Effects of CO\textsubscript{2} Addition to Raw Milk on Microbial, Physiochemical and Sensory Properties of Probiotic Set Yoghurt

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ABSTRACT

The suitability of milk preserved by refrigeration and CO\textsubscript{2} addition for the manufacture of plain yoghurt using two commercial strains of \textit{Lactobacillus delbrueckii} ssp. \textit{Bulgarius} and \textit{Streptococcus thermophilus} and probiotic yoghurt by \textit{Lactobacillus acidophilus} was evaluated. Yoghurts (plain and probiotic) manufactured after milk pasteurization, from fresh and refrigerated CO\textsubscript{2} treated samples (pH= 6.2), were compared with the corresponding controls (fresh and refrigerated). The multiplication and acidification capacity of the starter were neither affected by the previous refrigeration and CO\textsubscript{2} addition of raw milk nor by the residual CO\textsubscript{2} present in the pasteurized milk. CO\textsubscript{2} addition of raw milk slightly enhanced viscosity and reduced syneresis of yoghurts. The taste panel preferred yoghurts made from CO\textsubscript{2} treated milks to the corresponding controls during cold storage. These results support the suitability of CO\textsubscript{2}-addition in preservation of milk for manufacturing of yoghurts.

Keywords: Carbon dioxide, Probiotic, Refrigerated milk, Syneresis, Viscosity, Yoghurt.

INTRODUCTION

Milk refrigeration at farms and dairy processing plants maintains the quality of milk by reducing the growth rate of mesophilic bacteria and extends the storage time of raw milk before processing. However, it does not prevent the growth of psychrotrophic bacteria, which are present as normal contaminants of raw milk. Although psychrotrophs are killed by most of the industrial heat treatments of milk, the organisms can produce extracellular enzymes (proteases and lipases) that are not completely inactivated by heat treatments (Ruas-Madiedo et al., 1996; Barbano et al., 2006). These enzymes are capable of degrading various milk components, affecting the storage life of heat-processed milk, and the quality of dairy products (Champagne et al., 1994). One procedure for the control of psychrotrophic bacteria in raw milk involves the treatment of refrigerated milk with CO\textsubscript{2} (King and Mabbit, 1982; Roberts and Torrey, 1988; Amigo et al., 1995; Hotchkiss et al., 1999). Several theories explaining the mechanism of CO\textsubscript{2} action on microorganisms have been proposed. The exclusion of oxygen by replacement with CO\textsubscript{2} may contribute to the overall effect by slowing the growth rate of aerobic bacteria. Carbon dioxide can readily pass through cell membranes and form carbonic acid within the cell with a resultant decrease in intracellular pH, which slows intracellular enzyme activities. Carbon dioxide has been demonstrated to be inhibitory of certain enzymes, especially decarboxylating enzymes. The effect of CO\textsubscript{2} is enhanced at lower temperatures (Hass et al., 1989; Rajagopal et al., 2005; Hotchkiss et al., 2006). Vacuum degasification prior to pasteurization prevents milk coagulation and

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volume changes of the occluded gas in the pasteurizer and also renders milk acceptable for liquid consumption (Ruas-Madiedo et al., 1996).

The consumption of yoghurt and fermented milks is currently increasing in industrialized countries due to the expanding variety, sensory aspects, and proposed therapeutic properties of these products. Several works have recently been carried out on the use of CO$_2$ for carbonation of pasteurized milk prior to the manufacture of yoghurt (Calvo et al., 1999) as well as for carbonation of cooled finished product (Karagul-Yuceer et al., 1999). However, there is little information yet on the use of milk preserved by refrigeration and CO$_2$ addition for the manufacture of yoghurt. Also CO$_2$ has been used effectively in extending the shelf life of a variety of cold stored dairy products, including cottage cheese (Werner and Hotchkiss, 2002). Among the potential advantages for the cheese industry are the lower concentrations of rennet needed to achieve coagulation in milks to which CO$_2$ has been added as a consequence of the decrease in clotting time (de la Fuente, 1998). CO$_2$-treatment effectively prevents the decrease in cheese yield caused by the microorganisms present in raw milk of poor microbial quality (Ruas-Maddiedo et al., 1998).

In recent years, there has been an increasing interest in the addition of Lactobacillus acidophilus and bifidobacteria to fermented milks and a great variety of products containing them have been formulated world-wide. After ingestion, these cultures must overcome biological barriers including acid in the stomach and bile in the intestine (Gilliland, 1978; Lankaputhra and Shah, 1995), implant in the intestinal tract and exert health promoting effects (Kailasapathy and Rybka, 1997). In order to produce therapeutic benefits, a suggested minimum level for probiotic bacteria in fermented milk is from $10^6$ to $10^8$ CFUml$^{-1}$. Lactic acid bacteria seem to be relatively tolerant to CO$_2$. Furthermore, there is a reason to suspect that the lactobacilli might react positively to CO$_2$ injection in milk. It is known that CO$_2$ produced by Streptococcus thermophilus stimulates Lactobacillus delbrueckii ssp. bulgaricus (Vinderola et al., 2000).

The present work was undertaken to determine the effect of the refrigeration and CO$_2$ addition to raw milk on the subsequent chemical, physical, microbiological, and sensory properties of plain yoghurt and probiotic yoghurt.

**MATERIALS AND METHODS**

**Starters and Probiotic Cultures**

The starter cultures used in this study were Y 621 LYO 502 (Freeze dried culture, Copenhagen K, Danisco). Both cultures contained S. thermophilus and L. delbrueckii subsp. Bulgaricus. Probiotic culture L. acidophilus La5TM (freeze-dried DVS) was obtained from Chr. Hansen (Hørsholm, Danmark) company representative (Tehran, Iran) and prepared according to the manufacturer’s instructions (direct addition).

**Milk Treatment and Processing**

Cow raw milk samples with initial microbial loads between lower $10^6$ and $10^7$ CFUml$^{-1}$ and initial pH from 6.80 to 6.75 (3.09% protein; 3.4% fat & 11.65% total solid) were collected from the farm of University of Tabriz in Iran and were preserved by refrigeration and CO$_2$ addition in June 2007. After cold storage, milk was used to manufacture plain and probiotic yoghurt. In order to ascertain the influence of the combined use of milk preservation by refrigeration and CO$_2$ addition on the subsequent characteristics of yoghurt, fresh (24h at 4°C) and refrigerated (4 days at 4°C) CO$_2$-treated samples were compared with untreated controls: Fresh and Refrigerated. CO$_2$-treated samples were acidified to pH 6.20 with food grade CO$_2$ by means of a manual injection system. CO$_2$ addition was
performed under sterile condition and slowly (68 kpa and 4°C) so as to prevent precipitation of proteins from the milk. CO\textsubscript{2} addition to milk was controlled by pH. After injection, milk was held at 4°C. Then, milk (control: without CO\textsubscript{2} and treatment: with CO\textsubscript{2}) was pasteurized at 85°C for 30 minutes, cooled to 42°C and inoculated (3% w/v) with mixture of \textit{Streptococcus thermophilus} and \textit{Lactobacillus delbrueckii} ssp. \textit{bulgaricus} (plain), (0.2446% w/v) \textit{Lactobacillus acidophilus} (probiotic) to produce yoghurt. Milk was distributed to plastic retail containers and incubated at 42°C (Gueimond et al., 2003) and 40°C (optimum growth temperature for La-5 is 37-40°C, according to the manufacturer’s instructions; Yeganehzad et al., 2007) for plain and probiotic yoghurt, respectively. After 3 hours and 6 minutes (termination of fermentation process in method of rapid fermentation at production of yoghurt, Tamime and Robinson, 1999), yoghurts were stored at 4°C for 28 days. These processes were repeated three times. Microbial counts and physicochemical analysis were carried out at 1, 9, 18 and 28 days of cold storage.

**Microbial Experiments**

Initial total viable counts of microbes in raw milk were obtained by deep plating on PCA (Merck, Darmstade, Germany) and aerobic incubation at 30°C for 48-72 hours (Gueimond et al., 2003). Samples for counts of \textit{Streptococcus thermophilus}, \textit{Lactobacillus acidophilus} and \textit{Lactobacillus delbrueckii} ssp. \textit{bulgaricus} were spread plated on M17 (Lab M\textsuperscript{TM}, Bury, Lancs BL, UK) and MRS agar (Lioflichem, Roseto d. A. [TE], Italy), respectively. The first culture (\textit{S. thermophilus}) was incubated for 48 hours at 42°C and the second (\textit{L. acidophilus}) and third cultures (\textit{L. delbrueckii} ssp. \textit{bulgaricus}) were incubated aerobically and anaerobically for 3-4 days at 37°C, respectively. Microbiological count data were expressed as a log of colony-forming units per milliliter of yogurt (Birolo et al., 2000; Donkor et al., 2006).

**Physicochemical Properties of Yoghurt**

**pH**

The pH change of yoghurt batches during storage was obtained by direct measurement with a pH meter (HANNA Instruments).

**Viscosity**

The measurements were carried out using a Haake viscometer (VT 24, Haake, Karlsruhe, Germany) fitted with a MV\textsubscript{2} Cup. Prior to analysis, samples were brought to room temperature (controlled at 20°C) and all determinations were performed at this temperature. About 106 gr of sample was placed in a cup viscometer and, before loading, was left at cup viscometer for 15 minutes, then, it was loaded to achieve a homogenous and equilibrium mixture for 20 min. After this time, the viscosity (cpoise) was recorded at shear rates of 1 and 4 s\textsuperscript{-1}.

**Syneresis**

Whey that separated from samples during storage was removed using a syringe (Lucey, 2001). The syneresis was expressed as the weight of the whey over the initial weight of the yoghurt sample.

**Sensory Evaluation**

Yoghurt samples were subjected to sensory evaluations (taste, firmness and texture, and overall acceptability) by a trained 20-member panel from Food Science and Technology Students, University of Tabriz, during cold storage. Samples were scored on a hedonic scale of 1-5. For each evaluation, yoghurt made from fresh and refrigerated milk was used as the control.

**Statistical Methods and Analyses**

Effects of four factors on pH, syneresis, viscosity, survival of \textit{Lactobacillus acidophilus}, \textit{Streptococcus thermophilus}, \textit{Lactobacillus bulgaricus} and sensory properties were studied using factorial split plot design based on randomized complete
Table 1. Titrable acidity and pH values of fresh and refrigerated control milk and fresh and refrigerated CO₂-treated milk before (BP) and after (AP) pasteurization.

<table>
<thead>
<tr>
<th>Milk sample</th>
<th>Pasteurization</th>
<th>pH</th>
<th>Titrable acidity (Dornic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh control</td>
<td>BP</td>
<td>6.8±0.02</td>
<td>15±0.03</td>
</tr>
<tr>
<td>Fresh CO₂ treated milk</td>
<td>BP</td>
<td>6.2±0.03</td>
<td>31.2±0.02</td>
</tr>
<tr>
<td>Refrigerated control</td>
<td>BP</td>
<td>6.75±0.07</td>
<td>17±0.04</td>
</tr>
<tr>
<td>Refrigerated CO₂ treated milk</td>
<td>BP</td>
<td>6.15±0.05</td>
<td>31.4±0.03</td>
</tr>
<tr>
<td>Fresh control</td>
<td>AP</td>
<td>6.44±0.08</td>
<td>15±0.03</td>
</tr>
<tr>
<td>Fresh CO₂ treated milk</td>
<td>AP</td>
<td>6.39±0.06</td>
<td>16±0.04</td>
</tr>
<tr>
<td>Refrigerated control</td>
<td>AP</td>
<td>6.39±0.03</td>
<td>15.3±0.03</td>
</tr>
<tr>
<td>Refrigerated CO₂ treated milk</td>
<td>AP</td>
<td>6.33±0.07</td>
<td>17±0.02</td>
</tr>
</tbody>
</table>

Data are Means±S.D., n = 3.

*Before pasteurization, †After pasteurization.

blocks with three replications. The factors were: 1) CO₂ (treatment or control), 2) preservation of milk (fresh and refrigerated), 3) type of starter (Y 621 LYO 502 [Streptococcus thermophilus and Lactobacillus delbruekii ssp. bulgaricus] or Lactobacillus acidophilus), and 4) storage time of yoghurt samples (1, 9, 18 and 28 days). The factorial combinations of factors 1 to 3 were assigned to the main plots and the storage times of yoghurt were included in the subplots. Means were compared by Dunkan’s multiple range test.

**RESULTS AND DISCUSSION**

**Physicochemical and Microbiological Evolution**

As expected, just before pasteurization, the refrigerated milk presented lower pH and higher acidity values than the fresh control samples (Table 1). Pasteurization increased pH and decreased acidity in the preserved milk. However, after pasteurization, pH and acidity values remained lower and higher, respectively, than in the control fresh milk because CO₂ was not totally removed from the preserved milk by heat-vacuum (Ruas-Madiedo et al., 2002; Ruas-Madiedo et al., 2003; Hotchkiss et al., 2006).

Figure 1(a, b, and c) shows the microbiological profile of yoghurt made from pasteurized milk. The population of *S. thermophilus, L. delbruekii ssp bulgaricus and L. acidophilus* during cold storage did not show significant change (P> 0.05). With respect to the CO₂ treatment, it was interesting to note that levels of rods and cocci/rods ratio (Figure 1-d) in plain yoghurt were slightly higher in CO₂-treated samples than in the control samples during cold storage period, although the difference was not significant (P> 0.05). In this respect, *Lactobacillus delbruekii ssp bulgaricus* population of the control samples (fresh) decreased (P> 0.05) from 9th day of refrigeration until the end of the storage period, whereas in yoghurts made from CO₂-treated milk, the decrease of *L. delbruekii ssp bulgaricus* levels (P> 0.05) started at the 28th day of refrigeration. CO₂ addition caused the population of *S. thermophilus* in yoghurts made from CO₂-treated milk to be higher than the control samples (P> 0.05). On the other hand, *S. thermophilus* population in yoghurts made from fresh CO₂-treated milk was always higher than the other samples (refrigerated CO₂-treated milk and controls, Figure 1-b). Also, by CO₂ addition to raw milk, *Lactobacillus acidophilus* population increased in probiotic yoghurts made from CO₂-treated milk (P > 0.05). According to Figure 1c, *Lactobacillus acidophilus* population started to decrease from the 9th day of refrigeration and the decrease continued at slow rate until the end of the storage period. In probiotic yoghurt made from fresh milk
**Figure 1(a-d).** Evaluation of cell counts of S. Thermophilus (a), L. Bulgaricus (b), L. acidophilus (c) and Changes cocci/rods ratio (S. thermophilus/L. bulgaricus ratio) during cold storage of plain yoghurts. (Data are Means±SD, n= 3).

(controls) *Lactobacillus acidophilus* population obviously decreased from the 18th day of storage in comparison with the others (P< 0.05). In addition, count of these bacteria (> 10⁹ CFU ml⁻¹) always remained higher than a suggested minimum level needed for producing a therapeutic effect (10⁶ CFU ml⁻¹). Results indicated a slight growth stimulation of these microorganisms during fermentation and an improvement of viability during cold storage of yoghurts. Similarly, Gueimonde *et al.* (2003) showed that acidification of raw milk by carbonation prior to the manufacture of plain yoghurt enhanced growth and metabolic activity of the starter. Similar results were obtained by Vinderola *et al.* (2000) in the fermented milks made from carbonated pasteurised milk.

**pH**

During the manufacture of yoghurt, as a result of the activity of microorganisms, the pH dropped in the control and CO₂-treated samples. As expected, the storage time affected the pH level significantly (P< 0.01) and the pH decreased, but CO₂ addition did not have any significant effect. The decrease in pH slowed down during the first days of cold storage of the product, but the decrease was slight afterwards. In the plain yoghurt made from CO₂-treated milk (fresh) the highest correlation between pH and time storage of yoghurts was observed. No significant difference (P> 0.05) for pH was
obtained between yoghurt made from the CO₂-treated milk and the control (Figure 2). As stated before, since CO₂ addition did not have any significant effect (P > 0.05) on the activity of microorganisms, the pH of samples were not affected. Gueimonde et al. (2003) showed lack of significant differences (P > 0.05) for pH and titratable acidity between yoghurt made from CO₂-treated and the control milks. Similar results were obtained by Vinderola et al. (2000) in fermented milks made from carbonated pasteurised milk.

Viscosity and Syneresis

CO₂ addition enhanced viscosity of plain and probiotic yoghurts, (Figure 3). Probably, CO₂ addition to raw milk decreases the pH of milk and progressively dissolves the CCP and reduces the binding of Ca to casein. Decreasing pH also leads to an increase in Ca²⁺ activity. It is well known that this ion plays an important role in reducing repulsion between negatively charged caseins and, consequently, increases the aggregation rate during the coagulation of milk (De La Fuente, 1998). Decreasing pH also controls psychrotrophic bacteria in raw milk and their enzyme activity (Ma et al., 2003; Martin et al., 2003; Rajagopal et al., 2005; Hotchkiss et al., 2006). This causes an increase in viscosity and reduces syneresis of yoghurts (Calvo et al., 1993 and 1999). The extent of viscosity and syneresis during the cold storage was also significantly (P < 0.05) affected by the type of strain. Viscosity values were higher in plain yoghurts in comparison to probiotic samples during cold period (Figure 4). Whey separation was lower in probiotic samples in comparison plain yoghurts (Figure 5). This could be attributed to the low acidity of these samples. Higher acidity stimulates the syneresis in yoghurt (Tamime and Robinson, 1999). Syneresis in the samples decreased during cold storage. This could be due to metabolic activity of yoghurt starter cultures and decrease in net pressure in the protein matrix, which decrease syneresis (Güler-Akin and Sedar-Akin, 2007). As previously
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**Sensory Analysis**

Sensory evaluation was carried out during cold storage period. In terms of panelists’ scores, there was significant difference (P<0.01) between the products made from carbonated and non-acidified milk. Purwandari et al. (2007) stated that use of CO₂ treated milk for the production of yoghurts resulted in fresher or cleaner taste, firmer texture (Figures 7 and 8) and higher overall acceptability (Figure 6) as compared with the control samples. They preferred, therefore, yoghurts made from CO₂-treated milk to the control samples. According to Figure 6, the panelists gave the highest scores of overall acceptability to the plain yoghurts made from fresh CO₂ treated milk followed by plain (refrigerated) and probiotic yoghurts (fresh and refrigerated) made from CO₂ treated milk, respectively. The lowest score was given to the controls.

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**Figure 3.** Evaluation effect of CO₂ addition on viscosity of plain and probiotic yoghurts made from refrigerated and fresh milk at 20°C and shear rate of 4s⁻¹. Since the combined effects of CO₂, time of preservation of raw milk, type of starter, and storage of yoghurts did not have significant (P> 0.05) effect on viscosity and syneresis of yoghurts, they are not shown in this figure. (Data are Means±SD, n= 3).

**Figure 4.** Viscosity values for plain and probiotic yoghurts (made from fresh and refrigerated milk) as the function of storage time with and without CO₂ addition (control) at temperature 20°C and shear rate of 4 s⁻¹. a, b, c and d states significant differences P< 0.05. Since the combined effects of CO₂, time of preservation of raw milk, type of starter, and storage of yoghurts did not have significant (P> 0.05) effect on viscosity and syneresis of yoghurts, they are not shown in this figure. (Data are Means±SD, n= 3).
Figure 5. Synthesis values for plain and probiotic yoghurts as the function of storage time with and without CO₂ addition (control) at temperature of 20°C (Combination effects of CO₂ type of starter and storage of yoghurts on synersis). Since the combined effects of CO₂ time of preservation of raw milk, type of starter, and storage of yoghurts did not have significant (P > 0.05) effect on viscosity and synersis of yoghurts, they are not shown in this figure. (Data are Means±SD, n= 3).

Figure 6. Evaluation of overall acceptibility during cold storage of yoghurts.
Data are means ± S.D., n=3.

Figure 7. Evaluation of taste during cold storage of yoghurts. (Data are Means±SD, n= 3).
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CONCLUSIONS

The CO₂-treatment of milk had no significant effect on pH of yoghurts. CO₂-treatment improved technological properties of yoghurts: syneresis was reduced and viscosity was increased. Also CO₂-treatment slightly improved growth and viability of the microorganisms during cold storage. Panelists preferred yoghurts made from CO₂-treated milk to the control samples. Therefore, it was concluded that refrigerated milk preserved by CO₂ addition could be satisfactorily used in the manufacture of yoghurts. This method could also be extended to the production of other fermented milks.

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