Antagonism of Lactic Streptococci Toward *Staphylococcus aureus* in Associative Milk Cultures

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The inhibition of growth of *Staphylococcus aureus* by lactic streptococci in associative cultures in milk was not due to hydrogen peroxide produced by the streptococci. Dialyzed whey from the milk culture of lactic streptococci was more inhibitory than dialyzed whey from milk acidified with lactic acid, indicating that material other than lactate was also involved. Analyses of cation and anion exchange fractions from the dialyzed whey showed that only the neutral fraction was inhibitory.

We have shown (6) that commercial lactic streptococcus starter cultures are antagonistic toward staphylococci and salmonellae during associative growth in milk. The intensity of the antagonism varied among starter cultures and could not be correlated with the rapidity of acid production. Furthermore, the antagonism was still evident when the milk was automatically maintained at pH 6.5. Others (2, 8, 11) have also shown that the inhibition of staphylococci by lactic streptococci in milk is not entirely due to low pH. Certain strains of lactic streptococci have been reported to produce antibiotics (4, 7). Others also produce peroxide (5), which has been implicated in the inhibition of *Staphylococcus aureus* (3). Volatile fatty acids produced by some lactic acid bacteria can also cause inhibition of undesirable bacteria in foods (9, 12). The mode of action involved in the inhibition of food-borne pathogens by the lactic streptococci may include one or more of the aforementioned possibilities. A more thorough understanding of factors involved in the antagonism should make it possible to utilize the lactic streptococci more efficiently in controlling food-borne pathogens. The purpose of this investigation was to characterize the substance(s) produced in milk by lactic streptococci which inhibits food-borne pathogens.

The multiple-strain cultures of lactic streptococci used in these experiments were obtained from a commercial culture supplier. The *S. aureus* B925 was from the North Carolina State University Food Microbiology culture collection. The cultures of streptococci were maintained by subculturing in litmus milk using 1% inocula and incubation at 22 C for 18 h. *S. aureus* was subcultured in sterile 10% nonfat milk solids using 1% inoculum and incubation at 32 C for 18 h. All cultures were stored in a refrigerator between subcultures.

The required volume of sterile 10% nonfat milk solids was inoculated with approximately 10^8 colony-forming units (CFU) of *S. aureus* per ml and aseptically divided into two flasks. One flask was additionally inoculated (1%) with an 18-h milk culture of lactic streptococci. The samples were then incubated 6 h at 32 C, after which requisite dilutions of both were plated (spread technique) onto selective media. Mannitol salt agar was used for enumerating *S. aureus*. The plates were incubated 24 h at 37 C. Percentages of inhibition were determined using the following formula: % inhibition = [(CFU/ml in control) – (CFU/ml in sample inoculated with streptococcus)]/(CFU/ml in control) × 100. To determine if hydrogen peroxide was responsible for the antagonistic interaction, catalase (Nutritional Biochemical Corp., Cleveland, Ohio) was added (30 U/ml) to a duplicate set of milk cultures prior to incubation.

The acidity of 1 liter of an 18-h (22 C) milk culture of lactic streptococci was determined as percent lactic acid by titration with 0.1 N NaOH. The titratable acidity of 1 liter of sterile 10% nonfat milk solids was adjusted to that of the cultured milk using 30% lactic acid. Each was centrifuged 30 min at 4,080 × g to remove the curd. The supernatant fluid (whey) was collected, and 700 ml from each sample was dialyzed against 1,400 ml of distilled water at 5 C for 24 h. The dialysis tubing (Fisher Scientific Co.) was approximately 3.2 cm wide (flat) and was prepared for use by soaking in hot (100 C) distilled water. The tubes were long enough so that each could contain approximately 50 ml of whey. The dialysates were concentrated at 45 C under vacuum to the
original volumes of whey, adjusted to pH 6.5 with NaOH, filtered through sterile membrane filters (0.45-μm pore size; Millipore Corp.) into sterile flasks, and stored at 5 C until assayed.

Samples of whey-dialysate were prepared for ion exchange chromatography in a similar manner, except the pH was not adjusted nor were the samples filter sterilized. Samples (100 ml) of dialysate were passed over an Amberlite IR 120 cation (H+ form) exchange column (bed volume, 200 ml). The samples were washed through the column with distilled water until the effluent was negative to the molisch test. The flow rate was 2 ml/min. The column was then eluted with 6.5 bed volumes of 2 N NH4OH. The NH4OH was removed from the eluate by repeated evaporation (at 45 C under vacuum) and dilution. Both effluent and eluate were concentrated by evaporation to a final volume of approximately 5 ml, the pH was adjusted to 6.0 with NaOH or HCl, and the volume was adjusted to 10 ml with distilled water. Both samples were passed through a sterile membrane filter (0.45-μm pore size; Millipore Corp.) and stored at 5 C in a sterile container.

Five milliliters of the concentrated effluent fraction from the cation exchange column was applied to an Amberlite IR 400 anion (Cl− form) exchange column having a 100-ml bed volume. The sample was washed through the resin with distilled water (2 ml/min) until the effluent was negative to the molisch test. The effluent fraction was concentrated to 5 ml and adjusted to pH 6. The column was eluted with 10 bed volumes of 2 N acetic acid. The acetic acid was removed by repeated evaporation (at 45 C under vacuum) and dilution. The final volume was adjusted to 5 ml at pH 6.0. Both anion effluent and eluate fractions were passed through sterile membrane filters (0.45-μm pore size; Millipore Corp.) into sterile containers and stored in a refrigerator.

Sterile 20% nonfat milk solids was inoculated with approximately 2 × 10^4 CFU of S. aureus B925 per ml. The inoculated milk was dispensed in 1.5-ml portions into sterile screw-capped test tubes (13 by 100 mm). The desired dialysate or ion exchange fraction from lactic streptococcus milk cultures were aseptically added to the tubes (1.5 ml each). Tubes were also prepared for the appropriate control dialysates and ion exchange fractions. The comparative effect of an aqueous solution of sodium lactate (pH 6.0) was evaluated in a similar manner. The lactate was at the same concentration as in the dialysate samples. All tubes were incubated 6 h at 32 C. The samples were then placed in an ice-water bath and plated on Trypticase soy agar (BBL). The plates were incubated 24 h at 37 C.

The data presented in Table 1 show the inhibitory effect of three lactic streptococcus cultures on S. aureus B925 during a 6-h incubation period. Catalase had no effect on the inhibition of S. aureus.

Dialysate from the whey of a milk culture of lactic streptococcus D was inhibitory to S. aureus B925 (Table 2). Since the preparation of the control whey dialysate involved adjusting milk to the same acidity as that of milk culture D with lactic acid, these data show that inhibition of S. aureus B925 by lactic streptococcus D was not entirely due to the presence of lactic acid. An aqueous solution of sodium lactate (pH 6) equal in lactate concentration (1.1%) to the dialysate samples was also evaluated. More growth occurred in this sample than in either of those containing the dialysate samples. However, the amount of growth was less in the sample containing lactate than in a sample to which only water was added, indicating that lactate was responsible for part of the inhibition. Throughout the remainder of the study, dialysates or fractions thereof from both milk cultures of lactic streptococci and milk acidified with lactic acid were compared in order to study inhibition due to factors other than lactate.

Assay of fractions obtained from cation exchange chromatography of the dialysate samples revealed that the inhibitory substance(s) produced by streptococcus culture D was associated with the effluent (Table 3). The number of

| Lactic Streptococcus culture | S. aureus Control | Catalase (30 U/ml) |
|-----------------------------|------------------|------------------|
| A                           | 98.0             | 97.8             |
| D                           | 98.9             | 98.5             |
| G                           | 97.6             | 97.8             |

| Sample                      | CFU/ml           |
|-----------------------------|------------------|
| Control whey dialysate      | 1.5 × 10^4       |
| Culture whey dialysate      | 8.4 × 10^4       |
| Water                       | 4.4 × 10^4       |
| Sodium lactate              | 2.3 × 10^4       |
CFU per milliliter in the sample containing the cation effluent fraction of whey dialysate from Streptococcus D was only 32% of that in the sample containing the effluent from the control dialysate. More growth was observed in the sample containing the eluate fraction from culture D than in the sample containing the control eluate. Analyses of fractions obtained by anion exchange chromatography of the cation effluent fractions revealed that the inhibitory material was in the anion effluent fraction (Table 3). The amount of inhibition was greater than that observed from the cation effluent fraction. The anionic material in the eluate fraction from whey dialysate of culture D allowed more growth of the staphylococci than did the eluate from the control. Ion exchange fractions from dialysates of several additional batches of whey from control milk and milk cultures of the lactic streptococci had similar effects on the staphylococci.

Results from a previous study (6) showed that the inhibition of staphylococci and salmonellae by lactic streptococci was not caused entirely by an acidic environment created during growth of the streptococci. Certain lactobacilli have been shown to produce sufficient hydrogen peroxide to inhibit the growth of staphylococci (1) and *Pseudomonas* species (10). The lactic streptococci when grown in milk produce auto-inhibitory levels of hydrogen peroxide (5). Haines and Harmon (3) reported that hydrogen peroxide was involved in the inhibition of *S. aureus* by lactic streptococci in associative culture in a broth medium. However, in the present study peroxide was apparently not produced in sufficient quantities by the lactic streptococci to inhibit *S. aureus* B925 in associative milk cultures.

Sodium lactate, at a concentration equal to that in the culture and control whey dialysates, exerted some inhibitory action on *S. aureus* B925. This indicated that lactate produced by the streptococci during growth was partially responsible for inhibiting the pathogen. The lower growth response of *S. aureus* in milk containing whey dialysate from the lactic streptococcus milk culture than in milk containing the control dialysate suggested that an inhibitor(s) other than lactate was involved in the antagonistic action. The dialyzability of the inhibitor(s) indicated that it had a relatively small molecular weight. Results from analyses of fractions obtained from ion exchange chromatography revealed that the inhibitor(s) was neutral (i.e., not absorbed by cation or anion exchange resins). The inhibitor(s) did not resemble nisin (4) or diplococcin (7), which are produced by some lactic streptococci. Nisin should be absorbed by cation exchange resins (4) and diplococcin is isolated only by extraction directly from cells of the streptococci (7).

The antagonistic action of the lactic streptococci toward *S. aureus* in milk cultures apparently results from several factors. After sufficient growth of the streptococci has occurred, certainly the low pH and the lactic acid produced during growth are somewhat inhibitory. Other inhibitors produced by the lactic streptococci in milk are also important, one of which is a neutral material of low molecular weight.

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| Whey dialysate sample | Ion exchange fraction | *S. aureus* B925 CFU/ml | Inhibition (%) |
|-----------------------|-----------------------|--------------------------|---------------|
| Control               | Cation effluent        | 1.6 x 10⁴                 |               |
| Culture D             | Cation effluent        | 5.1 x 10⁴                 | 68            |
| Control               | Cation eluate          | 1.6 x 10⁴                 |               |
| Culture D             | Cation eluate          | 2.5 x 10⁴                 |               |
| Control               | Anion effluent         | 6.2 x 10⁴                 |               |
| Culture D             | Anion effluent         | 1.7 x 10⁴                 | 97            |
| Control               | Anion eluate           | 2.5 x 10⁴                 |               |
| Culture D             | Anion eluate           | 9.3 x 10⁴                 |               |

* Effluent fractions from cation exchange chromatography were fractionated on anion exchange resin.
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