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Free Fatty Acid Accumulation by Mesophilic Lactic Acid Bacteria in Cold-Stored Milk

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This study was aimed to determine the accumulation of free fatty acid by mesophilic lactic acid bacteria (*Lactococcus lactis* subsp. *lactis* 1471, *Lactococcus lactis* subsp. *cremoris* 1000 and *Lactobacillus casei* 111) in cold-stored milk. According to the results, all cold-stored milks had higher acid degree values than those of fresh milk. This phenomenon showed that a slight increase occurred in the accumulation of free fatty acids as a result of spontaneous lipolysis during cold storage. All lactic acid bacteria showed good performance in production of titratable acidity, which increased during fermentation of the milk (fresh and stored milks). Moreover, as the storage time was prolonged, more free fatty acid accumulation was obtained from the fermentation of the cold-stored milk by the investigated lactic acid bacteria. The control milk, which was without lactic acid bacteria, showed no change in the accumulation of free fatty acid during fermentation. From this result, it can be suggested that longer cold-storage time can induce higher free fatty acid accumulation in milk by lactic acid bacteria.

Key words: free fatty acid, lactic acid bacteria

Accumulation of free fatty acids (FFAs) in milk mainly originates from three sources; those directly diffused from blood to milk, those not esterified in milk-secretory cells of the mammary gland, and those liberated by enzyme action (Metin, 1999).

FFAs in milk and dairy products are formed mainly as a result of indigenous lipase action found in milk (Anonymous, 1991). This lipase is called lipoprotein lipase, and a portion of it is found together with casein micelles (plasma lipase), and the rest is found in the fat globule membrane (membrane lipase). When milk is cooled without any mechanical agitation, spontaneous lipolysis occurs in milk through the membrane lipase. Any agitation or homogenization may damage the fat globule membrane, and the lipolysis level is accelerated by the plasma lipase, which is called as induced lipolysis (Gönç and Gahun 1979, Atamer 1993).

On the other hand, the most important effect on lipolysis comes from the lipases of psychrotrophs (certain strains of *Pseudomonas* spp., *Enterobacteriaceae* and *Acinetobacter*) that are grown in cold-stored milk. This effect may become noticeable when cell counts exceed 10^6 or 10^7/ml. The indigenous enzyme of milk and the cells of psychrotrophs are inactivated through heat treatment. However, the exocellular lipases from psychrotrophs cannot be inactivated since they are heat-stable (Kiliç *et al.* 2000). Additionally, many other direct or indirect factors such as feed, lactation period, mastitis, estrus period, piping of the milk to the tanks, agitation, homogenization, thermal manipulation, and somatic cell count may cause FFA accumulation in milk (Weinrauch 1988). The release of short-chain fatty acids by hydrolysis of ester bands in lipids is responsible for the development of the rancid flavor in raw milk and also in dairy products.

Lactic acid bacteria may be an additional contributor in the accumulation of FFAs in milk and dairy products. Kurmann (1988) reported that *L. acidophilus, L. brevis, L. fermentum*, and *L. plantarum* are lipolytically active species among lactic acid bacteria. Dellagio (1988) stated that *Streptococcus thermophilus* and *L. bulgaricus* have partial lipolytic activity. However, *L. bulgaricus* is more lipolytically active than *Str. thermophilus*. Menéndez *et al.* (2000) studied the effects of various lactic acid bacteria on the proteolysis and lipolysis of cheese, and they found that tested *Lactobacillus* spp. showed higher lipolytic activity than classic mesophilic *Lactococcus*. The cheeses that are made with *Lactobacillus* spp. as the starter bacteria had a more rancid flavor than the others. Early studies reported that the growth of *Streptococcus lactis* was inhibited in rancid milk possibly due to FFA accumulation (Sellars and Babel, 1985). Sausa and Malkata (1997) studied the effects of pasteurization of milk and the addition of rennet and starter on the lipolysis in cheese, and they measured

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lipolysis in raw milk cheese as 3,538 mg/kg, in pasteurized-no starter cheese as 3,002 mg/kg, and in pasteurized and starter added cheese as 4847 mg/kg.

Since milk is a rich medium for the growth of microorganisms, it must be cooled and stored under refrigeration conditions. During this storage, some degradation of proteins by the enzymes of psychrotrophs occurs and results in the loss of cheese yield and smooth texture (Fox, 1989). This is one of the disadvantages with using cold-stored milk for the production of cheese. However, no study was monitored for the relationship between cold-stored milk and lactic acid bacteria activity in terms of FFA accumulation.

This study was conducted to determine the occurrence of spontaneous lipolysis in cold-stored milk and to reveal how mesophilic lactic acid bacteria behave in fresh milk and in cold-stored milk in terms of FFA accumulation.

Materials and Methods

Materials

Cow milk from a private producer in Van city, Turkey, was used in the study. Immediately after milking, the morning milks were taken and transported to the laboratory without agitation. Three mesophilic starter lactic acid bacteria, *Lactococcus lactis* subsp. *lactis* 1471, *Lactococcus lactis* subsp. *cremoris* 1000 and *Lactobacillus casei* 111, were obtained from the Food Microbiology Laboratory of the Food Engineering Department, Agricultural College of Ankara University, Turkey. Cultures were activated as described by Sellars and Babel (1985).

Fermentation

The milk samples were divided into three parts. The first part of the milk (fresh milk) was analyzed chemically (dry matter, fat, TA %) and microbiologically (total aerobic count). Also, acid degree value (ADV) as total free fatty acids was determined. After that, the first part of the milk was autoclaved at 120°C for 15 min and was cooled to 30°C. Then, it was inoculated with one of the mesophilic lactic acid bacteria at the rate of 2%. During fermentation, changes in titratable acidity (TA %) and ADV were monitored (Fig. 1).

The cell count in the inoculated volume was 5.04×10^8 cfu/ml for *L. lactis* 1471, 5.70×10^8 cfu/ml for *L. cremoris* 1000, and 1.80×10^8 cfu/ml for *L. casei* 111, respectively. Fermentation time was 48 h for *L. lactis* 1471 and *L. casei* 111 and 24 h for *L. cremoris* 1000. Analysis periods were the 0, 8th, 24th, 48th h for *L. lactis* 1471 and *L. casei* 111 and 0, 4th, 8th, 24th h for *L. cremoris* 1000.

The second part of the milk was stored at 3 ± 0.5°C for one day. After storage, the chemical, microbiological analysis and ADV were carried out, and the milk was autoclaved as described above. Then, the milk was inoculated with one of the starter bacteria. During fermentation, changes in TA and ADV were monitored. The third part of the milk was kept at 3 ± 0.5°C for two days, and after, the same procedure was followed as described above (Fig. 1).

The control milk was not inoculated with any bacteria; however, the rest of the procedure was the same. The experiment was performed in duplicate for both cultured milk and non-cultured (control) milk, using a fresh sample of milk each time.

Analysis

Dry matter (DM), fat, titratable acidity (TA %), and total mesophilic aerobic count in the milk samples were determined using the methods given by Kurt *et al.* (1993).

To determine FFA, 50 ml of a milk sample was placed in a modified butyrometer; 10 ml of BDI reagent (a solution of 30 g Triton X-100 and 70 g of sodium tetra phosphate in 1 L of distilled water) was added, and the butyrometers were placed in a gently boiling water bath for 20 min to liberate the fat. The mixture was centrifuged for 1 min, and enough aqueous methanol was added to bring the fat into the column neck of the butyrometer.

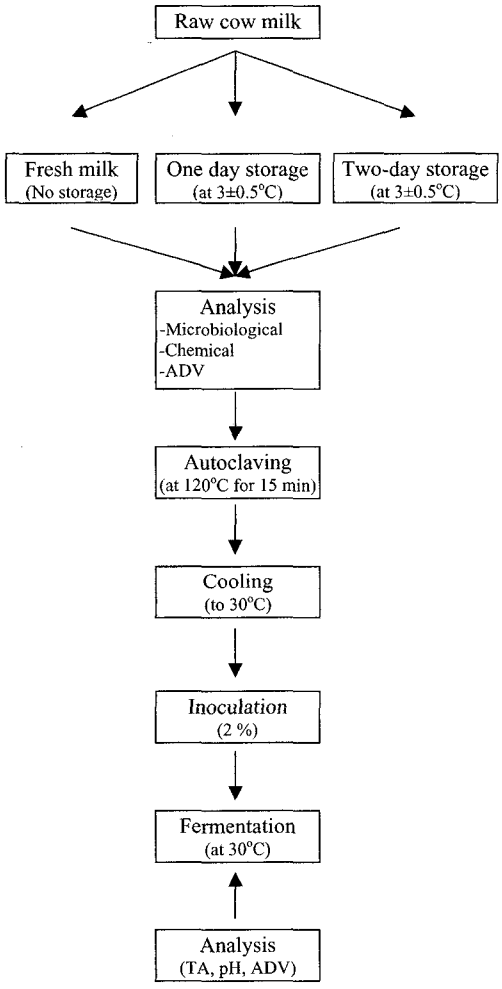

Fig. 1. Procedures applied to the milk samples in the experiment.

Table 1. The properties of fresh and cold-stored milk samples used for fermentation with mesophilic lactic acid bacteria*

Properties	Storage time (Days)			
	Milks used for	0 (Fresh)	1	2
TA (%)	*L. lactis* 1471	0.14±0.007	0.14 ± 0.014	0.14 ± 0.007
	L. cremoris 1000	0.16±0.014	0.17±0.021	0.14±0.014
	L. casei 111	0.16±0.021	0.16±0.007	0.16±0.014
	Control	0.16±0.014	0.16±0.000	0.17±0.000
DM (%)	*L. lactis* 1471	11.54±0.332	11.54±0.332	11.54±0.332
	L. cremoris 1000	12.9 ±0.000	12.9 ±0.000	12.9 ±0.000
	L. casei 111	12.35±1.767	12.35±1.767	12.35±1.767
	Control	12.80±1.131	12.80±1.131	12.80±1.131
Fat (%)	*L. lactis* 1471	3.95±0.070	3.95±0.070	3.95±0.070
	L. cremoris 1000	4.00±0.000	4.00±0.000	4.00±0.000
	L. casei 111	3.90±0.141	3.90±0.141	3.90±0.141
	Control	4.10±0.141	4.10±0.141	4.10±0.141
ADV	*L. lactis* 1471	0.41±0.007	0.66±0.014	0.58±0.183
	L. cremoris 1000	0.37±0.056	0.41±0.070	0.40±0.077
	L. casei 111	0.47±0.014	0.48±0.007	0.52±0.028
	Control	0.48±0.000	0.47±0.000	0.51±0.042
Total aerobic count (Log/ml)	*L. lactis* 1471	6.18±0.516	6.35±0.395	6.19±0.622
	L. cremoris 1000	5.89±0.091	6.43±0.056	6.47±0.007
	L. casei 111	5.81±1.145	6.37±0.523	6.78±0.042
	Control	5.94±1.329	7.00±0.007	7.42±0.558

*Each value in the table shows the mean of two repetitions and standard deviation.

Then the mixture was centrifuged for another 1 min. The fraction of liquid fat was transferred into a 50 ml flask and was weighed. Five ml of fat solvent (4 : 1 petroleum ether and *n*-propanol) was added to the flask. Then, 5 drops of 1% phenolphthalein (=1 g, dissolved in pure methanol) was added. The content was titrated with 0.02 N alcoholic KOH. Total free fatty acid was calculated as described by Case *et al.* (1985). The amount of total free fatty acids was expressed as Acid Degree Value (ADV).

Data obtained from fermented milks were statistically evaluated using a 3×4×2 factorial experimental design. Analysis of variance was performed using the Super ANOVA package program. Duncans multiple range test was used to compare significant means of parameters (Anonymous, 1989).

Results

Table 1 shows the properties of fresh milk and two day old cold-stored milk that were used for the fermentations of mesophilic lactic acid bacteria and the control.

Milks used for the lactic acid bacteria and the control were from different batches for the experiments. Each batch of milk had its own properties, and among these properties TA (%), dry matter (DM), and fat contents were almost stable during cold storage. In terms of ADV value, all cold-stored milks had higher ADV values than those of fresh milks. This phenomenon showed that a slight increase occurred in ADV values as a result of spontaneous lipolysis during cold storage. Also, a slight increase was observed in total aerobic counts of the cold-stored milks.

All lactic acid bacteria (LAB) that were inoculated into the milks (fresh and stored) increased titratable acidity (TA) from ~0.15 to a range between >0.30 and <0.65% during fermentation (Table 2). Increasing the TA values is important in terms of showing that the LAB tested is alive. Relatively higher TA values were obtained from fermentation of those milks stored for two days. However, the differences between TA values of fresh milk and stored milk were not significant ($p>0.05$). Differences between fermentation periods for all tested LAB were found to be significant, but the control milk without LAB did not develop TA during fermentation.

Table 3 demonstrates total free fatty acid accumulation through the use of lactic acid bacteria in fresh and cold stored milks.

The control milk during all fermentation periods for the tested LAB showed no change in FFA accumulation between fresh and stored milks ($p>0.05$) and also between 0, 4, 8, and 24 fermentation hours ($p>0.05$) (Table 3). However, the ADV value of each day milk slightly increased within 48 h ($p<0.05$). This increase can be explained by partial reactivation of the enzymes (Metin, 1999).

Table 2. Changes in titratable acidity (%) during fermentation of fresh and stored milks that were inoculated with mesophilic lactic acid bacteria*

LAB	Storage (Days)	Fermentation times (h)				
		0	8	24	48	
L. lactis 1471	0 (Fresh)	0.14 ± 0.000	0.19 ± 0.007	0.31 ± 0.007	0.46 ± 0.000	a
	1	0.14 ± 0.007	0.19 ± 0.014	0.34 ± 0.028	0.53 ± 0.049	a
	2	0.13 ± 0.014 a**	0.20 ± 0.021 b	0.29 ± 0.056 c	0.53 ± 0.028 d	a
L. casei 111	0 (Fresh)	0.16 ± 0.014	0.21 ± 0.007	0.28 ± 0.007	0.55 ± 0.042	a
	1	0.17 ± 0.021	0.19 ± 0.014	0.31 ± 0.042	0.52 ± 0.035	a
	2	0.16 ± 0.014 a**	0.21 ± 0.014 a	0.44 ± 0.134 b	0.65 ± 0.169 c	a
		Fermentation times (h)				
		0	4	8	24	
L. cremoris 1000	0 (Fresh)	0.16 ± 0.007	0.18 ± 0.007	0.19 ± 0.000	0.32 ± 0.035	a
	1	0.15 ± 0.000	0.18 ± 0.007	0.19 ± 0.000	0.31 ± 0.028	a
	2	0.15 ± 0.014 a**	0.19 ± 0.007 b	0.19 ± 0.007 b	0.37 ± 0.021 c	a
		Fermentation times (h)				
		0	4	8	24	48	
Control	0 (Fresh)	0.17 ± 0.000	0.16 ± 0.014	0.16 ± 0.014	0.16 ± 0.014	0.17 ± 0.000	a
	1	0.17 ± 0.007	0.17 ± 0.007	0.17 ± 0.000	0.17 ± 0.000	0.17 ± 0.000	a
	2	0.17 ± 0.000 a**	0.17 ± 0.007 a	0.17 ± 0.007 a	0.17 ± 0.007 a	0.17 ± 0.000 a	a

*Each value in the table shows the mean of two repetitions and standard deviation.
**The means bearing different letters differ from each other at level of $p<0.05$, all others not.

Table 3. The accumulation of free fatty acid (as ADV value) during fermentation of fresh and stored milks inoculated with mesophilic lactic acid bacteria*

LAB	Storage (Days)	Fermentation times (h)				
		0	8	24	48	
L. lactis 1471	0 (Fresh)	0.34 ± 0.141	0.60 ± 0.077	0.50 ± 0.000	0.90 ± 0.325	a
	1	0.51 ± 0.106	1.00 ± 0.113	0.76 ± 0.318	0.86 ± 0.388	ab
	2	0.60 ± 0.035 a**	0.90 ± 0.261 bc	0.73 ± 0.247 ab	1.20 ± 0.318 c	b
L. casei 111	0 (Fresh)	0.44 ± 0.091	0.54 ± 0.035	0.43 ± 0.113	0.64 ± 0.098	a
	1	0.47 ± 0.091	0.61 ± 0.176	0.65 ± 0.084	0.93 ± 0.049	b
	2	0.46 ± 0.063 a**	0.61 ± 0.063 ab	0.95 ± 0.148 b	0.97 ± 0.296 c	b
		Fermentation times (h)				
		0	4	8	24	
L. cremoris 1000	0 (Fresh)	0.33 ± 0.000	0.36 ± 0.035	0.39 ± 0.021	0.58 ± 0.035	a
	1	0.39 ± 0.042	0.49 ± 0.021	0.47 ± 0.014	0.65 ± 0.070	b
	2	0.51 ± 0.007 a**	0.49 ± 0.021 a	0.56 ± 0.084 a	0.79 ± 0.120 b	c
		Fermentation times (h)				
		0	4	8	24	48	
Control	0 (Fresh)	0.46 ± 0.056	0.46 ± 0.014	0.45 ± 0.021	0.45 ± 0.007	0.60 ± 0.028	
	1	0.50 ± 0.042	0.47 ± 0.028	0.44 ± 0.000	0.44 ± 0.028	0.53 ± 0.000	
	2	0.40 ± 0.014 a**	0.44 ± 0.000 a	0.47 ± 0.028 a	0.42 ± 0.042 a	0.46 ± 0.000 b	

*Each value in the table shows the mean of two repetitions and standard deviation.
**The means bearing different letters differ from each other at level of $p<0.05$, all others not.

L. lactis 1471 increased ADV values in fresh milk during fermentation. This increase was observed for the stored milks as well, but with higher values; and the increase was found to be significant for two day-cold stored milks ($p<0.05$), in which the highest ADV value was obtained in the last fermentation hour. *L. casei* 111 produced higher ADV values in those milks stored for 1 and 2 days. The amount of FFA accumulation was close to each other for 1 and 2 day-storages of the milks. However, the amounts from the stored milks were higher than the fresh ones ($p<0.05$). Similar developments were obtained for *L. cremoris* 1000, and when more milks were stored, more FFA accumulation occurred by LAB tested. This accumulation was also significant on days 1 and 2 with a level of $p<0.05$. Meyers *et al.* (1996) studied lipase production by lactic acid bacteria, and the activity on butter oil and a total of 29 lipase-producing strains were identified. Assays using extracellular material and intracellular extracts showed that lipases were produced as intracellular enzymes in the strains among the lactic acid bacteria that were studied.

Discussion

According to the results obtained, LAB used in the study had an increasing effect on FFA accumulation when milk was kept in cold conditions. This may be due to the fact that indigenous lipoprotein lipases and bacterial lipases partly hydrolyzed triglicerides into mono and diglicerides during the cold storage. Thus, mono and diglicerides served as substrates for the lipases produced by lactic acid bacteria. According to this study, lactic acid bacteria may be another factor that increases FFA in milk and in dairy products when the milk is stored in cold conditions for a prolonged period of time. This phenomenon implies that milk should be processed when it is fresh to avoid further lipolysis. Kondyli *et al.* (2002) studied FFAs and volatile compounds of low-fat Feta-type cheese made with a commercial adjunct culture. They found that the adjunct-treated (containing *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*) low-fat cheeses had higher total free fatty acid levels than the low-fat control cheese. Similar results were reported later on in Kefalograviera-type cheese also made with commercial adjunct cultures (LBC 80 (*Lactobacillus casei* subsp. *rhamnosus*) and CR-213 (containing *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*)) (Kondyli *et al.*, 2003).

Conclusion

The results of the present study suggested that cold-storage of milk resulted in an increase in the accumulation of free fatty acids. In addition, when storage time was prolonged, more free fatty acid accumulation by the investigated lactic acid bacteria was obtained. From this result, it can be suggested that longer cold-storage time of milk can induce the free fatty acid accumulation in milk by lactic acid bacteria, and this phenomenon reveals that milk should be freshly processed for better product quality. Otherwise, lactic acid bacteria can contribute or increase the amount of free fatty acid, which affect the taste and flavor of many dairy products.

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