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Biochemical Properties and Viable Probiotic Population of Yogurt at Different Bacterial Inoculation Rates and Incubation Temperatures

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Combined effects of inoculation rate [standard inoculation (S), 2S, 4S and 8S (representing two-fold, four-fold and eight-fold of standard inoculation, respectively] and incubation temperature (40 or 44 °C) on biochemical and microbiological characteristics of yogurt milk during and immediately after fermentation were investigated. Two probiotic bacteria (*Lactobacillus acidophilus* LA-5 and *Bifidobacterium lactis* BB-12), along with the yogurt bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*), were used. Acidification-related parameters during fermentation, incubation time and concentrations of lactic and acetic acids, as well as probiotic bacteria viability at the end of fermentation (final pH of 4.5), were determined. Incubation time and inoculation rate showed interactive effects on biochemical parameters and incubation time, as well as on viability of both probiotic bacteria. The greatest and the lowest pH drop, acidity increase and redox potential increase rates were observed in the treatments 8S-44 (eight-fold inoculation and fermentation at 44 °C) and S-40 (standard inoculation and fermentation at 40 °C), respectively. The concentration of acetic acid was significantly greater ($p<0.05$) in the treatments incubated at 40 ºC compared with those incubated at 44 ºC. The longest and the shortest incubation times were observed in the treatments S-40 and 8S-44, respectively. The greatest viability for each probiotic bacteria as well as total probiotic bacteria was observed in the treatment 8S-40. The lowest viabilities of *L. acidophilus* and bifidobacteria were seen in the treatments 8S-44 and S-44, respectively.

Key Words: Bifidobacteria, incubation, inoculation, *Lactobacillus acidophilus,* probiotic, viability, yogurt

INTRODUCTION

The manufacture of fermented milks containing probiotic microorganisms (especially yogurt) has become popular and commercially significant, resulting in the availability of many products of this kind in the world market (Shah 2001; Tamime et al. 2005; Korbekandi et al. 2011). Presently, the species *Lactobacillus acidophilus* and *Bifidobacterium lactis* are frequently used in production of probiotic fermented milks (Tamime et al. 2005; Mortazavian and Sohrabvandi 2006a; Korbekandi et al. 2011).

The most important qualitative parameter of probiotic microorganisms is their viability in the final product until time of consumption. Generally, the values 10^6 and 10^7-10^8 cfu mL^{-1} have been accepted as the minimum and the satisfactory levels, respectively, for probiotics (Shah 2000; Tamime et al. 2005; Anon. 2009; Korbekandi et al. 2011). Various compositional and process factors significantly affect the viability of probiotic microorganisms in fermented milks; among them, incubation temperature and inoculation rate possess remarkable impacts (Tamime et al. 2005; Mortazavian and Sohrabvandi 2006a; Cruz et al. 2007; Champagne and Rastall 2009). An important point is the interactive effects of these variables on the viability of

probiotic organisms compared with their individual influences. Although the single effect of incubation temperature or its combined effects with some other processing variables (such as heat treatment and refrigerated storage temperature) on viability of probiotic bacteria in fermented milks has been the subject of several studies (Singh 1983; Fernandez 1995; Kneifel et al. 2005; Mortazavian et al. 2006b, 2006c), there has been no report on the interrelated effects of the these variables with inoculation rate. The hypothesis is that in mixed probiotic cultures containing several probiotic organisms and yogurt bacteria, changing inoculation rate along with the incubation temperature would lead to selective growth conditions in favor of certain populations, considerably affecting the viability of probiotic bacteria. This study therefore investigated the interactive effects of inoculation rate and incubation temperature on biochemical characteristics of fermented milk and the viability of probiotic bacteria in yogurt.

MATERIALS AND METHODS

Starter Culture

Pouches of commercial lyophilized ABY culture (A = *Lactobacillus acidophilus* LA-5, B = *Bifidobacterium lactis* BB-12, and Y = yogurt bacteria, including *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*) labeled as 'FD-DVS ABY-1' were supplied by Chr-Hansen (Horsholm, Denmark). The cultures were maintained according to manufacturer's instructions at -18 °C until use.

Study Design and Sample Preparation

Eight yogurt treatments with four inoculation rates of the ABY culture (Standard = S, 2S = two-fold inoculation, 4S = four-fold inoculation, 8S = eight-fold inoculation) and two incubation temperatures (40 and 44 °C) 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 15 min. After inoculation, fermentation was carried out at 40 or 44 °C until pH 4.5±0.02. Biochemical parameters including pH drop, acidity increase and redox potential increase were measured during fermentation period. These parameters were recorded every 30 min. Parameters of incubation time (in min), final titratable acidity, pH drop, acidity increase and redox potential increase rates were determined immediately after fermentation. The final samples were cooled down and kept at 5 °C until the probiotic organisms were counted and the concentrations of lactic and acetic acids were also determined.

Microbiological Analysis

MRS-bile agar medium (MRS agar from Merck, Darmstadt, Germany and bile from Sigma-Aldrich, Inc., Reyde, USA) was used for the selective count of *L. acidophilus* and *Bifidobacterium lactis* in ABY culture composition according to the method of Mortazavian et al. (2007b). The plates were incubated aerobically and anaerobically at 37 °C for at least 72 h. Anaerobic conditions were produced using the GasPac system (Merck, Darmstadt, Germany).

Growth proportion index (GPI) of probiotic microorganisms at the end of fermentation was calculated as follows (Mortazavian et al. 2010):

$$GPI = \frac{\text{Final cell population (cfu mL}^{-1})}{\text{initial cell population (cfu mL}^{-1})}$$

Chemical Analysis

The pH values and the redox potential of the yogurt samples were measured at room temperature using a pH meter. Titratable acidity was determined after mixing 10 mL of the sample with 10 mL of distilled water and titrating with 0.1 N NaOH using 0.5% phenolphthalein according to the method of Dave and Shah (1997).

The pH drop rate [pH value min^{-1}], acidity increase rate [Dornic degree min^{-1}] and redox potential increase rate [mV min^{-1}] were calculated as follows (Mortazavian et al. 2010):

$$\text{pH drop rate} = \frac{\text{(final pH value – initial pH value)}}{\text{incubation period}}$$

$$\text{Acidity increase rate} = \frac{\text{(final acidity value – initial acidity value)}}{\text{incubation time}}$$

$$\text{Redox potential increase rate} = \frac{\text{(final value – initial value)}}{\text{incubation time}}$$

Quantification of lactic and acetic acids was carried out by High Performance Liquid Chromatography (CE 4200- Instrument, Cecil, Milton Technical Center, Cambridge CB46AZ, UK) according to Mortazavian et al. (2010). Briefly, for extraction of acids, 4.0 g of sample was diluted to 25 mL with 0.1 N H_2SO_4, homogenized and centrifuged at 5000 *g* for 10 min. The supernatant, filtered through Whatman #1 filter paper and through a 0.20 µm membrane filter, was immediately analyzed. A Jasco UV-980 detector and a Nucleosil 100-5C_{18} column (Macherey Nagel, Duren, Germany) were used. The mobile phase was 0.009 N H_2SO_4 at a flow rate of 0.5 mL min^{-1}. The wavelength of detection was optimized at 210 nm. 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 3.58 min and the standard curve regression coefficients were 0.989 and 0.991, respectively.

Statistical Analysis

Experiments were performed in triplicate and were set up using a completely randomized design. Data were subjected to analysis of variance and comparison of the means was done using LSD at 5% level.

RESULTS AND DISCUSSION

Biochemical Characteristics of Yogurt Treatments during and at the End of Fermentation

Table 1 presents the biochemical changes in the different treatments as affected by inoculation rate and temperature. Fermentation at 44 ºC at different levels of inoculation resulted in significantly greater pH drop rates compared with those fermented at 40 ºC. At various incubation temperatures, 8S and 4S inoculation rates resulted in significantly higher pH drop rates compared with 2S and S. The fastest and the slowest pH drop rates were observed in the treatments 8S-44, and S-40 and 2S-40, respectively. An increase in each variable alone would enhance the mean pH drop rate during fermentation. For instance, the effect of 4 ºC increase in fermentation temperature on pH drop rate during fermentation was equal to a two-fold reduction in inoculation rate, e.g., treatments 8S-40 and 4S-44. In treatments with the same incubation temperatures, an increase in inoculation rate enhanced increase in acidity and in the mean redox potential rates. On the other hand, in treatments with equal inoculation rates, incubation at 44 ºC led to a significant increase in the mentioned parameters compared with incubation at 40 ºC. The greatest and lowest acidity and redox potential increase rates were observed in treatments 8S-44 and S-40, respectively.

Clearly, raising the temperature from 40 to 44 ºC would considerably increase the growth and activity of yogurt starter cultures, e.g., *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*, especially the former. The optimum temperature for the growth of *L. delbrueckii* ssp. *bulgaricus* is 44–45 ºC compared with about 42 ºC for *S. thermophilus* (Korbekandi et al. 2011). *L. delbrueckii* ssp. *bulgaricus* is more active than the other yogurt bacteria in terms of glycolytic and proteolytic activities at the optimum temperature range (Gomes and Malcata 1999). Increasing the fermentation temperature from 40 to 44 ºC therefore would lead to a remarkable increase in pH drop, acidity increase and redox potential increase rates during fermentation. The rate of increase in redox potential during fermentation is linearly proportional to the rate of titratable acidity increase due to organic acids produced during the mentioned period (Korbekandi et al. 2011). The optimum temperatures for growth and activity of *L. acidophilus* and most bifidobacteria are 38–40 ºC and 37–38 ºC, respectively (Gomes and Malcata 1999; Korbekandi et al. 2011). Therefore, increasing fermentation temperature from 40 to 44 ºC results in unsuitable growth and activity conditions for probiotic cells in terms of their growth temperature and would reduce their contribution in acidification and rate of decrease in pH. Probiotic bacteria, especially bifidobacteria, grow poorly in milk compared with yogurt bacteria due to the lack of proteolytic and glycolytic activities as well as the relatively high nutritional demand for non-protein nitrogen and B-group vitamins (Gomes and Malcata 1999; Tamime et al. 2005). Therefore, their reduced contribution to acidification compared with the noticeable increased acidification rate due to yogurt bacteria would be statistically negligible.

Treatments S-40 and 8S-44 had the significantly longest and shortest incubation times, respectively. In treatments with the same incubation temperatures (40 or 44 ºC), the incubation time was reduced in parallel with the increase in inoculation rate. Similarly, in treatments with equal inoculation rate, an increase in incubation temperature resulted in a decrease in fermentation period. As earlier mentioned, higher incubation temperature resulted in optimum activation of yogurt bacteria, especially *L. delbrueckii* ssp. *bulgaricus*, and led to sharper and faster acidification during fermentation.

The concentrations of lactic and acetic acids among treatments were significantly different, representing different growth ratios of yogurt bacteria to probiotic bacteria in the mixed starter culture. The smallest amount of lactic acid was observed in the treatment 8S-40, indicating production of greater amounts of organic acids other than lactic acid. The concentration of acetic acid was significantly greater in the treatments that had smaller amounts of lactic acid, namely, those fermented at 40 ºC compared with those fermented at 44 ºC. The highest and lowest levels of acetic acid were related to the treatments 8S-40 and 4S-40, and 4S-44 and 8S-44, respectively. The greater amount of acetic acid in the former treatments could represent higher growth and activity rate of bifidobacteria. These ranges in concentration of organic acids were consistent with the results reported in yogurt by Heydari (2010).

Viability of Probiotic Bacteria at the End of Fermentation

Table 2 shows the viability of probiotic microorganisms (*L. acidophilus* LA-5 and *B. lactis* BB-12) and the relevant growth proportion index (GPI) in the different treatments immediately at the end of fermentation. The viability of *L. acidophilus* regardless of inoculation rate was significantly higher in the treatments incubated at 40

Table 1. Biochemical changes in yogurt as a function of culture inoculation rate and incubation temperature.

Treatment		Parameters						
Inoculation Rate*	T (°C)	pH Drop Rate (pH/min)	Acidity Increase Rate (°D/min)	Redox Potential Increase Rate (mV/min)	Incubation Time (min)	Final Acidity (°D)^{ns}	% Lactic Acid	% Acetic Acid
S	40	0.006^{d}	0.23^{fg}	0.34^{e}	330^{a}	93.9	0.81^{b}	0.10^{c}
2S	40	0.006^{d}	0.24^{f}	0.37^{d}	310^{b}	94.2	0.79^{bc}	0.11^{c}
4S	40	0.007^{c}	0.29^{e}	0.41^{c}	270^{d}	93.6	0.78^{c}	0.14^{ab}
8S	40	0.008^{b}	0.34^{c}	0.48^{b}	240^{f}	94.2	0.71^{d}	0.15^{a}
S	44	0.007^{c}	0.29^{e}	0.41^{c}	300^{c}	93.8	0.85^{ab}	0.07^{d}
2S	44	0.007^{c}	0.33^{cd}	0.48^{b}	250^{e}	94.7	0.86^{a}	0.06^{d}
4S	44	0.008^{b}	0.37^{ab}	0.49^{ab}	240^{f}	94.6	0.86^{a}	0.04^{e}
8S	44	0.009^{a}	0.38^{a}	0.51^{a}	210^{g}	94.8	0.87^{a}	0.05^{de}

Means in the same column with different letters are significantly different ($p<0.05$) using LSD.
ns = not significant
*S = standard or base inoculation; 2S = 2-fold, 4S = 4-fold, 8S = 8-fold of standard inoculation

Table 2. Viability of probiotic microorganisms in yoghurt as a function of inoculation level and incubation temperature.

Treatment			Initial Population (log cfu mL^{-1})			Final Population (log cfu mL^{-1})			Growth Proportion Index (GPI)		
Inoculation Rate*	T (°C)	pH	A**	B	A+B	A	B	A+B	A	B	A+B
S	40	4.5	6.21	6.44	6.64	7.57^{cdB}	7.69^{eA}	7.93^{cd}	23.1^{aA}	17.5^{aB}	19.5^{a}
2S	40	4.2	6.51	6.75	6.94	7.59^{cB}	7.71^{deA}	7.95^{c}	12.2^{bA}	9.1^{cB}	10.2^{c}
4S	40	4.5	6.81	7.05	7.24	7.69^{bB}	7.84^{bA}	8.08^{b}	7.6^{cA}	6.2^{eB}	6.8^{d}
8S	40	4.2	7.11	7.35	7.55	7.84^{aB}	8.00^{aA}	8.23^{a}	5.4^{deA}	4.2^{gB}	4.8^{f}
S	44	4.5	6.21	6.44	6.64	6.95^{eB}	7.62^{fA}	7.71^{f}	5.6^{dB}	15.0^{bA}	11.6^{b}
2S	44	4.2	6.51	6.75	6.94	6.84^{efB}	7.65^{eA}	7.72^{f}	2.2^{fB}	8.1^{dA}	5.9^{e}
4S	44	4.5	6.81	7.05	7.24	5.94^{gB}	7.74^{dA}	7.75^{ef}	0.1^{gB}	4.9^{fA}	3.2^{g}
8S	44	4.2	7.11	7.35	7.55	5.30^{hB}	7.77^{cA}	7.78^{e}	0.01^{hB}	2.7^{hA}	1.7^{h}

Means with different small and capital letters are significantly different ($p<0.05$) within a column (among the treatments) and within a row (between the two probiotic bacteria), respectively.
*S = standard inoculation, 2S = two-fold, 4S = four-fold, 8S = eight-fold of standard inoculation
** A = *Lactobacillus acidophilus*, B = *Bifidobacterium lactis*, A + B = total probiotic bacteria

ºC compared with those fermented at 44 ºC. In the treatments incubated at 40 ºC, increasing the inoculation rate led to a significant increase in viable population in both probiotics, while at 44 ºC, an increase in the inoculation rate resulted in the lower viability of *L. acidophilus*. Considering the optimum growth temperatures of yogurt and probiotic bacteria, incubation of milk at 44 ºC compared with 40 ºC enhanced the antagonistic effect of the latter bacterium against probiotic microorganisms, particularly *L. acidophilus*. *L. delbrueckii* ssp. *bulgaricus* became the dominant species in yogurt, producing a large amount of acid (sharp acidification), hydrogen peroxide and possibly, bacteriocins, resulting in the suppression of the probiotic microorganisms. In the composition of the ABY-type culture, loss of viability of *L. acidophilus* has been reported to be mainly due to the hydrogen peroxide produced by this bacterium (Shah et al. 1994; Tamime et al. 2005; Mortazavian et al. 2007a, 2008; Korbekandi et al. 2011). Furthermore, the optimum growth range of the pH of *L. acidophilus* was 5.5–6.0. Bifidobacteria are also sensitive to the level of pH variations and their growth is restricted at pH<5 (Korbekandi et al. 2011). Therefore, a rapid drop in pH below such level due to the fast growth of *L. delbrueckii* ssp. *bulgaricus* led to the slower growth rate and faster inactivation of *L. acidophilus*, resulting in lower viable counts of this organism after the fermentation period. Not only the low pH values but also the rate of drop in pH affects the viability of probiotic bacteria. Higher acidification rates until a certain final pH

of fermentation would lead to pH drop (fast pH decline) in probiotic cells and their significantly lower viability (Mortazavian et al. 2010). When only probiotic bacteria (not adjunct cultures) are used for the fermentation of milk, as these organisms grow poorly in milk, a large inoculum size would considerably enhance their growth, activity and viability. However, in ABY-type culture compositions, such an inoculation rate might be unfavorable to probiotic bacteria especially when the incubation temperature is near the optimum temperature for growth of *L. delbrueckii* ssp. *bulgaricus*. The inoculation ratio of the probiotic culture to the yoghurt culture in the inoculum is another key factor. Not to be overlooked is the fact that an excessive increase in the inoculation rate in a medium lacking in nutritional value might lead to opposite results with regard to viability. Therefore, the viability of the starter organisms might be reduced due to competition in nutrients and antagonistic relationships between them.

In the case of bifidobacteria, at the same inoculation rates, increasing fermentation temperature from 40 to 44 ºC led to lower viability. However, this decrease in viability was not as remarkable as that of *L. acidophilus* and could be attributed to the greater antagonistic effects of *L. delbrueckii* ssp. *bulgaricus* against *L. acidophilus* than bifidobacteria, which has been previously reported in ABY-type culture composition (Mortazavian et al. 2006b, 2007a, 2008, 2010). The viable population of bifidobacteria in all treatments was significantly higher than that of *L. acidophilus*. Apart from the inherent greater resistance of bifidobacteria cells compared with *L. acidophilus,* the result could be due to the initially higher population of the former bacteria in the inocula (Table 2). The greatest viability of each probiotic bacteria as well as the total probiotic bacteria was observed in the treatment 8S-40. The lowest viability of *L. acidophilus* was seen in the treatment 8S-44. The lowest viability of bifidobacteria was observed in the treatment S-44, followed by 2S-44 and S-40. The lowest viability of the total probiotic bacteria was observed in treatments S, 2S and 4S at 44 ºC. In the samples incubated at 44 ºC, regardless of the rate of inoculation, the viable count of *L. acidophilus* was lower than the standard limit of 10^7 cfu mL^{-1}.

The growth proportion index (GPI) for *L. acidophilus* was significantly greater than for bifidobacteria in yoghurt incubated at 40 ºC (Table 2). An opposite trend was noted at 44 ºC. At 40 ºC incubation, the antagonistic effect of *L. delbrueckii* ssp. *bulgaricus* on *L. acidophilus* was not appreciably felt, the latter bacterial cells were able to adequately proliferate and increase their viable population. On the other hand, bifidobacteria which generally possess slower cell metabolic activity and growth in milk but with higher resistance to fermenting environment of mixed cultures, retain their initial viability appropriately. This fact has been previously reported (Mortazavian et al. 2010). The GPI data revealed that increasing inoculation rate in order to increase viability of probiotic cells at the end of fermentation was not feasible in ABY-type cultures in terms of efficiency. The GPI (representing growth efficiency of starters during fermentation) for both probiotic bacteria considerably decreased with an increase in inoculation rate at constant incubation temperature. The greatest value of GPI for *L. acidophilus* and bifidobacteria was observed in the treatment S-40. The initial inoculated populations of probiotic bacteria therefore did not proliferate in a linear proportion during fermentation, and in ABY-cultures, the death rate might be greater than the growth rate depending on the environmental conditions. For instance, viable population of *L. acidophilus* in the treatment S-40 reached 23-fold of its initial inoculated population but this was reduced to 5.4-fold in the treatment 8S-40. The greater population of *L. acidophilus* in the latter treatment was most probably due to the higher inoculation rate than the higher growth rate during fermentation. Similar to *L. acidophilus*, GPI of bifidobacteria considerably decreased with an increase in inoculation rate.

In general, there appears to be a relationship between the concentration of acetic acid and viability of bifidobacteria. Because these bacteria produce appreciable amounts of acetic acid during fermentation, the amount of this acid represents bifidobacteria growth and/or activity. The same results have been previously reported in ABY cultures (Heydari 2010; Mortazavian et al. 2010). For example, the treatment 8S-40 which had the greatest viability of bifidobacteria, also exhibited the highest concentration of acetic acid.

CONCLUSION

Both incubation temperature and inoculation rate had significant effects on biochemical characteristics of fermenting milk as well as on the viability of probiotic bacteria and incubation time. At 40 ºC, an increase in the inoculation rate led to a significant increase in probiotic viable population, while at 44 ºC, an increase in the inoculation rate resulted in lower viability of *L. acidophilus*. In contrast to *L. acidophilus*, fermentation temperature (40 or 44 ºC) compared with inoculation rate was not critical for bifidobacteria in terms of viability probably because of the inherent resistance of bifidobacteria cells to detrimental conditions of fermenting milk as well as to antagonistic effects of *L. delbrueckii* ssp. *bulgaricus* compared with *L. acidophilus*. Apart from incubation temperature, inoculation rate also plays a determinable role in the viability of the cells in bifidobacteria; compared with *L. acidophilus*, an increase in the inoculation rate led to

significantly greater viability in incubation temperature either at 40 or 44 ºC. Increasing the inoculation rate to increase viability of probiotic cells at the end of fermentation was not feasible due to a decrease in GPI for both probiotic bacteria parallel to an increase in inoculation rate (at constant incubation temperature). In conclusion, incubation temperature and inoculation rate had interrelated effects on the viability of probiotic bacteria. Choosing the best incubation temperatures and inoculation rates depend on culture composition and the presence of *L. delbrueckii* ssp. *bulgaricus*.

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