Bacteriological Studies of Cultured Buttermilk

III. The Effect of Additions of Citric Acid, Sodium Citrate and Lactic Acid on the Progressive Changes in the Numbers of *Leuconostoc citrovorum* and *Streptococcus cremoris* as Associated with Acetylmethylcarbinol Plus Biacetyl and pH Levels[1,2]

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Citric acid is the major source of biacetyl contributing to the flavor of cultured buttermilk. Templeton and Sommer (1929) first suggested addition of citric acid and sodium citrate to milk to increase biacetyl production.

Michaelian *et al.* (1938), using pure cultures of biacetyl-producing organisms, studied the relation between production of acetylmethylcarbinol plus biacetyl and the pH level of the milk. They also showed the unfavorable effect of lactic acid as an additive. Cox (1945), likewise using pure cultures, investigated the relation between pH and biacetyl production. In addition, he made observations on the rate of growth at various fixed pH levels as determined by frequent microscopic examinations of stained preparations.

The present study explores the effect of added citric acid, sodium citrate, and lactic acid on the associated growth of *Leuconostoc citrovorum* and *Streptococcus cremoris* in relation to production of acetylmethylcarbinol plus biacetyl. Comparisons have been made with milk containing these additives and with milk containing no additive. Detailed observations on bacteriological and biochemical studies of cultured buttermilk containing no additives were described in a previous publication (Glenn and Prouty, 1954).

The data presented in this paper are the first record of numerical changes of the associated *Leuconostoc* and *Streptococcus* through the progressive course of the fermentation in milk containing citric acid and sodium citrate as additives.

Experimental Methods

Cultures. Two buttermilk starter cultures designated as Hs and Fs and composed of *Leuconostoc citrovorum* and *Streptococcus cremoris* were used. These were used in previous studies by Prouty and Glenn (1954) and Glenn and Prouty (1954).

Additives. Citric acid was added at the rate of 0.15 per cent. Sodium citrate was added in an amount in which the citrate radical was equivalent to 0.15 per cent citric acid. These are the amounts usually added when this practice is employed commercially. Lactic acid was added in an amount sufficient to provide the same pH level as that of the milk to which citric acid was added. These additions were made to the pasteurized nonfat milk immediately prior to inoculation with the starter culture.

Culturing. A 1-liter portion of fresh nonfat milk, in a 2-liter flask, was heated in flowing steam for 45 minutes, cooled to 22 C and inoculated at the 0.1 per cent level with a culture which had been incubating at 22 C during the preceding 24 to 30 hours. The inoculation was made late at night, and with the small amount used the culture was at the proper stage for observation 10 to 12 hours later, thus permitting maximum convenience in relation to time for the subsequent observations. A sample was taken for analysis immediately after inoculation. Beginning at 10 hours after inoculation, samples were taken at 1-hour intervals during the following 10 hours. This permitted a coverage of the period in which the most important developments of the fermentation occurred. An additional sample was examined at the end of 35 hours of incubation. A temperature of 22 C was maintained throughout the period of observation.

Analytical procedure. Observations at each sampling period included plate counts for *L. citrovorum* and total bacterial flora, and determinations of pH value and acetylmethylcarbinol plus biacetyl content.

L. citrovorum count. The count of this species was made by a method described by Prouty and Glenn (1954). This consisted of a plate culture method using a medium capable of supporting the growth of *L. citrovorum* but not that of *S. cremoris*.

Total count. The total count was considered to consist entirely of *S. cremoris* and *L. citrovorum* and was made

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[2] These data were taken in part from a thesis presented by the senior author to the faculty of the State College of Washington in partial fulfillment of the requirements for the Ph.D. degree.

by the plate method using a tomato juice-peptonized milk agar as described by Turner and Nelson (1951). Both species grew well on this medium. Incubation was at 26 to 27 C for 5 days.

Acetylmethylcarbinol plus biacetyl. The acetylmethylcarbinol plus biacetyl content was determined by using the method proposed by King (1948) as modified by Beutler (1951). This analysis makes use of the Evelyn Photoelectric Colorimeter with a 540 mμ filter and standard biacetyl curves for determining the milligrams of acetylmethylcarbinol plus biacetyl per 100 g of the sample. The analysis was made on a 4.0 g sample.

Results and Discussion

Because the results using the two buttermilk starter cultures followed the same general pattern, only the data from trials in which the Hs starter were used are presented. These represent the averages of four separate trials. Data of plate counts of both *Leuconostoc citrovorum* and total bacterial flora are presented as logarithmic averages.

L. citrovorum. Data showing the growth of *L. citrovorum* throughout the course of the fermentation are presented in figure 1. At the observation period 10 hours after inoculation, the numbers of this species were of a similar magnitude in all samples. The more rapid rate of growth in the samples to which citric and lactic acids had been added was evident early in the subsequent observation periods and this trend increased for the next several hours.

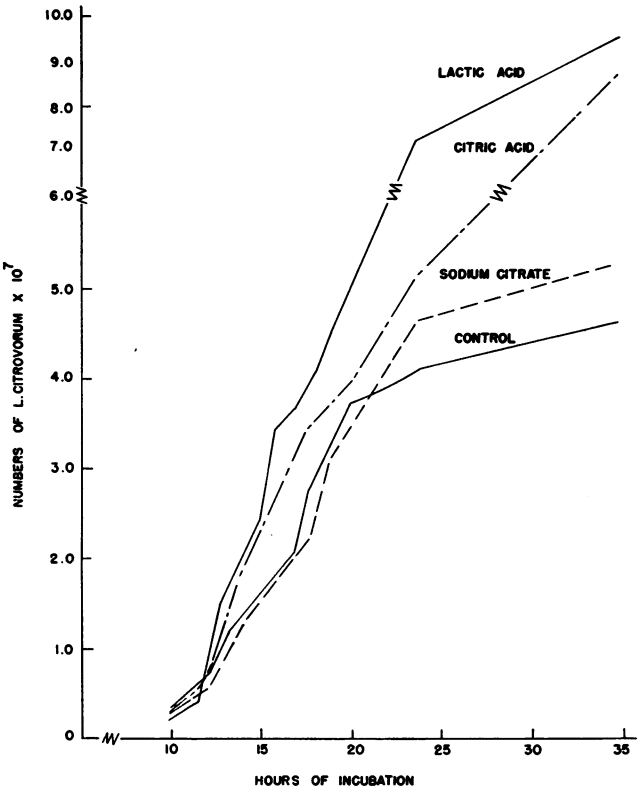

FIG. 1. Effect of citric acid, sodium citrate and lactic acid on the numbers of *Leuconostoc citrovorum*.

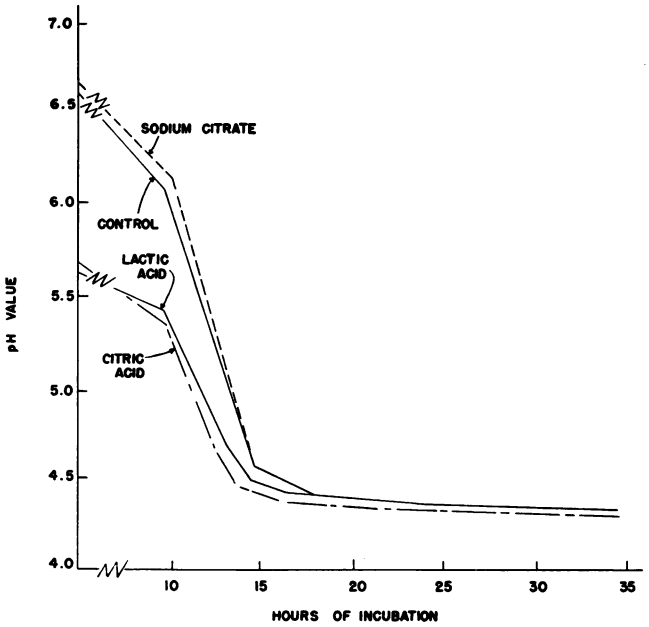

FIG. 2. Effect of citric acid, sodium citrate, and lactic acid on pH value.

The increased rate of growth of this species in these samples appears to have been associated with the lower pH levels, as shown in figure 2, these being 5.62 and 5.60 respectively at the time of inoculation as compared to 6.68 and 6.57 for the sodium citrate-treated and control samples. The samples to which citric acid was added maintained the lowest pH level at all of the observation periods. At the first sampling period (10-hour period) these had an average pH of 5.12 as compared to 5.39, 6.15 and 6.04 respectively for the samples treated with lactic acid, sodium citrate and the control. At the 18-hour period the samples treated with lactic acid, sodium citrate and the control had reached the common pH level of 4.45 with little or no further change occurring beyond this point. After the fermentation was in progress, the samples to which citric acid was added maintained a lower pH level in each instance than did the other samples.

By direct microscopic examinations of pure cultures of biacetyl producing organisms, in fixed pH environments ranging from 5.5 to 4.4, Cox (1945) concluded that the lowered pH levels had a retarding effect on the rate of growth of the organisms. In the present study, the fermentations which started at the lower pH level produced greater numbers of *L. citrovorum* than did the fermentations starting at the higher pH level. However, the lowest initial pH level of the cultures used in this study was approximately the same as the highest pH level used by Cox. It is very likely that the pH level of 5.62 to 5.5 more nearly approximated an optimum pH environment for the biacetyl-

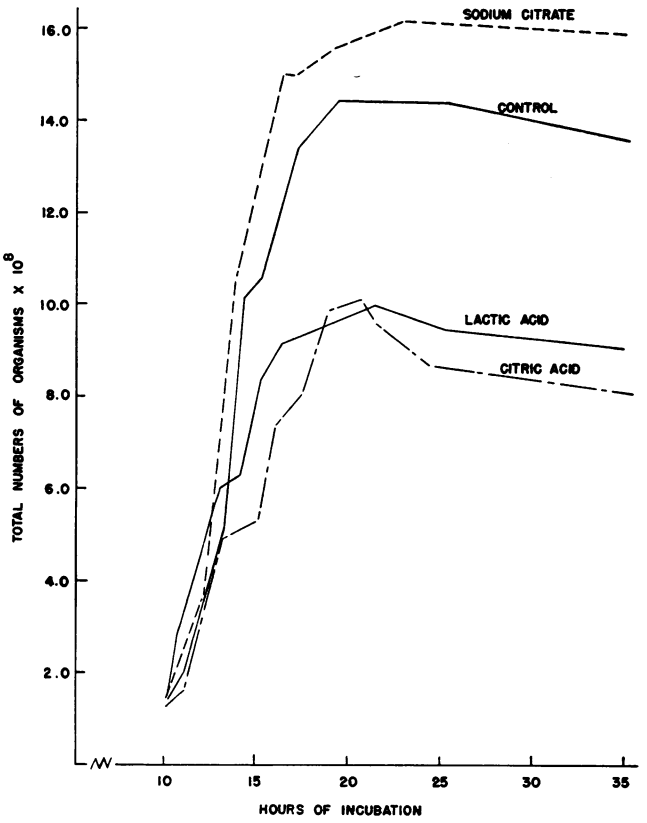

FIG. 3. Effect of citric acid, sodium citrate, and lactic acid on the total bacterial flora (*Leuconostoc citrovorum* plus *Streptococcus cremoris*).

producing organisms than did the higher or lower levels.

Total bacteria. Data showing total bacterial growth throughout the course of the fermentation are presented in figure 3. *S. cremoris* constituted the vast majority of the bacterial population in this association. At the 10-hour observation period this species made up approximately 98 to 99 per cent of the flora of all samples. As the fermentation progressed to the point where the pH value reached a constant level the numbers increased, after which they remained relatively constant. Maximum numbers were present in the sodium citrate-treated samples followed closely by the controls, being 45 to 60 per cent greater than in the citric and lactic acid-treated samples. The greater numbers associated with these samples were attributed to the higher initial pH values which served to extend favorable growth conditions over a longer period of time, particularly for *S. cremoris*.

Comparison of L. citrovorum and total numbers. At the 10-hour observation period *L. citrovorum* made up from 1.0 to 2.0 per cent of the bacterial population. As the fermentation progressed this percentage increased, the increase being greater in the samples to which citric and lactic acids were added. At the 24-hour period these percentages for the samples treated with citric acid, lactic acid, sodium citrate and the control were 11.0, 9.4, 2.9 and 3.5 respectively.

Acetylmethylcarbinol plus biacetyl. Data showing levels of acetylmethylcarbinol plus biacetyl are presented in figure 4. The production of these compounds, at all observation periods, was highest in the samples to which citric acid had been added, and except for the final observation period, the lactic acid-treated samples were next.

The increased production of acetylmethylcarbinol, plus biacetyl in these samples, undoubtedly was favored by the lower pH levels. Michaelian *et al.* (1938) and Cox (1945) showed that a definite relation exists between the production of these compounds and the pH level of the fermenting milk, greater production occurring within limits, at the lower pH levels.

In addition to providing a lower and more favorable pH level to the fermenting milk, the added citric acid increased the substrate for the production of these compounds. This may have attributed to the higher acetylmethylcarbinol plus biacetyl values, particularly as they prevailed toward the end of the observation period in the citric acid-treated samples.

It will be noted from figure 4 that, while the production of acetylmethylcarbinol plus biacetyl in the lactic acid-treated samples began sooner and proceeded at a slightly faster rate than in the sodium citrate-treated and control samples, the rate of decline in the production of these compounds was rapid and relatively abrupt. Michaelian *et al.* (1938) found that when milk was acidified with lactic acid and then inoculated with the citric acid fermenting streptococci the yields of acetylmethylcarbinol plus biacetyl were lower than when other acids were used. They suggested that the frequent failure to obtain a high yield of these com-

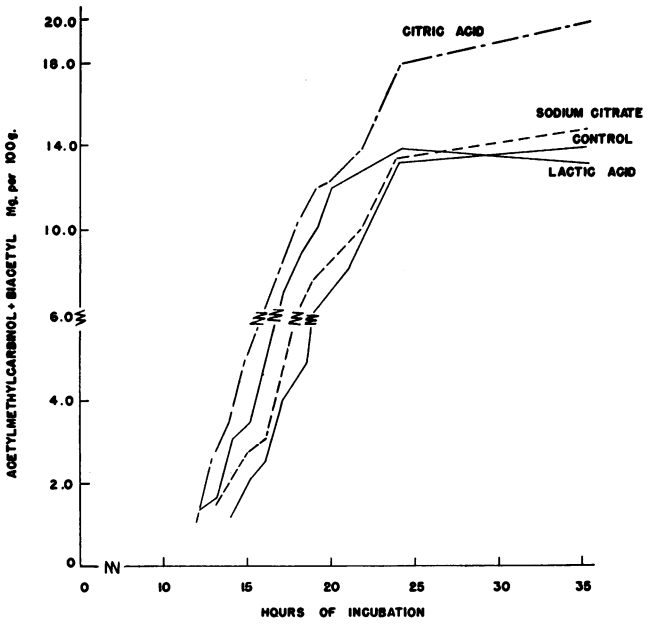

FIG. 4. Effect of citric acid, sodium citrate, and lactic acid on the production of acetylmethylcarbinol plus biacetyl.

pounds with culture of citric acid fermenting streptococci may be due to the relatively unfavorable character of lactic acid.

The addition of sodium citrate exerted a small stimulating effect upon the production of acetylmethylcarbinol plus biacetyl, the values for these compounds being somewhat higher, throughout the course of the fermentation, than the control samples. Compared with the samples to which citric acid was added, a lag period of two to three hours developed in the production of acetylmethylcarbinol plus biacetyl. Apparently, this was associated with the relatively high pH level resulting from the use of sodium citrate as an additive.

ACKNOWLEDGMENT

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SUMMARY

A study was made of the effects of adding citric acid, sodium citrate, and lactic acid on the numerical relationships of *Leuconostoc citrovorum* and *Streptococcus cremoris* as these species developed throughout the course of the fermentation and as they were associated with changing levels of pH and acetylmethylcarbinol plus biacetyl.

The greater numbers of *L. citrovorum* were present in the samples to which citric acid and lactic acid had been added. On the other hand, the greatest numbers of *S. cremoris* were present in the control and sodium citrate-treated samples. These trends were apparent relatively early in the fermentation process.

The percentage relationship of *L. citrovorum* to *S. cremoris* decreased during the early period of the fermentation process and then increased following coagulation of the milk, and was considerably higher in the samples to which citric and lactic acids were added.

The production rate of acetylmethylcarbinol plus biacetyl was greatest in the citric acid-treated samples. Except toward the end of the observation period, the sample treated with lactic acid gave the next greatest production of these compounds. The addition of sodium citrate exerted a small stimulating effect upon the production of acetylmethylcarbinol plus biacetyl.

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