Inhibitory Action of *Lactobacillus bulgaricus* Toward Psychrotrophic Bacteria from Raw Milk

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Story in Brief

Frozen concentrated cultures were prepared from selected lactobacilli isolated from three commercial samples of yogurt. Portions of the concentrated cultures were added to milk containing a psychrotrophic bacteria which had been isolated from raw milk. The milk was stored at 5.5°C. A significant inhibition of growth of the psychrotrophic culture occurred when at least 2.5 x 10^7 lactobacilli were added per ml. Higher numbers of lactobacilli resulted in greater inhibition of psychrotroph. The intensity of inhibition of a psychrotrophic culture varied among the cultures of lactobacilli tested. In most cases the more active inhibitory cultures produced more peroxide in refrigerated milk than less inhibitory ones.

Introduction

Psychrotrophic bacteria can grow readily in refrigerated raw milk and seriously limit the keeping quality of the milk. While most psychrotrophs are easily inactivated by pasteurization, some produce proteolytic enzymes and lipolytic enzymes that are not inactivated by pasteurization (Law *et al.*, 1976, and Speck and Adams, 1976). These heat resistant enzymes can cause spoilage in heat processed milk and in some products made from it. It would be desirable to prevent the growth of psychrotrophic microorganisms in raw milk, thus decreasing the amount of such enzymes.

Concentrated suspensions of certain starter culture bacteria, when added to some refrigerated foods, have been shown to suppress growth at refrigeration temperatures of spoilage bacteria (Gilliland and Speck, 1975). Hydrogen peroxide was identified as the antagonistic agent produced by the lactobacilli included in the starter cultures tested. Commercial starters containing mixed species of lactic cultures have also been used to restrict growth of psychrotrophs in raw milk and in autoclaved milk inoculated with a psychrotrophic culture stored at 3.5 and 7°C (Juffs and Babel, 1975).

The purpose of this study was to isolate lactobacilli from commercial yogurt which, when added to refrigerated milk, would control the growth of psychrotrophic bacteria.

Experimental Procedure

Commercial brands of plain yogurt were purchased from local supermarkets and health food stores. All samples of yogurt were subcultured twice in sterile reconstituted 10 percent NFMS using 1 percent inocula and incubation at 37°C for 24 hr. With the aid of a sterile glass hockey stick, 0.1 ml of an appropriate dilution of a milk culture was spread onto the surface of a milk base agar medium containing peroxidase (0.002

mg/ml) and 0-tolidine (0.2 mg/ml). The plates were incubated 24 hr at 37°C. Various colony types were selected from each yogurt culture, including those with no color zones surrounding them, and those with intermediate and large brown zones. The brown color was due to hydrogen peroxide produced by the lactobacilli. Cells from selected colonies were inoculated with the aid of a sterile inoculating needle into lactobacilli MRS broth (Difco) and incubated at 37°C until growth was evident. The isolates were identified using procedures described by Gilliland and Speck (1977). Gram positive, catalase negative rod-shaped bacteria were assumed to be lactobacilli.

Each culture of lactobacilli was inoculated (1 percent) into MRS broth and incubated at 37°C until the culture reached the late exponential phase of growth. The cells were harvested by centrifugation, resuspended in small volumes of cold sterile 10 percent NFMS and dispensed into sterile plastic freezing vials (2 g each). The vials were immediately submerged in liquid nitrogen (−196°C) for storage.

Psychrotroph RM was a gram negative rod-shaped bacterium isolated from raw milk obtained from the Oklahoma State University Dairy Farm. The milk was held 5 days at 5.5°C then plated on Plate Count Agar (Difco). Cells from a predominating type of colony were inoculated into sterile 10 percent NFMS and incubated 24 hr at 21°C. For the interaction studies, the psychrotroph was subcultured twice on successive days in 10 percent NFMS, using 1 percent inocula and incubation for 19 hr at 21°C just prior to use.

Desired populations of lactobacilli were prepared by aseptically adding the required amount of thawed and appropriately diluted cultures (diluted in cold sterile 10 percent NFMS) into sterile bottles contained in an ice bath. The volume in each bottle was adjusted to 10 ml with cold sterile 10 percent NFMS. Ten ml of autoclaved 10 percent NFMS, containing the desired number of cells of a freshly prepared psychrotrophic culture, were added to each bottle. From each test sample, 5 ml were removed for day 0 bacterial counts (facultative lactobacilli and non-lactobacilli). The remainder of each sample was stored for 6 days in a 5.5°C water bath, and the numbers of non-lactobacilli were then determined by plate count. The pH of each sample was measured on the initial and final day of the experiment.

Lactobacilli were enumerated by plating the appropriate dilutions on MRS agar and incubating the plates 48 hr at 37°C. Non-lactobacilli were enumerated by plating required dilutions on Plate Count Agar (PCA) (Difco) and incubating the plates 5 days at 21°C. The lactobacilli used in this study did not form colonies on this medium at 21°C. All colonies visible with the aid of a Quebec Colony Counter were counted.

The following formula was used for comparing the inhibition of psychrotrophs by cultures of lactobacilli:

$$\% \text{ inhibition} = \left[\frac{A_x - B_x}{A_x} \right] 100$$

A_x = number of non-lactobacilli in control on day x.
B_x = number of non-lactobacilli in sample containing added lactobacilli on day x.

Duncan's new multiple range test was used to compare the means of percent inhibition produced by cultures of lactobacilli. It was also used to compare the effect of different numbers of lactobacilli on the growth of the psychrotroph.

To test for peroxide production by cells from the frozen concentrated cultures, the required amount of each thawed culture was added to 25 ml volumes of cold 10 percent

NFMS contained in 50 ml Erlenmeyer flasks to yield a population of 1 x 10^8/ml. The samples were incubated for 22 hr at 5.5°C on a platform shaker (76 strokes per min). Hydrogen peroxide was measured using the method of Gilliland (1969). For the experiments in this study, the relative amounts of peroxide formed by the cultures are represented as absorbance at 400 nm.

Results and Discussion

Those cultures which exhibited differences in their ability to produce hydrogen peroxide in refrigerated milk in preliminary experiments were selected for further study. They were identified as *Lactobacillus bulgaricus*. After storage for 1 month, the frozen concentrated cultures were thawed and tested for hydrogen peroxide production in milk at 5.5°C. The relative amount of peroxide produced by the cultures varied; culture F1 produced the lowest level, cultures E1 and E2 produced intermediate amounts of peroxide and cultures D1, D3 and F2 produced higher levels of peroxide (Table 1). The number of lactobacilli in all samples was 1 x 10^8/ml.

To determine a reasonable population for comparing different cultures of lactobacilli for the ability to inhibit the growth of a psychrotrophic culture in refrigerated milk, increasing numbers of *L. bulgaricus* E2 were added to autoclaved 10 percent NFMS inoculated with 2 x 10^3 psychrotroph RM per ml (Figure 1). The data from three trials are presented graphically as the $\log_{10}$ of the numbers of non-lactobacilli per ml after 6 days of storage at 5.5°C plotted against the numbers of lactobacilli added per ml. The growth of the psychrotroph was not significantly affected ($P>.05$) by populations of lactobacilli up to 1 x 10^7/ml (increase noted in Trial 1), but at 2.5 x 10^7 lactobacilli per ml there was significant inhibition ($P<.05$) of the psychrotrophic culture. This antagonism against the psychrotroph increased significantly ($P<.05$) as higher numbers of lactobacilli were added.

A comparison was made of the inhibition of psychrotroph RM by five cultures of *L. bulgaricus* in autoclaved 10 percent NFMS (Table 2). The number of lactobacilli added to the milk was 2.5 x 10^7/ml for all cultures tested. There was considerable variation in the percentages of inhibition produced by cultures of lactobacilli in the three trials. A comparison of the average percentages of inhibition indicated that *L. bulgaricus* D1 and D3 were the most active inhibitors; *L. bulgaricus* E2 and F2 were intermediate inhibitors and *L. bulgaricus* F1 was the least active inhibitor. Statistical analysis of the data

Table 1. Hydrogen peroxide production in 10% NFMS by cells from concentrated cultures of *Lactobacillus bulgaricus*[a].

Yogurt isolates	H_2O_2[b] $A_{400\ nm}$
L. bulgaricus D1	.33
L. bulgaricus D3	.33
L. bulgaricus E1	.24
L. bulgaricus E2	.24
L. bulgaricus F1	.15
L. bulgaricus F2	.47

[a]Stored 1 month in liquid nitrogen; tested at 1 x 10^8 cells/ml.
[b]After 22 hr incubation at 5°C with continuous agitation; the higher the absorbance reading, the more peroxide is present.

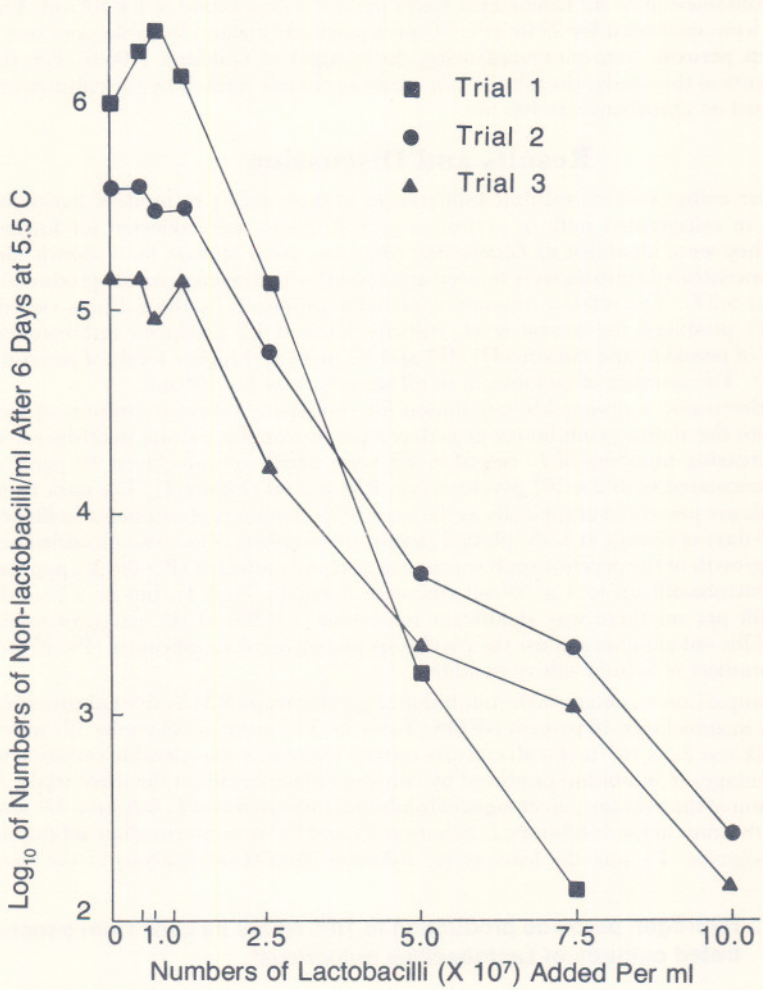

Figure 1. Effect on numbers of *Lactobacillus bulgaricus* E2 on the growth of psychrotrophic culture in 10% NFMS.

revealed that the differences among the cultures D1, D3, E2, and F2 were not significant ($P>.05$). However, culture D1 was significantly ($P<.05$) more inhibitory than culture F1.

With the exception of strain F2, the ranking of the lactobacilli with respect to the relative percentages of inhibition was similar to that observed for peroxide formation as shown in Table 1. Strain F2, which produced the highest level of peroxide, did not cause the greatest amount of inhibition of the psychrotroph. In related experiments, we have observed that the amount of detectable peroxide formed by some strains of

Table 2. Inhibition of growth of psychrotroph RM in autoclaved 10% NFMS by cells of different cultures of *Lactobacillus bulgaricus*.

Culture	Percent inhibition			
	Trial 1	Trial 2	Trial 3	Avg
L. bulgaricus D1	48	99	99	82
L. bulgaricus D3	62	91	75	76
L. bulgaricus E2	30	83	40	51
L. bulgaricus F1	−48	77	49	26
L. bulgaricus F2	48	83	32	54

[a]2.5 x 10^7 lactobacilli added/ml for each culture; initial population of psychrotroph - 1 x 10^3/ml.

lactobacilli in refrigerated milk reached a peak after 12 to 14 hr of storage, then dissipated slowly during subsequent storage. For other cultures of lactobacilli, similar amounts were produced in 12 to 14 hr, but the amount did not appear to dissipate during subsequent storage (unpublished data). This might explain some of the differences observed among the strains of *L. bulgaricus* with respect to the intensity of antagonism toward the psychrotroph in refrigerated milk.

The lactobacilli isolated from yogurt did not grow at refrigeration temperature and thus caused very little change in the pH of the milk during refrigerated storage. No samples exhibited decreases in pH of more than 0.1 to 0.2 units. Such cultures would be useful in aiding in the preservation of certain refrigerated foods since they would be expected to cause little or no change in the organoleptic quality of the food. In order for the lactobacilli to be used advantageously to inhibit psychrotrophs in raw milk, they must produce sufficient hydrogen peroxide either to overcome peroxidase or to activate the lactoperoxidase-thiocyanate system that is present in raw milk. This system has been shown to inhibit psychrotrophs in raw milk if sufficient hydrogen peroxide is present (Reiter, 1978). Preliminary experiments indicated that the strains of lactobacilli used in the present study did not produce sufficient peroxide at the populations tested to inhibit psychrotrophs. If the lactobacilli are to be used in raw milk to inhibit the growth of psychrotrophs, then strains must be selected to produce sufficient peroxide or the storage conditions modified to inhance peroxide production.

Literature Cited

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