

Effects of CO₂ 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 CO₂ addition for the manufacture of plain yoghurt using two commercial strains of *Lactobacillus delbrueckii* ssp. *Bulgaricus* and *Streptococcus thermophilus* and probiotic yoghurt by *Lactobacillus acidophilus* was evaluated. Yoghurts (plain and probiotic) manufactured after milk pasteurization, from fresh and refrigerated CO₂ 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 CO₂ addition of raw milk nor by the residual CO₂ present in the pasteurized milk. CO₂ addition of raw milk slightly enhanced viscosity and reduced syneresis of yoghurts. The taste panel preferred yoghurts made from CO₂ treated milks to the corresponding controls during cold storage. These results support the suitability of CO₂-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 CO₂ (King and Mabbitt, 1982; Roberts and Torrey, 1988; Amigo *et al.*, 1995; Hotchkiss *et al.*, 1999). Several theories explaining the mechanism of CO₂ action on microorganisms have been proposed. The exclusion of oxygen by replacement with CO₂ 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 CO₂ 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 CO₂ 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 CO₂ addition for the manufacture of yoghurt. Also CO₂ 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 CO₂ has been added as a consequence of the decrease in clotting time (de la Fuente, 1998). CO₂-treatment effectively prevents the decrease in cheese yield caused by the microorganisms present in raw milk of poor microbial quality (Ruas-Madiedo *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 10⁵ to 10⁶ CFUml⁻¹. Lactic acid bacteria seem to be relatively tolerant to CO₂. Furthermore, there is a reason to suspect that the

lactobacilli might react positively to CO₂ injection in milk. It is known that CO₂ 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 CO₂ 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, Denmark) 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 10⁶ and 10⁷ 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 CO₂ 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 CO₂ addition on the subsequent characteristics of yoghurt, fresh (24h at 4°C) and refrigerated (4 days at 4°C) CO₂-treated samples were compared with untreated controls: Fresh and Refrigerated. CO₂-treated samples were acidified to pH 6.20 with food grade CO₂ by means of a manual injection system. CO₂ addition was

performed under sterile condition and slowly (68 kpa and 4°C) so as to prevent precipitation of proteins from the milk. CO₂ addition to milk was controlled by pH. After injection, milk was held at 4°C. Then, milk (control: without CO₂ and treatment: with CO₂) was pasteurized at 85°C for 30 minutes, cooled to 42°C and inoculated (3% w/v) with mixture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* (plain), (0.2446% w/v) *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 *Streptococcus thermophilus*, *Lactobacillus acidophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* were spread plated on M17 (Lab MTM, Bury, Lancs BL, UK) and MRS agar (Lioflichem, Roseto d. A. [TE], Italy), respectively. The first culture (*S. thermophilus*) was incubated for 48 hours at 42°C and the second (*L. acidophilus*) and third cultures (*L. delbrueckii* ssp. *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 MV₂ 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⁻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 *Lactobacillus acidophilus*, *Streptococcus thermophilus*, *Lactobacillus bulgaricus* and sensory properties were studied using factorial split plot design based on randomized complete



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 Duncan'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

Table 1. Titrable acidity and pH values of fresh and refrigerated control milk and fresh and refrigerated CO₂-treated milk before (BP)^a and after (AP)^b pasteurization.

Milk sample	Pasteurization	pH	Titrate acidity (Dornic)
Fresh control	BP	6.8±0.02	15±0.03
Fresh CO ₂ treated milk	BP	6.2±0.03	31.2±0.02
Refrigerated control	BP	6.75±0.07	17±0.04
Refrigerated CO ₂ treated milk	BP	6.15±0.05	31.4±0.03
Fresh control	AP	6.44±0.08	15±0.03
Fresh CO ₂ treated milk	AP	6.39±0.06	16±0.04
Refrigerated control	AP	6.39±0.03	15.3±0.03
Refrigerated CO ₂ treated milk	AP	6.33±0.07	17±0.02

Data are Means±S.D., n= 3.

^a Before pasteurization, ^b After pasteurization.

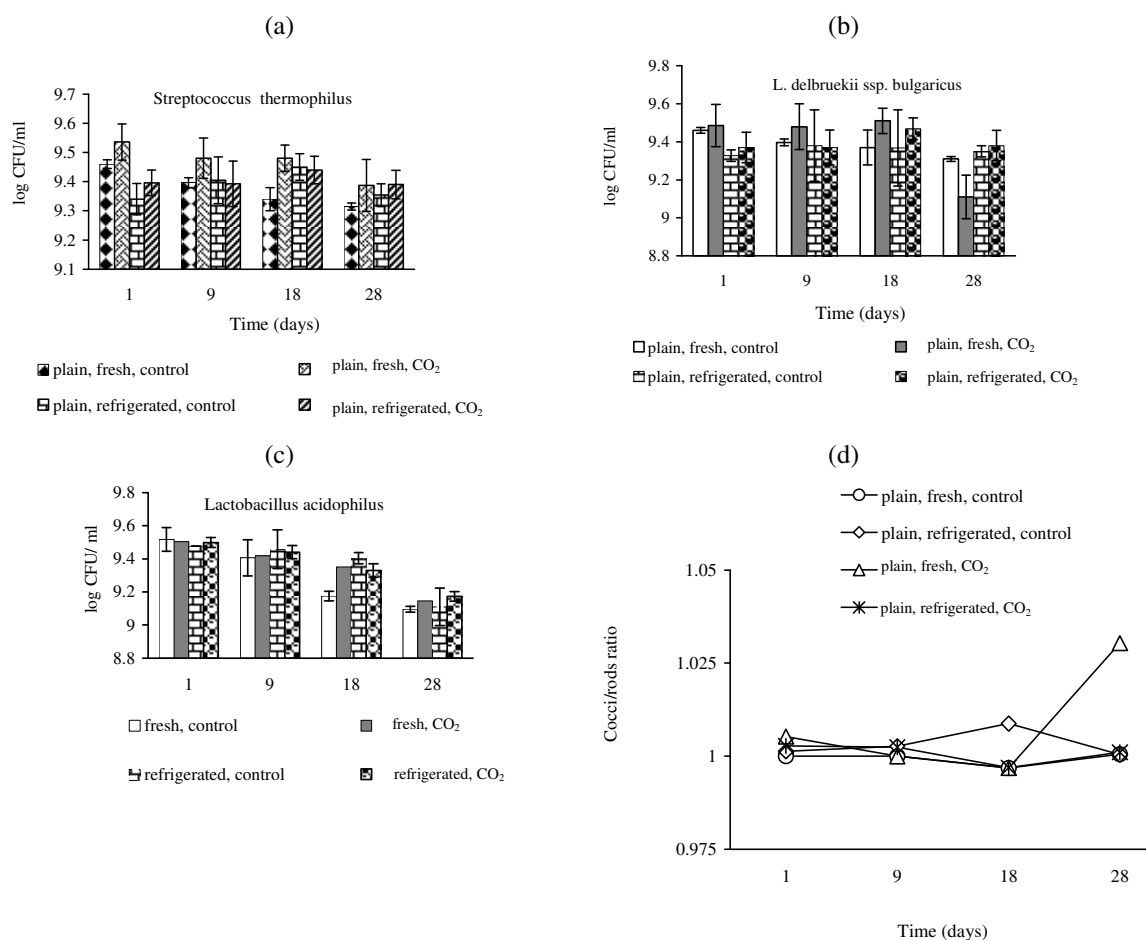


Figure 1(a-d). Evaluation of cell counts of *S. Thermophilus*(a), *L. Bulgaricus*(b), *L. acidophilus* (c) and Changes cocci/rods ratio (*S. thermophilus*/*L. bulgaricus* ratio) during cold storage of plain yoghurts. (Data are Means \pm SD, n= 3).

(control) *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 ($> 10^9$ CFU ml⁻¹) always remained higher than a suggested minimum level needed for producing a therapeutic effect (10^6 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-Akın and Sedar-Akın, 2007). As previously

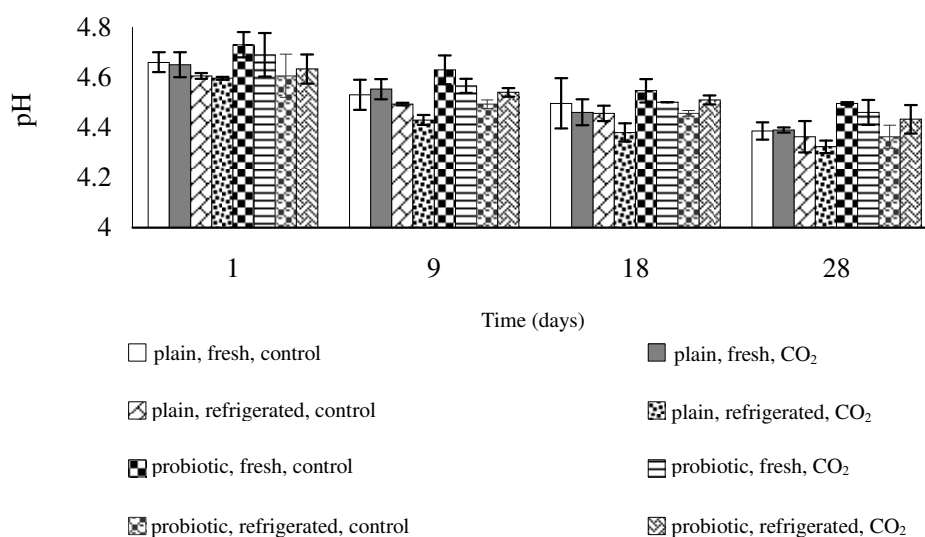


Figure 2. Evaluation of pH during cold storage of yoghurts. (Data are Means \pm SD, n= 3).

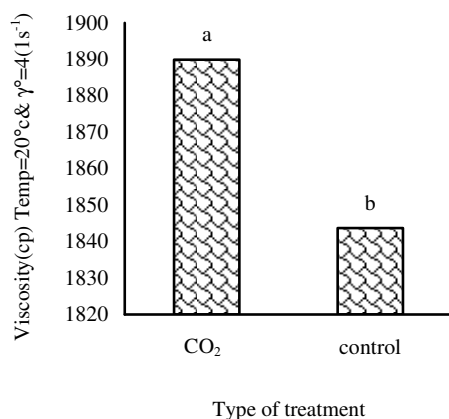


Figure 3. Evaluation effect of CO₂ addition on viscosity of plain and probiotic yoghurts made from refrigerated and fresh milk at 20°C and shear rate of 4 s⁻¹. Since the combined effects of CO₂, time of preservation of raw milk, type of starter, and storage of yoghurts did not have significant ($p >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

indicated by Calvo *et al.* (1993), higher curd hardness and whey losses were also obtained in preserved milk.

Figure 5 shows that prolonged storage affected the extent of syneresis greatly ($P < 0.01$) with a general tendency towards

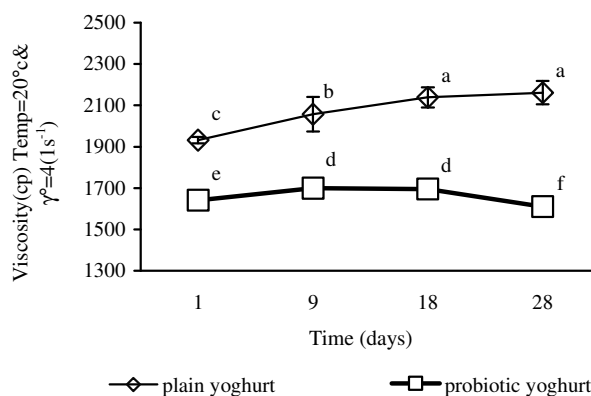


Figure 4. Viscosity values for plain and probiotic yoghurts (made from fresh and refrigerated milk) as the function of storage time with and without CO₂ addition (control) at temperature 20°C and shear rate of 4 s⁻¹. a, b, c and d states significant differences $P < 0.05$. Since the combined effects 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 \pm SD, $n = 3$).

reduction, although exceptions were noticed. On the other hand, Purwandari *et al.* (2007) stated that the duration of the storage of yoghurts had a more pronounced effect on the parameters of viscoelastic and flow behaviour, and on syneresis, than did the type of culture.

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. Panelists 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

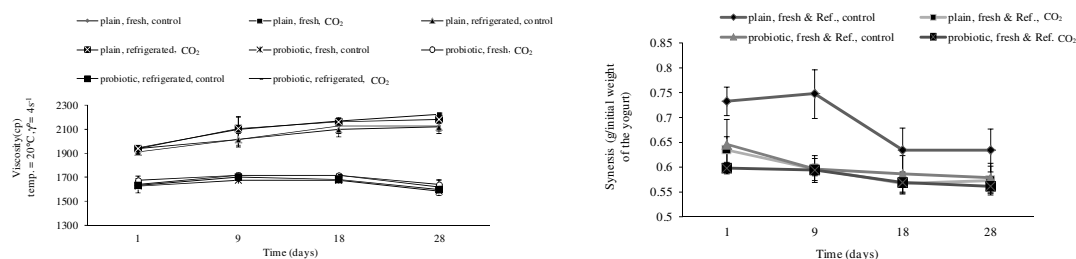


Figure 5. Syneresis values for plain and probiotic yoghurts as the function of storage time with and without CO₂ addition (control) at temperature of 20°C (Combination effects of CO₂, type of starter and storage of yoghurts on syneresis). Since the combined effects of CO₂, time of preservation of raw 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 \pm SD, $n = 3$).

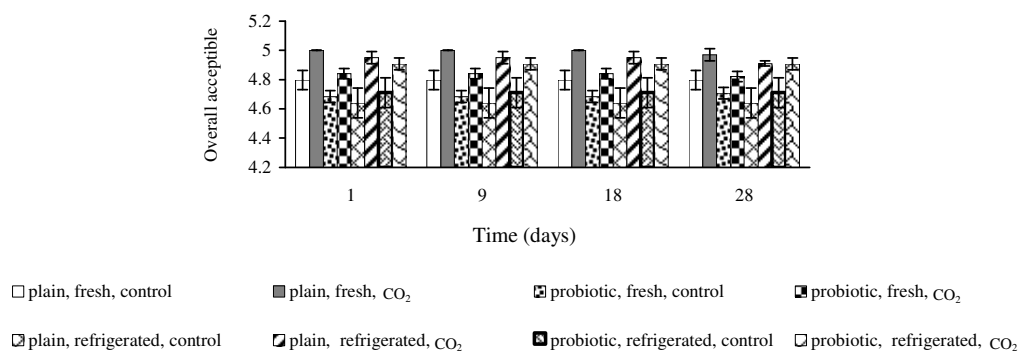


Figure 6. Evaluation of overall acceptability during cold storage of yoghurts. Data are means \pm S.D., $n = 3$.

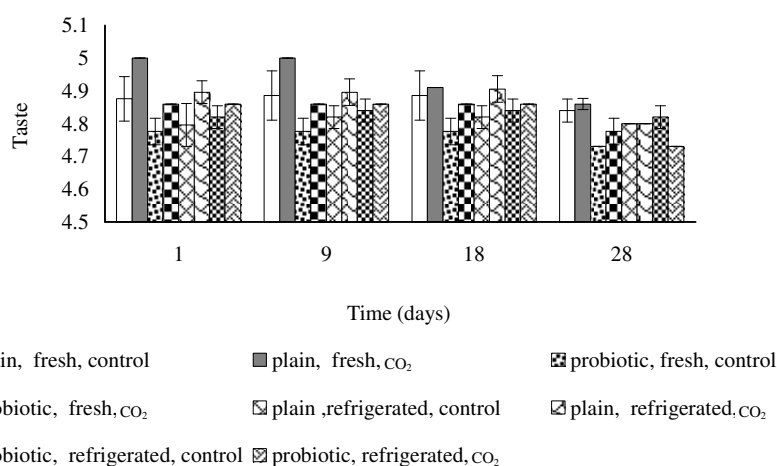


Figure 7. Evaluation of taste during cold storage of yoghurts. (Data are Means \pm SD, $n = 3$).

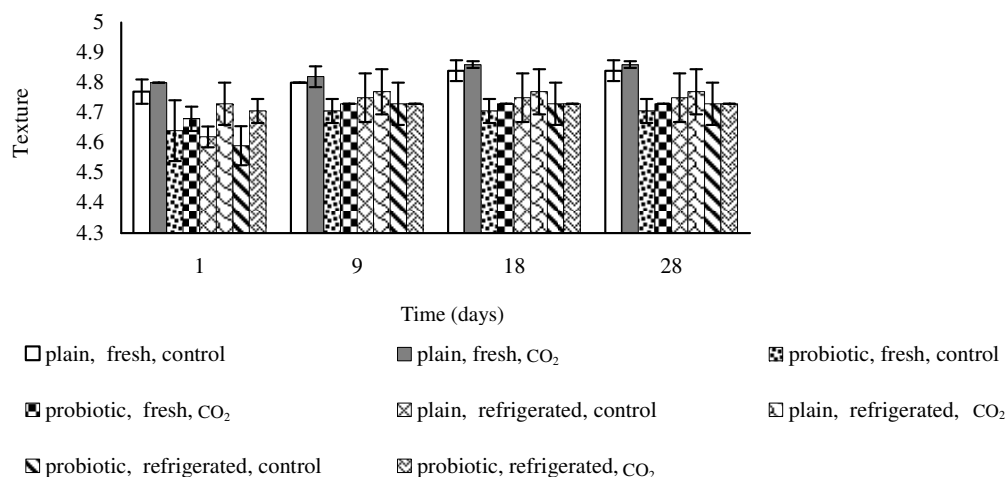


Figure 8. Evaluation of texture and firmness during cold storage of yoghurts. (Data are Means \pm SD, n= 3).

(plain and probiotic). Plain yoghurts were significantly different ($P < 0.05$) in terms of overall acceptability at 28th day of cold storage, but samples of probiotic did not show significant differences ($P > 0.05$) during cold storage. In contrast, Gueimonde *et al.* (2003) detected no differences between controls (both fresh and refrigerated) and CO₂ treated samples at the end of the legal period of storage (24 days). Also Karagul-Yuceer *et al.* (1999) demonstrated that the carbonated yoghurt at the end of the manufacturing process had a refreshing taste. They reported that panelists liked the carbon dioxide in the product because of its perceived fresher or cleaner taste. Also, Vinderola *et al.* (2000) indicated that the use of milk acidified with CO₂ had no detrimental effects on the sensory properties of ABT (*L. acidophilus*, *B. bifidum* and *S. thermophilus*) fermented milk.

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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اثرات افزودن دی اکسید کربن به شیر خام روی ویژگیهای فیزیوشیمیایی و حسی ماست پروبیوتیک قالبی

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چکیده

در این پروژه امکان تولید ماست ساده بوسیله سویه های دلبروکی زیرگونه بولگاریکوس و استرپتوکوکوس ترموفیلوس و ماست پروبیوتیک با استفاده از لاکتوباسیلوس اسیدوفیلوس از شیر خام خنک که با تزریق دی اکسید کربن نگهداری شده است مورد بررسی قرار گرفت. نمونه های ماست ساده و پروبیوتیک تولید شده از شیرهای خام تازه و یخچالگذاری شده تحت دی اکسید کربن با نمونه های کنترل (تهیه شده از شیر خام تازه و یخچالگذاری شده) مقایسه شدند. تکثیر و ظرفیت اسیدسازی آغازگرها نه بوسیله سرد کردن اولیه و تزریق دی اکسید کربن و نه دی اکسید کربن باقیمانده در شیر پاستوریزه تحت تاثیر قرار نگرفت. افزودن دی اکسید کربن به صورت قابل توجهی موجب افزایش گرانی و کاهش آب اندازی ماست شد. ارزیابی های حسی ماستهای تهیه شده از شیر حاوی دی اکسید کربن را به ماست های معمولی ترجیح دادند. این مطالعه مناسب بودن شیر نگهداری شده تحت تزریق دی اکسید کربن را برای تولید ماست نشان داد.