop rate [pH value min^{-1}], acidity increase rate [$\text{Dornic degree min}^{-1}$], and redox potential increase rate [Mv.min^{-1}] were calculated as follows [5, 13]:

$$\text{pH drop rate} = \frac{(\text{final pH value} - \text{initial pH value})}{\text{incubation period}} \quad (\text{Eq. 2})$$

$$\text{Acidity increase rate} = \frac{(\text{final acidity value} - \text{initial acidity value})}{\text{incubation time}} \quad (\text{Eq. 3})$$

$$\text{Redox potential increase rate} = \frac{(\text{final value} - \text{initial value})}{\text{incubation time}} \quad (\text{Eq. 4})$$

Quantification of acetaldehyde was carried out by static headspace gas chromatographic method [5]. Ten grams of sample was transferred in a 20 ml headspace vial (Agilent, USA), which was sealed with PTFE/BYTL headspace septa (Agilent, Germany) and aluminum cap (Agilent, USA). The samples were kept at -20°C until analysis. Prior to analysis, frozen samples of yogurt (10g) were thawed at 4°C overnight. The vials with samples were held at 60°C for 1h, then at 75°C for 10min and stirred 5 times, and subsequently kept for 5min. Headspace (250 μl) was injected with gas-tight syringe onto the GC column using split mode (10:1). The temperature of syringe was kept at 80°C . All glass materials (HS vials, and volumetric flasks) were sterilized before use. Double distilled water to be used for preparation of standard solutions was boiled for 20min to remove residual volatiles, and subsequently stored in a Stopped glass container. All chemicals were of analytical grade, supplied by Merck Ltd. (Merck House Poole, Dorset England BH15 ITD, UK).

The volatile compounds were separated on a HP-INNOWAX capillary column (30m x

0.32mm id x 0.25 μm film thickness) under the following conditions: injector temperature 200°C ; carrier gas helium at a flow rate of 1.4 ml.min^{-1} ; and oven temperature program initially held at 50°C for 6min, then programmed from 50°C to 180°C at $8^{\circ}\text{C.min}^{-1}$, and held at 180°C for 5min. The GC column was connected to the Agilent 5973N model mass selective (MS) detector (Agilent, USA), which was operating in the scan mode within a mass range of 33 to 330 m.z^{-1} at 1 scan s^{-1} . The interface line to MS was set at 250°C .

Quantification of lactic and acetic acids was carried out by High Performance Liquid Chromatography (CE 4200- Instrument, Cecil, Milton Technical Center, Cambridge CB46AZ, UK) [5, 10, 13]. For extraction of acids, 4.0g of the sample was diluted to 25ml with 0.1N H_2SO_4 . Then it was homogenized and centrifuged at 5000g for 10min. The supernatant was filtered through Whatman #1 filter paper and through a 0.20 μm membrane filter, and analyzed immediately. A Jasco UV-980 detector and a Nucleosil 100-5C18 column Macherey Nagel, Duren, Germany) were used. The mobile phase was 0.009N H_2SO_4 at a flow rate of 0.5 ml.min^{-1} . The wavelength of detection was optimized at 210nm. The standard solutions of lactic and acetic acids (Merck, Darmstadt, Germany) were prepared in distilled water. The retention times for lactic and acetic acids were 3.45 and 3.58min, and the standard curve regression coefficients were 0.989 and 0.991, respectively.

2.5 Statistical analysis

All experiments were performed in triplicate, and the significant differences among the means ($P < 0.05$) were analyzed using the ANOVA test from SAS software.

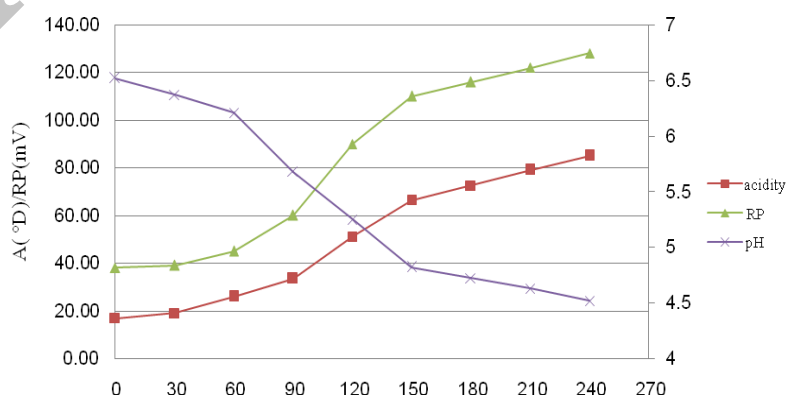


Figure 1. Changes in pH drop, acidity (A) increase, and redox potential (RP) increase during the fermentation period.

3. Results and discussion

3.1 Biochemical characteristics of probiotic yogurts during the fermentation and refrigerated storage

Figure 1 shows the changes in pH drop, titrable acidity increase, and redox potential increase during the fermentation period in the treatments. Table 1 presents mean pH drop rate, mean acidity increase rate, mean redox potential increase rate, incubation time, and final titrable acidity in the treatments during the fermentation and refrigerated storage. When the initial pH of milk (pH 6.51) decreased to 4.50, the fermentation was finished. The fermentation time to reach pH 4.50 for yogurt was 4 h. The titrable acidity values during the fermentation period ranged from 16.90°D to 85°D. The redox potential values varied from 38mv to 128mv. As shown in Figure 1, three distinguished phases, namely lag and pre-log phases, log phase, and late log and stationary phases could be observed [5, 13].

According to Figure 1, the sharpest pH decline in the treatments was observed within 60-90h of fermentation. In other words, the steepest decline of pH during fermentation was between the 60th and 90th minutes in which the pH reached 5.68

The minimum decrease rate of pH as well as the minimum increase rates of acidity and redox potential were observed within the initial steps of fermentation, representing the late lag/early log phase of bacterial growth. Another reason for the considerable slow decline in pH at the start of fermentation is the buffering capacity of the product. The rate of increase in redox potential

during the fermentation is linearly proportional to the rate of titrable acidity increase due to organic acids produced during the mentioned period. The redox potential of medium is supposed to affect the bacterial metabolism [12, 16]. It has been also demonstrated that this parameter increases the production of aroma compounds [12].

Table 1 shows an increase in titrable acidity to 120.2°D during the storage; this is the main evidence for post-acidification. Table 2 presents the concentration of acetic acid, lactic acid and acetaldehyde at the end of fermentation and storage time. As shown, there is a decrease in acetaldehyde level in the yogurt from the d 0 up to the end of storage. At lower pH values, acetaldehyde can easily be oxidized to acetate; therefore, during the storage, the level of acetaldehyde is decreased [3, 16, 21]. Additionally, acetaldehyde can easily be degraded to ethanol by alcohol dehydrogenase synthesized by *S. thermophilus*, and this is more likely at the later stages of storage since alcohol dehydrogenase is more active at lower pH values [3, 21]. During the manufacturing, production of acetaldehyde is only highlighted when a certain level of acidification is reached (pH 5.0). The acetic acid concentration at d 0 was 0.09%, which was increased to 0.10% at d 21. Further slight increases were observed throughout the storage period.

Bifidobacteria forms acetic acid during the fermentation, and this observation coincides with the changes in bifidobacteria population because the optimum pH for growth of bifidobacteria is 6.5-7.0, and the growth of this bacterium is significantly retarded below pH 5.5 [2, 9, 22].

Table 1. Biochemical characteristics of the treatments throughout the fermentation and refrigerated storage

Treatment	Mean pH-DR*		Mean A-IR		Mean RP-IR		Final acidity		Incubation time (min)
	F** (pH.min ⁻¹)	S (pH.day ⁻¹)	F (°D.min ⁻¹)	S (°D.day ⁻¹)	F (mV.min ⁻¹)	S (mV.day ⁻¹)	F (°D)	S (°D)	
Probiotic yogurt	0.008	0.008	0.28	0.34	0.37	1.62	85	97.50	240

* pH-DR= pH drop rate, A-IR= acidity increase rate, RP-IR= redox potential increase rate.

** F = during fermentation, S = during storage

Table 2. The concentration of acetic acid, lactic acid and acetaldehyde at the end of fermentation and storage time*

Treatment	Parameters					
	Lactic acid (%)		Acetic acid (%)		Acetaldehyde (mg.l ⁻¹)	
	d 0	d 21	d 0	d 21	d 0	d 21
Probiotic yogurt	0.75 ^b	0.92 ^a	0.09 ^a	0.11 ^a	22 ^a	13 ^b

* Means in a row related to a certain chemical compound (lactic acid, acetic acid or acetaldehyde), between the days 0 and 21, shown with different English letters, are significantly different.

3.2 Viability of probiotic bacteria at the end of fermentation and storage time

The changes in the counts of probiotic and yogurt microorganisms at the end of fermentation and during the cold storage are presented in Table 3. As shown, there are significant differences ($P < 0.05$) among the viable counts of the mentioned bacteria. The initial viable cell counts of the starter cultures (inoculation rate) ranged from 6.84 (*S. thermophilus*) to 6.30 \log_{10} cfu.ml⁻¹ (*B. lactis*). The average counts after 4h incubation (d 0) were 8.72, 8.48, 7.38 and 6.91 (\log_{10} cfu.ml⁻¹) for *S. thermophilus*, *L. bulgaricus*, *L. acidophilus* and *B. lactis*, respectively. Throughout the fermentation period, the bacterial populations were increased and *S. thermophilus* showed the fastest growth. *L. bulgaricus* showed a decline in viability at d 7. The viable counts of *L. acidophilus*, *B. lactis* and *S. thermophilus* increased up to the d 7, and then declined during the subsequent storage. The decline was found to be the highest for *L. delbrueckii* ssp. *bulgaricus*, especially during the final days of storage. Among the four organisms enumerated, *S. thermophilus* was found to be the most stable in yoghurt, and its counts were >8.70 (\log cfu.ml⁻¹) throughout the storage. *L. delbrueckii* ssp. *bulgaricus* lost its viability considerably until the end of storage. The viability loss of bifidobacteria was gradual and steady during the storage, and the organisms exhibited good stability during the storage as compared to lactobacilli.

Table 4 represents the viability proportion index (VPI) in the treatments immediately at the end of fermentation and during the refrigerated

storage. Among the probiotic bacteria, the most survivability throughout the storage belonged to *B. lactis*. As mentioned, the survival rate of *S. thermophilus* was greater compared to that of *L. delbrueckii* ssp. *bulgaricus* and probiotic organisms. This bacterium maintained 96% of its initial viable population at the end of storage (d 21 d 0⁻¹), whilst this amount was 76%, 35% and 0.7% for *B. lactis*, *L. acidophilus* and *L. bulgaricus*, respectively. The results showed that the viability of yogurt bacteria and pH of the yogurt had significant effects on the viability of probiotic bacteria.

Considering the optimum growth temperatures of yogurt and probiotic bacteria, incubation of milk at 44°C enhanced the antagonistic effect of the latter against probiotic microorganisms, particularly *L. acidophilus*.

L. delbrueckii ssp. *bulgaricus* produces a large amount of acid (sharp acidification), hydrogen peroxide and possibly bacteriocins resulting in the suppression of probiotic microorganisms. Viability of *L. acidophilus* is adversely affected by the presence of *L. delbrueckii* ssp. *bulgaricus*. Viability loss of *L. acidophilus* has been reported to be mainly due to the hydrogen peroxide produced by *L. delbrueckii* ssp. *bulgaricus* [6, 13-14].

Both *L. acidophilus* and *Bifidobacterium* spp. are classified as microaerophilic and strictly anaerobic, respectively. The molecular oxygen induces cell death or poor viability of probiotics [16]. The oxygen susceptibility of bifidobacteria could, however, be strain dependent. The result of this study showed appropriate survival of bifidobacteria compared to *L. acidophilus* [3, 22].

Table 3. Viable counts (\log cfu.ml⁻¹) of probiotic and yogurt bacteria at the end of fermentation and during the storage period*

Treatment	Type of bacteria	Inoculation rate	Viable count (\log cfu.ml ⁻¹)			
			d 0***	d 7	d 14	d 21
Probiotic yogurt	St**	6.84 ^b	8.72 ^{aBC}	8.81 ^{aA}	8.77 ^{aB}	8.70 ^{aC}
	Lb	7.16 ^{aC}	8.48 ^{bA}	8.18 ^{bB}	7.04 ^{cD}	6.30 ^{dE}
	La	6.48 ^{cE}	7.38 ^{cB}	7.43 ^{cA}	7.22 ^{bC}	6.93 ^{bD}
	Bl	6.30 ^{dD}	6.91 ^{dA}	6.93 ^{dA}	6.84 ^{dB}	6.79 ^{cBC}

* Means in the same column and row (respectively) shown with different small and large English letters are significantly different.

** St = *Streptococcus thermophilus*, Lb = *Lactobacillus delb. ssp. bulgaricus*, La = *Lactobacillus acidophilus*, Bl = *B. lactis*.

*** Immediately after fermentation

4. Conclusion

This study demonstrated that the survival of probiotic and yogurt bacteria is dependent on the strain and storage time. Differential survival behavior for *L. acidophilus* and *B. lactis* strains was further analyzed. *S. thermophilus* and *B. lactis* showed higher resistance than other bacteria when yogurt was stored at 4°C (21

days). Probiotic bacteria were able to maintain the recommended viable cell concentration (10^6 cfu.ml⁻¹) until the end of the storage time (21 day). The survival loss of *B. lactis* was gradual and steady during the storage, and the organisms exhibited better stability during the storage compared to *L. acidophilus*. *S. thermophilus* showed the greatest viability among the starter

bacteria. Acetaldehyde level significantly decreased in yogurt during the storage. It is concluded that the most remarkable changes in

acetaldehyde level occurs at the end of the storage period. This may be due to the prolonged storage as a result of enzymatic reactions.

Table 4. Viability proportion index (VPI) of probiotic and yogurt bacteria at the end of fermentation and during the storage time

Treatment	Type of bacteria	Viability proportion index (VPI)					
		d 0/I**	d 7/d 0	d 14		d 21	
				d 14/d 0	d 14/d 7	d 21/d 0	d 21/d 14
Probiotic yogurt	St*	74.28	1.23	1.13	0.92	0.96	0.85
	Lb	20.55	0.50	0.04	0.07	0.007	0.18
	La	8.07	1.11	0.68	0.61	0.35	0.51
	Bl	4.05	1.05	0.86	0.82	0.76	0.88

*St = *Streptococcus thermophilus*, Lb = *Lactobacillus delb. ssp. bulgaricus*, La = *Lactobacillus acidophilus*, Bl = *B. lactis*.

**I = Immediately after fermentation

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