Effect of Seeding Raw Milk with *Lactobacillus lactis* on Growth of Spoilage Organisms during Refrigerated Storage

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Story in Brief

Cells of *Lactobacillus lactis* from frozen concentrated cultures were added to refrigerated raw milk to determine their effect on growth of psychrotrophic spoilage bacteria in the milk during storage at 5 or 7 C. Some strains of *L. lactis* were significantly inhibitory at both temperatures. Two selected strains of *L. lactis* were added along with potassium sorbate to refrigerated raw milk stored at 5 C to determine their combined effect on growth of the psychrotrophs. The combination of cells of lactobacilli and sorbate was more inhibitory than either lactobacilli or sorbate alone.

Introduction

The growth of psychrotrophic microorganisms in raw milk during refrigeration in bulk storage tanks can reduce the quality of the milk. While they are easily killed by heat treatment, some of them produce heat stable enzymes that can survive pasteurization or sterilization treatments and adversely affect the quality of products made from the heat processed milk.

Lactic acid bacteria including lactobacilli can inhibit the growth of food-borne pathogens and spoilage microorganisms in various foods at refrigeration temperatures. The inhibitory agent produced by the lactobacilli has been identified as hydrogen peroxide. Since cells of lactobacilli accumulate hydrogen peroxide in the suspending menstrum during refrigerated storage even though they do not grow, the possibility exists that they might produce sufficient peroxide to inhibit the growth of psychrotrophs in refrigerated food.

Sorbic acid has been used in many foods as a preservative. It is said to be more inhibitory to bacteria similar to those causing spoilage of refrigerated foods than to the lactobacilli. Therefore, a combination of sorbate and cells of *L. lactis* might be expected to exert greater inhibitory action on psychrotrophic spoilage microorganisms in raw milk than would either alone.

The objectives of this study were to determine if the growth of psychrotrophic microorganisms in refrigerated raw milk could be retarded by adding cells of *L. lactis* and to determine if the additions of potassium sorbate along with cells would enhance any inhibition caused by the lactobacilli.

Materials and Methods

The cultures of *L. lactis* were maintained by subculturing (1 percent inocula) weekly into sterile (121 C for 15 min) 10 percent non-fat milk solids (NFMS). The inoculated milk was incubated at 37 C for 24 hr. These cultures were stored in a refrigerator between weekly subcultures. Before use in experiments, the cultures were subcultured twice in 10 ml of sterile lactobacilli MRS broth (Difco) using a one percent inocula and incubating 18 hr at 37 C.

Each of the cultures of *L. lactic* was inoculated into 600 ml of lactobacilli MRS broth (Difco) and incubated at 37 C until the culture reached the late exponential or early stationary phase of growth. The cells were harvested by centrifugation and resuspended in 30 ml of cold, sterile 10 percent NFMS. The resulting concentrated cultures were dispensed in 2 g portions into sterile plastic freezing vials and placed immediately into liquid nitrogen (−196 C) for storage. For experimental use, each frozen concentrated culture was thawed by submerging in 1 liter of tap water (24 C) for 5 min.

Raw milk was obtained from the bulk storage tanks at the Oklahoma State University Dairy Cattle Center. The raw milk was aseptically placed into a sterile flask contained in an ice-water bath for transport to the laboratory and held there until used (never held longer than 2 hr). The raw milk was then dispensed in 100 ml volumes into sterile 250 ml flasks. Thawed concentrated cultures were added to the raw milk in the quantities necessary to obtain 1×10^8 lactobacilli per ml. One flask of raw milk was used as a control. The flasks were then placed in a refrigerated Gyrotory Water Bath Shaker for storage. The bath was at either 5 or 7 C, and agitation was adjusted to 160 RPM. Samples for microbial analyses were taken from each flask initially and daily for 6 days. In all trials pH values were determined on the final day of storage.

Lactobacilli were enumerated in the initial samples by plating the appropriate dilutions with MRS agar (Speck, 1975). The plates were incubated for 48 hr at 37 C. Non-lactobacilli were enumerated by plating appropriate dilutions with Plate Count Agar (PCA) and incubating the plates at 21 C for 5 days. The lactobacilli used in this study did not form colonies on PCA under these conditions. This enabled us to follow the growth of psychrotrophic bacteria in the milk during storage.

Thawed concentrated cultures of selected strains of L. lactis (39A1 and 39A2) were added to raw milk to yield populations of 1×10^8/ml. Three flasks of raw milk (100 ml) were prepared for each strain. A set of three flasks of raw milk without lactobacilli was also prepared. To each set of three flasks with and without lactobacilli, a sterile 10 percent solution of potassium sorbate was added to yield final concentrations of 0, 0.1 percent, and 0.2 percent. The samples were stored at 5 C as in previous experiments. Samples (10 ml) were taken from each flask initially and on the third and fourth days of storage for microbial analyses as indicated in the previous experiments.

Results

Table 1 is a summary of results from four trials in which the antagonistic action of six strains of *L. lactis* toward the growth of psychrotrophs in raw milk was measured at 7 C. The data are presented as the $\log_{10}$ of counts of non-lactobacilli per ml obtained after the milk samples had been stored 4 days at 7 C. When compared to the average count for the control samples, *L. lactis* 403E-15 was the most inhibitory followed in order by strains 39A1, 12315, B, 39A2, and Farr.

Table 1. Numbers of non-lactobacilli in raw milk with and without added cells of *Lactobacillus lactis* after 4 days of storage at 7 C

Sample	Days at 7 C	Non-lactobacilli count/ml[b]				
		Trial 1	Trial 2	Trial 3	Trial 4	Avg.
Control	0	3.56	3.63	4.04	3.40	
Control	4	6.20	7.20	7.65	5.64	6.67
L. lactis B	4	5.70	7.18	6.30	5.18	6.09
L. lactis Farr	4	5.60	7.04	7.11	5.60	6.34
L. lactis 12315	4	5.86	6.85	6.38	5.28	6.09
L. lactis 403E-15	4	5.62	6.53	6.45	5.08	5.92
L. lactis 39A1	4	5.85	6.64	6.88	4.43	5.96
L. lactis 39A2	4	6.15	6.91	6.20	5.08	6.09

[a]*L. lactis* counts were approximately 1 x 10^8/ml.
[b]Counts recorded as $\log_{10}$ of numbers of non-lactobacilli/ml.

There were no differences in the amounts of inhibition produced by strains B, 12315 and 39A2. Statistical analysis of the data revealed that strains B, 12315, and 39A2 were significantly inhibitory at $P<0.05$ and strains 403E-15 and 39A1 were significantly inhibitory at $P<0.01$. Little or no change in acidity was observed in any of the milk samples during storage at 7 C.

Table 2 is a summary of results from six trials in which the antagonistic action of the six strains of *L. lactis* toward the growth of psychrotrophs in raw milk was measured at 5 C. The data are presented as $\log_{10}$ of counts of non-lactobacilli per ml after the milk samples had been stored 4 days at 5 C. *L. lactis* 39A1 was the most inhibitory followed in order by strains B, 403E-15, Farr, and 39A2. Strains 403E-15, 12315, Farr, and 39A2 exhibited minimal differences in inhibition. Only *L. lactis* 39A1 and B were significantly inhibitory ($P<0.01$ and $P<0.05$ respectively). The pH of none of the milk samples changed during the storage period.

L. lactis in conjunction with potassium sorbate exhibited greater inhibition of the growth of psychrotrophs in raw milk at 5 C than did either treatment alone (Table 3). The data from four trials are represented as the $\log_{10}$ count of non-lactobacilli per ml after the milk samples had been stored 4 days at 5 C. Strains 39A1 and 39A2 combined with either 0.1 percent or 0.2 percent potassium sorbate were significantly ($P<0.01$) more inhibitory than either strain or sorbate alone. Sorbate alone had minimal inhibitory effect on the growth of the psychrotrophs. Both strains in combination with 0.2 percent sorbate were significantly more inhibitory than either strain combined with 0.1 percent sorbate ($P<0.01$).

Discussion

We observed in a previous study (Martin and Gilliland, 1980) that cells of *L. bulgaricus* had an antagonistic effect toward a psychrotrophic bacteria (isolated from raw milk) in autoclaved milk at 5.5 C. However, the *L. bulgaricus* did not inhibit growth of psychrotrophs in raw milk at 5.5 C. Apparently, the cultures of *L. bulgaricus* did not produce sufficient peroxide to inhibit psychrotrophs in raw milk. Since *L. lactis* reportedly produces more hydrogen peroxide than *L. bulgaricus*, it might be more likely to have an inhibitory effect on psychrotrophs in raw milk during refrigerated storage than would *L. bulgaricus*. In experiments related to the present study, the strains of *L. lactis* exerting the greatest inhibition of psychrotrophs in raw milk also produced the greatest amounts of peroxide.

Table 2. Numbers of non-lactobacilli in raw milk with and without added cells of *Lactobacillus lactis*[a] after 4 days of storage at 5°C

Sample	Days at 5°C	Non-lactobacilli/ml[b]						
		Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Avg.
Control	0	3.72	3.18	3.34	3.45	3.82	3.60	
Control	4	4.20	3.61	7.23	5.40	4.58	7.38	5.40
L. lactis B	4	4.00	3.34	5.85	5.40	4.20	6.76	4.93
L. lactis Farr	4	4.04	3.56	5.79	5.72	4.59	6.69	5.07
L. lactis 12315	4	3.96	3.77	5.92	5.23	4.53	6.70	5.02
L. lactis 403E-15	4	4.08	3.20	5.69	5.76	4.67	6.68	5.01
L. lactis 39A1	4	4.15	3.15	5.77	3.89	4.41	6.57	4.66
L. lactis 39A2	4	4.18	3.57	5.79	5.79	4.54	6.91	5.13

[a]*L. lactis* counts were approximately 1 x 10^8/ml.
[b]Counts recorded as $\log_{10}$ of numbers of non-lactobacilli/ml.

Table 3. Numbers of non-lactobacilli in raw milk with added cells of *Lactobacillus lactis* and/or sorbate after 4 days of storage at 5 °C

Treatment		Non-lactobacilli/ml[a]				
Culture[b]	Percent sorbate	Trial 1	Trial 2	Trial 3	Trial 4	Avg.
Control	0	3.85	3.96	4.26	3.88	3.99
	0.1	3.52	3.54	4.26	3.75	3.77
	0.2	3.52	3.49	4.26	4.90	3.79
L. lactis 39A1	0	3.54	3.63	4.20	3.57	3.74
	0.1	3.26	3.62	3.91	3.54	3.58
	0.2	2.70	2.59	3.91	3.59	3.20
L. lactis 39A2	0	3.65	3.70	4.11	3.71	3.79
	0.1	3.20	3.20	4.00	3.59	3.50
	0.2	3.26	3.00	3.98	3.60	3.46

[a]Counts recorded as $\log_{10}$ of numbers of non-lactobacilli on day four of storage; the counts on day zero were as follows: Trial 1 = 3.11; Trial 2 = 3.42; Trial 3 = 3.11; Trial 4 = 3.28.
[b]*L. lactis* counts were approximately 1 x 10^8/ml.

The differences in the intensity of the inhibition of psychrotrophs in raw milk by *L. lactis* among different trials in the present study may have been in part due to variations in the microflora present in the different lots of raw milk. There was no attempt to control types of microflora other than to include those organisms naturally present in the raw milk. These organisms might vary in susceptibility to the inhibitory action of lactobacilli. If the initial population of non-lactobacilli in the raw milk was too great and increased too rapidly, the high numbers may have overcome the inhibitory action of the lactobacilli. A slight variation in storage temperature can have considerable influence on the growth of psychrotrophs in raw milk. Storage of the raw milk samples at 7 C appeared to result in faster growth of the psychrotrophs than at 5 C. However, the degree of inhibition was similar at both temperatures.

Some have suggested that sorbate might inhibit catalase. If the catalase in raw milk were inhibited by the addition of sorbate, the hydrogen peroxide produced by *L. lactis* should be more inhibitory toward psychrotrophs in the milk. Thus, the use of sorbate in conjunction with *L. lactis* should significantly increase the inhibition of psychrotrophs in raw milk. The combined treatments of *L. lactis* with sorbate were more inhibitory to the growth of psychrotrophs in refrigerated raw milk than was either *L. lactis* or sorbate alone. At present we do not know whether or not this enhanced inhibition was due to reduced catalase activity in the raw milk.

The hydrogen peroxide production by the lactobacilli appears to be the most important means whereby they inhibit psychrotrophs in refrigerated raw milk. Additional research is needed to find means of improving the hydrogen peroxide production by *L. lactis* to increase the antagonism towards psychrotrophs.

Literature Cited

Martin, D. R. and S. E. Gilliland. 1980. J. Food Protect. 43:675-678.
Speck, M. L. 1976. Compendium of Methods for the Microbiological Examination of Foods. Am. Pub. Health Assoc.