Diacetyl, Acetoin, and Acetaldehyde Production by Mixed-Species Lactic Starter Cultures

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Citrate utilization and acetoin, diacetyl, acetaldehyde, and lactic acid production in milk at 21 °C by five different mixed-strain starters, containing Streptococcus diacetilactis (D type), Leuconostoc (B type), and S. diacetilactis and Leuconostoc (BD type), were measured. BD and D cultures utilized citrate more rapidly and produced more diacetyl, acetoin, and acetaldehyde than B types. All cultures produced much more acetoin than diacetyl, with the BD and D cultures producing four to five times larger amounts of acetoin than the B cultures. Reduction of diacetyl and acetoin toward the end of the normal incubation period was characteristic of BD and D cultures, whereas a similar reduction of acetaldehyde was characteristic of BD and especially of B cultures. Continued incubation of B cultures beyond 17 h also resulted in reduction of diacetyl and acetoin. Addition of citrate to the milk retarded diacetyl and acetoin reduction. Mn** had no effect on diacetyl production by a BD culture but increased citrate utilization and, as a consequence, caused greater diacetyl destruction in one of the B cultures.

The mixed-strain starter cultures used in the manufacture of many fermented dairy products contain lactic acid-producing bacteria, usually Streptococcus lactis or S. cremoris, or both, and diacetyl-producing bacteria, usually S. diacetilactis or different species of Leuconostoc. Depending on the nature of the aroma-producing bacteria, Galesloot and Hassing (4) divided starter cultures into three types: B type, containing only Leuconostoc species as an aroma producer; D type, containing only S. diacetilactis as an aroma producer; and BD type, containing both Leuconostoc and S. diacetilactis as aroma producers. Some differences in the behavior of these starters have been reported (4, 5), but in many studies no distinction was made regarding the nature of the aroma producers. Speckman and Collins (15) have shown that diacetyl and acetoin are synthesized by two distinct pathways and state that this finding places in question the results of a large number of published papers in which measurements of acetoin plus diacetyl have been considered to indicate the total production of diacetyl. According to de Man and Galesloot (10), the addition of trace amounts of Mn** to milk stimulated production and reduction of acetoin plus diacetyl and increased the utilization of citrate by B type but not D type cultures. High numbers of S. lactis and S. diacetilactis in comparison to Leuconostoc have been implicated as the cause of a green or yogurt-type flavor in buttermilk because of the accumulation of acetaldehyde (9). Since Leuconostoc species can convert acetaldehyde to ethanol in milk (6), it seems probable that B and D type cultures may behave differently in their ability to produce acetaldehyde.

The purpose of the present study was to compare flavor production by cultures containing different aroma producers.

MATERIALS AND METHODS

Cultures. The B type mixed cultures (FR8 and 21) were from J. Stadhouders, NIZO, Ede, The Netherlands, the D culture (H) was obtained from Chr. Hansen's Laboratory Ltd., London, and the BD cultures (V and PD) were from Visby Maelkeri Laboratory, Tonder, Denmark, and Flora Danica Laboratory, Odense, Denmark. All cultures were routinely cultivated in autoclaved (5 min, 121 C) 10% low-heat nonfat milk solids (NFMS) at 21 C by using a 1% inoculum.

Identification of mixed strain cultures. Whether the cultures were of the B, BD, or D type was determined by a modification (Leesment, personal communication) of the method of Nickels and Leesment (12, 13). In the modification, colonies surrounded by a clear halo were inoculated into litmus milk fortified with 0.3% yeast extract and incubated.
at 25 C for 6 days, after which citrate and acetoin were determined by the methods given below except that 1 ml of the culture was used directly in the estimation of acetoin. Cultures which produced acetoin and utilized citrate were considered to be strains of *S. diacetilactis*, whereas those utilizing citrate and not producing acetoin were considered to be *Leuconostoc*.

Experimental procedure. Previously chilled, autoclaved 10% NFMS was titrated to allow calculation of the percent developed lactic acid and inoculated with 2% of the culture being studied. After thorough mixing, the inoculated milk was divided into approximately 70-ml volumes in sterile screw-capped bottles (150-ml capacity) which were incubated at 21 C. One bottle was removed periodically for the various analyses. For the experiments containing added citrate, the control milk before inoculation was brought aseptically to the pH of the milk containing added citrate (pH 6.9). Citrate was added as sterile trisodium citrate to give a final concentration of added citrate in the milk of 0.5% (wt/vol) as citric acid.

Analyses. Lactic acid production was measured by titrating 10 g of culture to pH 8.3 with 0.11 N NaOH by using a Radiometer pH-stat and expressing the results as percent lactic acid. Diacetyl and acetoin were measured in steam distillates by modifications of the Prill and Hammer and Westerfeld procedures, respectively (B. Walsh and T. M. Cogan, J. Dairy Res., in press). The method of Lindsay and Day (8) was used to measure acetaldehyde except that 20 g of culture was steam distilled in the apparatus described by Cogan (2) and a sample of the first 10 ml of distillate was analyzed. Citric acid was measured in 5-g amounts of the NFMS by the method of Marier and Boulet (11). All results are expressed on a per gram basis.

RESULTS

Accumulation patterns. A comparison of diacetyl, acetoin, acetaldehyde, and lactic acid production, and citric acid utilization by cultures H (D type) and 21 (B type) is shown in Fig. 1. Similar rates of lactic acid production were obtained in both cultures. However, the D culture produced more acetoin, diacetyl, and acetaldehyde than the B culture, but none of these compounds was produced at the same rate as lactic acid in either culture. Slight reduction of diacetyl and acetoin were noted in the D culture at the end of the growth period, whereas the B culture did not appear to have reached maximum production of either compound. Little acetaldehyde accumulation occurred in the B culture. More rapid utilization of citric acid occurred in the D culture, with complete utilization obtained after about 14 h of incubation. Citrate was more or less completely utilized within the pH range of 4.8 to 5.0 in the case of the D and BD cultures. The latter cultures gave results similar to the D culture, whereas the other B culture behaved like the one shown in Fig. 1.

Acetoin accumulation patterns for all the cultures are shown in Fig. 2. Cultures FR8 and 21 (B type) produced about the same amount of acetoin which reached a maximum of about 85 µg/g, whereas cultures FD, V, and H (BD and D types) produced between 400 and 520 µg/g. Some reduction of acetoin was obtained in the

![Fig. 1. Comparison of the production of diacetyl (■), acetoin (□), acetaldehyde (●), and lactic acid (▲), and citrate utilization (○) at 21 C by cultures H (D type) and 21 (B type).](image-url)
Reduction of accumulated acetaldehyde was observed in the case of all cultures, although it is difficult to decide if reduction occurred in the B cultures since they produce so little. The maximum amounts of acetoin, diacetyl, and acetaldehyde were produced only late in the incubation period when the pH value of the milk reached about 4.8.

Effect of increased citrate. Addition of 0.5% citric acid to the milk resulted in increased levels of diacetyl and acetoin production by culture FD (BD type) without affecting the rates of lactic acid production (Fig. 5). The increase in acetoin production was much greater than in that of diacetyl. The pH of the culture BD and D cultures toward the end of the incubation period.

Diacetyl accumulation patterns are shown in Fig. 3. More diacetyl was produced by cultures FD, V, and H (BD and D types) than by cultures FR8 and 21 (B type), with culture H (D type) producing up to 12 µg/g at its peak. Reduction of diacetyl by D and BD but not B cultures was evident toward the end of the incubation period. In all cultures, much more acetoin than diacetyl was produced (e.g., culture V produced a maximum of 5.6 µg of diacetyl per g compared to 521 µg of acetoin per g).

Acetaldehyde production (Fig. 4) was much greater in cultures H, V, and FD (D and BD types) than in cultures FR8 and 21 (B type), with culture H (D type) producing up to 5 µg/g.
containing added citrate remained above 5.10 even after 16 h of incubation when the pH of the control culture was 4.70. In the absence of added citrate, reduction of accumulated diacetyl and acetoin occurred when the citrate level had fallen to approximately 100 μg/g (i.e., 94% utilization of citrate). The reduction of acetoin was retarded and diacetyl was delayed by increased levels of citric acid. A similar but not as dramatic effect was obtained in the case of the two B cultures.

**Effect of manganese.** Mn²⁺ addition at a level of 5 μg/ml increased the rate of utilization of citrate by culture FR8 (B type) but resulted in lower absolute amounts of diacetyl and acetoin being produced with subsequent greater rates of destruction (Fig. 6). Acetoin reduction was affected much more than diacetyl reduction. Similar rates of diacetyl and acetoin production were observed up to 15 h of incubation. Once the citrate level had fallen to 100 μg/g, reduction of both acetoin and diacetyl ensued. Mn²⁺ had no effect on diacetyl or acetoin production or citrate utilization by the FD culture (BD type). In the case of the other B type culture, addition of Mn²⁺ retarded citrate utilization and led to slightly lower absolute amounts of diacetyl and acetoin being produced.

**DISCUSSION**

The more rapid utilization of citrate by D and BD cultures compared to B cultures probably reflects faster growth of *S. diacetilactis* compared to leuconostoc strains. *S. diacetilactis* has been shown by Harvey and Collins (3) to possess an inducible citrate permease, which may be an additional factor in the utilization of citrate by D and BD cultures. In agreement with the present results, Galesloot and Hassing (4) also noted poor citrate utilization by B type cultures. In their studies, season had a pronounced effect on citrate utilization, which was lowest in April- and highest in October-produced milk. The D and BD starters also produced more diacetyl (up to 11 μg/ml) and acetoin (up to 500 μg/ml) than the B type, again reflecting rapid
I have shown that uptake of citrate by *S. diacetylactis*, which has been shown by many workers to act as a precursor for diacetyl and acetoin production in milk. The acetaldehyde may also originate in citrate since Speckman and Collins (15) have shown that "active" aldehyde is involved in diacetyl biosynthesis. Seitz et al. (14) have shown that *S. diacetylactis* contains diacetyl reductase, which probably explains the reduction of diacetyl by cultures containing *S. diacetylactis* (D and BD types) observed in the present study. If this species also contains an active acetoin reductase, a similar explanation of the present results can be invoked. In this connection, Bryn et al. (1) have shown that the same reductase enzyme carried both diacetyl and acetoin reduction in *Aerobacter aerogenes*.

Much greater amounts of acetoin than diacetyl were found in all cultures, but especially in those containing *S. diacetylactis*. Both diacetyl and acetoin are synthesized by different metabolic routes (15), and whether or not the present results reflect a more active pathway for acetoin than for diacetyl biosynthesis or the presence of a highly active diacetyl reductase enzyme is not known. A diacetyl reductaseless mutant of *S. diacetylactis* could be used with good effect to study this particular aspect.

Despite the fact that diacetyl and acetoin are synthesized by different pathways (15), any factor which had an influence on diacetyl production also influenced acetoin formation in a similar manner.

Acetaldehyde production by B cultures compared to BD and D cultures was low, probably due to the presence of an active alcohol dehydrogenase in the leuconostoc strains present, as suggested by the results of Keenan et al. (6). Unfortunately, no effort was made to quantify ethyl alcohol in the present study. Acetaldehyde production by BD cultures lies between B and D cultures, lending some weight to this conclusion. The acetaldehyde accumulation patterns for the BD and D cultures were similar to those obtained by Keenan et al. (7) for single-strain lactic streptococci.

Diacetyl and acetoin reduction occurred only in the BD and D cultures and was not usually noted until late in the incubation period when virtually complete utilization of citrate had occurred. These experiments were limited to the usual incubation period given to cultures in industry, namely 14 to 16 h. Continued incubation of B cultures beyond 17 h also led to slight diacetyl and acetoin reduction (Fig. 6) which coincided with the presence of low levels of citrate. These findings suggest that the rate of biosynthesis of diacetyl and acetoin is greater than the rate of destruction so that destruction is not evident until synthesis ceases when the precursor compound (citrate) is completely utilized. Supporting evidence for this conclusion is found in the effect of Mn²⁺. When Mn²⁺ increased the rate of utilization of citrate, destruction of diacetyl occurred at an earlier time in the incubation period.

That addition of citrate increased diacetyl production has been noted by numerous workers. No reduction of diacetyl or acetoin occurred in the presence of increased citrate (Fig. 5), and it is interesting to speculate that, if incubation had continued until all the added citrate were utilized, reduction of both diacetyl and acetoin may have occurred. Another explanation may be the effect of pH on diacetyl reductase, which may be more active at low pH than at high pH since the addition of citrate resulted in a final higher pH value of the milk. The compound(s) to which diacetyl and acetoin are reduced has not been identified, although Galesloot and Hassing (5) state that it is 2,3-butylene glycol.

In agreement with the results of Galesloot and Hassing (4), Mn²⁺ increased the rate of destruction of diacetyl and acetoin but in only one of the two B cultures examined. However, the effect is not a direct one on the rate of reduction...
of diacetyl and acetoin as suggested by these workers, but rather on the rate of utilization of citrate, since destruction of diacetyl, whether in the presence or absence of Mn²⁺, does not occur until all the citrate is utilized. Thus, as found in the present study, if Mn²⁺ retards citrate utilization, less destruction of diacetyl will occur, whereas if it promotes utilization a lower absolute amount will be produced and greater destruction ensues. This interpretation of the present results suggests that the role of Mn²⁺ and citrate in diacetyl and acetoin production by starter cultures needs further study.

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