Influence of Carbon Dioxide Addition to Raw Milk on Microbial Levels and Some Fat-Soluble Vitamin Contents of Raw and Pasteurized Milk

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The effect of the application of CO_2 to extend the cold storage of raw and pasteurized milk on the content of fat-soluble vitamins of milk was investigated. CO_2-treated milk (pH 6.2) was compared with a control (unacidified) milk. CO_2-treated and control raw milk samples were stored at 4 °C for 4 days. CO_2-treated milk was then vacuum degasified, and both control and treated samples were pasteurized and stored at 4 °C for 7 days. CO_2 addition inhibited the growth of microorganisms in raw milk without affecting the stability of vitamin A (retinol and β-carotene) and vitamin E (α-tocopherol). Acidity and pH data indicated that subsequent vacuum degasification and pasteurization on a pilot scale partially removed CO_2, making milk acceptable for liquid consumption. However, the residual CO_2 present extended the cold-storage period of pasteurized milk by inhibiting bacterial survivors without detrimental effects on retinol, β-carotene, and α-tocopherol. Slightly higher (not statistically significant, $p > 0.05$) concentrations of retinol, β-carotene, and α-tocopherol were detected during cold storage in raw and pasteurized CO_2-treated milk with respect to the control milk, which could be related to a certain protective effect of the CO_2.

Keywords: *Milk; carbon dioxide; psychrotrophs; cold storage; fat-soluble vitamin*

INTRODUCTION

Vitamins A, D, E, and K are associated with the fat component of milk. Milk is considered to be a major source of vitamin A but contains only small amounts of vitamins E, D, and K (McBean and Speckmann, 1988). Most of the vitamin A activity in milk is due to the *all-trans*-retinol content. The isomer 13-*cis*-retinol is present only in small amounts (Sierra et al., 1996). Carotenoids in milk are usually 11−50% of the total vitamin A activity, β-carotene being the most abundant and most active (McBean and Speckmann, 1988). α-Tocopherol is the main component of the vitamin E in milk, although small amounts of γ-tocopherol (Lehmann et al., 1986), δ-tocopherol, and β-tocopherol (Kanno et al., 1972; Sierra et al., 1996) can also be found. The vitamin content of milk can be modified by refrigeration during storage, transportation to factories, and certain thermal processes. Several authors indicate that fat-soluble vitamins are not affected by pasteurization (Lampert, 1975; Haroon et al., 1982). However, other heat treatments such as ultrahigh-temperature (UHT) processing and in-bottle milk sterilization can induce losses of fat-soluble vitamins due to the concomitant effect of other factors such as residual O_2, light exposure, fat content of milk, container material, or storage conditions (Burton et al., 1970; DeMan, 1981; Senyk and Shipe, 1981; Lau et al., 1986; McCarthy et al., 1986; Schaafsma, 1989; Vidal-Valverde et al., 1992).

Milk is also a good culture medium for microorganisms. Refrigeration of raw milk reduces the growth rate of mesophilic bacteria but favors the proliferation of psychrotrophic microorganisms. Although psychrotrophs are killed by the usual heat treatment of milk, they can produce heat-resistant exocellular proteinases and lipases that are able to degrade various milk components (Kraft, 1992). One procedure to prevent the proliferation of microorganisms involves the addition of CO_2 to refrigerated raw milk (King and Mabbit, 1982; Roberts and Torrey, 1988; Amigo et al., 1995; Ruas-Madiedo et al., 1996a; Espie and Madden, 1997). This is an affordable method to extend cold milk storage at the farm or processing plant. Acidification to pH 6.2 with periodic pH adjustments by gas bubbling has proved to be very efficient for extending the cold storage of raw milk. Vacuum degasification prior to pasteurization rendered milk acceptable for liquid consumption (Amigo et al., 1995; Ruas-Madiedo et al., 1996a).

Although considerable research has been carried out on the use of CO_2 to control the growth of microorganisms, few data are available about its influence on biochemical parameters of milk. The effect of CO_2 itself or combined with a heat treatment was reported on caseins and whey proteins (Eie et al., 1987; Chang and Zang, 1992; Olano et al., 1992; Ruas-Madiedo et al., 1996a), carbohydrate fraction (Olano et al., 1992), and organic acids and volatile compounds (Ruas-Madiedo et al., 1996a). A recent study by Sierra et al. (1996) demonstrated that *all-trans*-retinol, tocopherols, and β-carotene remained stable in refrigerated raw milk

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acidified with CO_2 (pH 6.0 and 6.4) after 7 days of storage. However, no studies are available on the influence of the CO_2 treatment of raw milk on the fat-soluble vitamins of the product after pasteurization. Therefore, the aim of the present work was to evaluate at pilot scale the effect of the CO_2 treatment of raw milk and the subsequent degasification and pasteurization on the fat-soluble vitamin content of milk during cold storage.

MATERIALS AND METHODS

Milk Processing. Raw milk samples with initial microbial loads of $\sim 1.06 \times 10^5$ were collected from one farm in Asturias (northern Spain). Four trials were carried out, each consisting of 120 L of raw milk distributed in three nonhermetically closed 40-L containers and stored for 4 days at 4 °C with constant stirring (14 rpm). One container was maintained as a control milk; the other two were acidified to pH 6.2 with food-grade CO_2 (Carburos Metálicos S.A., Barcelona, Spain). CO_2 was initially bubbled in until the pH of the milk ranged between 6.1 and 6.3. The pH variations were corrected daily by short-time CO_2 injections (5−10 min).

After 4 days of storage, the CO_2-treated milk was degasified through vacuum. Both refrigerated control and treated milk were separately pasteurized (72 °C, 15 s) in a plate pasteurizer heat-exchange unit as previously described by Ruas-Madiedo et al. (1996a) working at 260 L/h. Aliquots of each pasteurized control milk and pasteurized degasified CO_2-treated milk were respectively transferred to dark glass bottles (200 mL each) without leaving headspace and maintained at 4 °C for 7 days.

Samples for analyses were taken at different times of processing: raw milk at day 0 (0BP), raw milk stored for 4 days at 4 °C (4BP), milk just after pasteurization (0AP), milk after pasteurization and cold storage at 4 °C for 5 days (5AP), and milk after pasteurization and cold storage at 4 °C for 7 days (7AP).

Microbiological Counts. Serial dilutions of milk were made in a one-fourth strength Ringer's solution (Oxoid, Unipath Ltd., Basingstore, Hampshire, England) and deep plated on plate count agar (PCA) (Biokar Diagnostics, Beauvois, France) with incubation at 30 °C for 72 h. Plates were prepared in duplicate. Microbiological count data are expressed as log of colony forming units (CFU) per milliliter (CFU/mL).

Titratable Acidity and pH. Titratable acidity and pH were measured as previously described (Ruas-Madiedo et al., 1996a).

Fat-Soluble Vitamins. Retinol, vitamin E (α-tocopherol), and β-carotene were determined according to the following method: milk samples (10 mL) were submitted to overnight saponification at room temperature and extracted as described by Renken and Warthesen (1993) with some modifications. Volumes were readjusted to extract 10 mL of whole milk. After the two extraction steps with petroleum ether/diethyl ether (90:10, v/v) (Panreac S.A., Barcelona, Spain), the aqueous layer was re-extracted with 10 mL of petroleum ether/diethyl ether and then 14.5 mL of distillate water was added prior to a new extraction step with 14.5 mL of diethyl ether. The organic layers were washed three to four times with ice water to ensure neutrality and concentrated to 1−2 mL in a rotary evaporator under nitrogen atmosphere (≤40 °C). Absolute ethanol (2 mL) was added, and the solvent was evaporated under nitrogen until dry. The residue was reconstituted in 3 mL of methanol containing 0.0025% butylated hydroxytoluene (BHT; Fluka BioChemikal, Buchs, Switzerland), and 50−100-μL aliquots were injected into the HPLC system.

A short-time saponification procedure at high temperature (Brubacher et al., 1985) was also assayed as described by Medrano et al. (1994).

Recoveries of β-carotene by both methods were similar for control and CO_2-treated samples. However, considerable losses of retinol and α-tocopherol from CO_2-treated samples occurred when the short-time saponification was used. Therefore, overnight saponification at room temperature was routinely employed. This method gave average recoveries of 76.77 ± 0.92 and 96.87 ± 2.19% for retinol and α-tocopherol, respectively.

Table 1. Comparison of Total Plate Counts, pH, and Acidity in Untreated and CO_2-Treated Milk during Cold Storage before and after Pasteurization

		mean ± SD[a]		
storage time[b]	treatment	total counts (log CFU/mL)	pH	titratable acidity (g of acid/100 mL)
0BP	raw milk	5.03 ± 0.89	6.79 ± 0.02	0.15 ± 0.03
4BP	untreated	6.54 ± 0.58	6.73 ± 0.11	0.15 ± 0.01
	CO_2-treated	5.14 ± 0.59**	6.24 ± 0.09***	0.28 ± 0.03**
0AP	untreated	2.92 ± 0.26	6.76 ± 0.14	0.16 ± 0.01
	CO_2-treated	2.82 ± 0.35	6.55 ± 0.12	0.19 ± 0.02*
7AP	untreated	6.09 ± 0.90		
	CO_2-treated	4.90 ± 1.06		

[a] * $p < 0.1$; ** $p < 0.05$; *** $p < 0.001$. [b] 0BP, day 0 before pasteurization; 4BP, day 4 before pasteurization; 0AP, day 0 after pasteurization; 7AP, day 7 after pasteurization. Temperature during cold storage was 4 °C.

Standard solutions of *all-trans*-retinol (Fluka), DL-α-tocopherol (Sigma-Aldrich Quimica S.A., Madrid, Spain), and β-carotene (Sigma) were prepared in ethanol containing 0.025% BHT and stored at −20 °C. Working standard solutions were prepared by appropriate dilution of the stock solutions.

The vitamins were quantified by HPLC using Waters chromatographic equipment consisting of a model 600E solvent delivery system, a model 717 autosampler, a model 996 photodiode array detector, and Millennium 2010 software (Waters Corp., Milford, MA). The column used was a Lichrosorb RP-18 (5 μm, 250 × 4 mm) (Merck, Darmstadt, Germany). Retinol and α-tocopherol were eluted with methanol/water (98:2) at a flow rate of 1 mL/min. β-Carotene was eluted with methanol at 2 mL/min. The vitamins were quantified by integration of the corresponding peak areas obtained at 326 nm (retinol), 288 nm (α-tocopherol), or 452 nm (β-carotene).

Statistical Analysis. Statistical analysis was performed by using the SPSS-PC+ 4.0 software (SPSS, Inc., Chicago, IL). Data from total plate counts, pH, titratable acidity, and vitamin concentration were subjected to ANOVA test (Snedecor and Cochran, 1980). Results obtained at different times of sampling were compared using the CO_2 treatment as factor with two categories: untreated and CO_2-treated milk. The vitamin content of milk was also subjected to ANOVA test using the storage time as factor with five categories: 0BP, 4BP, 0AP, 5AP, and 7AP.

RESULTS AND DISCUSSION

Table 1 shows the pH values and titratable acidity of untreated and CO_2-treated milk at different times of processing. The pH of CO_2-treated raw milk remained within the range 6.1−6.3 during cold storage (Ruas-Madiedo et al., 1996b). Just before pasteurization, pH values of untreated and CO_2-treated milk were 6.72 and 6.24, respectively ($p < 0.001$) and those of the titratable acidity were 0.149 and 0.275 ($p < 0.05$). These data corroborated that residual CO_2 remained in treated samples after pasteurization, being not totally removed by degasification (Ruas-Madiedo et al., 1996a).

As previously indicated by several authors (King and Mabbit, 1982; Roberts and Torrey, 1988; Amigo et al., 1995; Ruas-Madiedo et al., 1996a), the growth of microorganisms in raw milk was slower in CO_2-treated samples than in the control ones. Differences between untreated control and CO_2-treated samples reached 1.39 log units after 4 days of refrigeration and corroborated

Table 2. Concentration of Retinol, β-Carotene, and α-Tocopherol in Untreated and CO_2-Treated Milk during Cold Storage before and after Pasteurization

storage time[a]	treatment	retinol	β-carotene	α-tocopherol
0BP	untreated	51.32 ± 5.53	8.68 ± 1.90	120.66 ± 10.94
4BP		49.76 ± 5.38	9.08 ± 1.25	121.13 ± 10.10
0AP		47.91 ± 4.28	8.67 ± 1.75	115.32 ± 13.63
5AP		48.23 ± 2.76	9.10 ± 1.13	121.11 ± 8.74
7AP		47.68 ± 3.91	8.41 ± 1.09	115.41 ± 8.79
0BP	CO_2-treated	51.32 ± 5.53	8.68 ± 1.90	120.66 ± 10.94
4BP		51.29 ± 5.64	8.59 ± 1.06	123.12 ± 11.99
0AP		49.63 ± 6.53	9.12 ± 0.94	121.14 ± 12.21
5AP		50.51 ± 0.23	9.30 ± 1.89	125.04 ± 12.46
7AP		50.98 ± 5.25	8.75 ± 0.59	122.70 ± 15.91

[a] 0BP, day 0 before pasteurization; 4BP, day 4 before pasteurization; 0AP, day 0 after pasteurization; 5AP, day 5 after pasteurization; 7AP, day 7 after pasteurization. Temperature during cold storage was 4 °C.

the inhibitory effect of the CO_2 treatment on the microbiological growth in milk. Total plate counts of control and CO_2-treated milk (Table 1) abruptly decreased after pasteurization. During cold storage after pasteurization, microbial growth rates were lower in the pasteurized CO_2-treated milk relative to the untreated control milk due to the inhibitory effect of the residual CO_2 still present after vacuum degasification and pasteurization in treated samples. The maximum level of total plate counts allowed in Spain for pasteurized milk is 1×10^5 CFU/mL (Pascual-Anderson, 1992). As shown in Table 1, at 7 days of storage only the pasteurized CO_2-treated milk was still within these values.

Concentrations of retinol, β-carotene, and α-tocopherol during the cold storage are summarized in Table 2. The values obtained for these vitamins were in the range of values previously published in the literature (Schaafsma, 1989; McBean and Speckmann, 1988). No significant variations were found ($p > 0.05$) in the content of retinol, α-tocopherol, and β-carotene of the untreated control and of the CO_2-treated samples (data not shown) during the cold storage, indicating a great stability of fat-soluble vitamins in milk. Neither was any significant variation found between the untreated control and the CO_2-treated milk in the retinol, β-carotene, and α-tocopherol contents ($p > 0.05$) (data not shown). However, most of the levels of these fat-soluble vitamins were slightly higher in the CO_2-treated milk (except for β-carotene at point 4BP) through the cold storage. These results are in accordance with those of Sierra et al. (1996), who reported no changes by CO_2 addition on vitamin retention during cold storage of raw milk. After 7 days of cold storage of pasteurized milk, differences of concentration between CO_2-treated and untreated samples were 3.3, 7.29, and 0.34 μg/100 mL for retinol, α-tocopherol, and β-carotene, respectively. We have not found information in the literature about the effect of the CO_2 addition to raw milk on the subsequent cold-stored pasteurized milk. However, Lau et al. (1986) indicated that in vitamin A fortified milk the degradation rates of the vitamin were lineal and varied inversely with the fat content of milk. The fat percentage of milk used in our work ranged from 3.6 to 3.9 and could have a certain protective effect on the fat-soluble vitamins. In contrast, Le Maguer and Jackson (1983) reported higher rates of vitamin A degradation in cartons of 2% UHT fortified milk with residual oxygen present. These authors also observed a doubling of the vitamin A degradation when storage temperatures increased from 20 to 35 °C. Several authors also indicated that pasteurization (Lampert, 1975; Haroon et al., 1982; Schaafsma, 1989) and degasification (Sierra et al., 1996) techniques did not affect fat-soluble vitamin content of milk. In our work, raw milk was stored at refrigeration with constant slow stirring, which can induce oxidation of milk. The reduction of oxygen content as a consequence of the CO_2 addition in raw milk and the residual CO_2 remaining in vacuum-degasified pasteurized milk could explain the slightly higher content of fat-soluble vitamins in CO_2-treated milk with respect to the control milk during the cold storage. The inhibitory effect of CO_2 on psychrotrophic bacteria during the storage at 4 °C could also avoid a destabilization and degradation of fat globules in milk by lipolytic microorganisms.

In conclusion, CO_2 addition to raw milk inhibited the growth of microorganisms without affecting the stability of fat-soluble vitamins. Subsequent vacuum degasification and pasteurization on a pilot-scale partially removed CO_2 and make milk acceptable for liquid consumption or dairy processing (Ruas-Madiedo et al., 1996a). The residual CO_2 not removed by degasification extended the shelf life of pasteurized milk by inhibition of survivors during cold storage without detrimental effects on retinol, α-tocopherol, and β-carotene.

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