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Title ACETALDEHYDE PRODUCTION AND UTILIZATION BY LACTIC CULTURES

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Acetaldehyde is known to be responsible for the green or yogurt-like flavor defect of lactic cultures. This study was undertaken to extend the knowledge of acetaldehyde production and utilization by microorganisms normally found in mixed-strain butter cultures. It is anticipated that the resulting information will contribute to a more thorough understanding of the development of a green flavor defect; hence, to methods of avoiding and overcoming this defect.

Acetaldehyde production by single-strain cultures of S. lactis, S. cremoris, and S. diacetilactis was found to parallel the increase in microbial population. S. lactis and S. cremoris were found to remove some of the acetaldehyde produced on continued incubation at 21°C. S. diacetilactis did not remove any of the acetaldehyde produced. The ratio of diacetyl to acetaldehyde in the strains of S. diacetilactis studied was found to be unfavorable for a good culture

flavor at all times up to 22-24 hr incubation. All of the cultures studied produced a distinct green flavor when grown in milk media.

All of the lactic streptococci studied produced both ethanol and acetone when grown in a boiled milk medium. No evidence of acetone utilization by S. diacetilactis was observed. A tentative mechanism for the formation of acetone from pyruvate via acetoacetate was proposed.

Single-strain cultures of Leuconostoc dextranicum and Leuconostoc mesenteroides were shown to be capable of utilizing added acetaldehyde under a variety of culturing conditions. These two organisms, along with L. citrovorum were combined into two-strain mixtures with various lactic streptococci. The production and utilization of acetaldehyde varied widely among different two-strain mixtures.

The ratio of different lactic organisms comprising the flora of a desirably flavored commercial mixed-strain butter culture was determined. The microbial shift occurring when this culture developed a green flavor defect was found to be an overgrowth of the homofermentative lactic streptococci by the S. diacetilactis population.

It was found that the concentration of acetaldehyde in a ripened single-strain lactic culture could be significantly reduced by adding a large inoculum of a culture of L. citrovorum and continuing incubation at 21° C or by cooling and holding the culture at 5° C after the addition of L. citrovorum.

ACETALDEHYDE PRODUCTION AND UTILIZATION BY LACTIC CULTURES

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INTRODUCTION

Under certain ill-defined conditions, lactic cultures develop a flavor defect described as green or yogurt-like. Acetaldehyde is known to be responsible for this defect. In low concentrations, adetaldehyde contributes to a good culture flavor by smoothing out the harsh flavor and contributing to the overall desirable flavor blend. However, in high concentrations this component imparts to the culture a distinct, almost pungent flavor very like that associated with green apples. The development of this flavor defect is one of the more serious problems plaguing the manufacture of cultured butter and buttermilk.

Up to the present time the production of acetaldehyde as a function of incubation time and microbial population has never been quantitated. There have been few studies made on acetaldehyde utilization; those that have were concerned almost wholly with acetaldehyde utilization by Leuconostoc citrovorum.

The purpose of this investigation was to extend the knowledge on the green flavor defect. It was especially desirable to determine the contribution of individual microbial species to the development of this defect and the contribution of other microorganisms to the prevention or removal of this flavor defect.

REVIEW OF LITERATURE

Flavor of Butter Cultures

The microbial species incorporated into butter cultures can be placed into three categories (52): 1. the lactic acid producing streptococci, Streptococcus lactis and Streptococcus cremoris; 2. the citrate fermenting aroma bacteria, Leuconostoc citrovorum and Leuconostoc dextranicum; and 3. the lactic acid and aroma producing strains of Streptococcus diacetilactis. It is an established fact that some of the metabolic products which occur from the associative growth of the above mentioned microorganisms impart the normal flavor and aroma to mixed-strain butter cultures.

Much of the early research on the flavor of butter cultures was concerned with the organic acid production by mixed-strain starters. Lactic acid has long been known to be the major metabolic end-product of homofermentative lactic streptococci. Pure lactic acid is odorless and non-volatile, thus it does not contribute to the odor, but is considered to be largely responsible for the acid taste of butter cultures (19).

Several workers have studied the volatile acids of butter cultures. It has been found that acetic acid comprises the major portion of the volatile acid fraction (18, p. 1-15). Hammer and Sherwood (18),

along with Knusden (29), have reported that propionic acid was produced in small quantities in desirably flavored cultures. Recently, butyric and valeric acids were tentatively identified in cultured buttermilks (7, p. 126).

Formic acid has been shown to be an end-product of lactose and glucose metabolism in non-milk media by S. cremoris, S. lactis, and L. dextranicum (14, 49). The amounts of formic acid were very small compared to the amount of acetic acid produced. Recently, Lindsay (30, p. 154) has identified formic acid in butter cultures and shown it to be present in small amounts compared to lactic and acetic acids. The volatile acids are considered to be important to the taste of butter cultures (19, 30, p. 206).

Since the recognition that diacetyl was a principle component of butter culture flavor by van Niel et al. (59), many investigations have been carried out on its production in butter cultures. Hammer and Babel (19) have cited work which indicates that concentrations of 1.5 to 2.5 ppm of this component are desirable in high quality butter cultures. Recently, Lindsay et al. (32) have confirmed this and shown that high concentrations of this component lead to a harsh flavor. The partial reduction products of diacetyl, acetoin and 2, 3-butanediol, have received considerable attention but these have no odor (19). These components are probably only rarely present in concentrations sufficient to affect the taste of butter cultures.

Carbon dioxide is known to have an effect on the flavor of butter cultures by imparting a "tangy" sensation to the culture. Several researchers consider this to be desirable (2, 19, 54).

Friedman (14), and Platt and Foster (49) have shown the production of ethanol by single-strain cultures of S. lactis, S. cremoris, and L. dextranicum when grown in non-milk media. Very recently, Bassette and Claydon (5) have demonstrated ethanol production by S. lactis in milk media. Ethanol has been isolated from mixed-strain butter cultures grown in milk media by several workers (4; 7,p. 126; 10).

Harvey (20) has reported that all strains of S. lactis, S. cremoris, and S. diacetilactis studied in his laboratory produced significant quantities of acetaldehyde when grown in milk media. He has suggested that the decarboxylation of pyruvate to produce acetaldehyde is a general property of these organisms. This worker has also found some strains of S. lactis and S. cremoris to produce acetone in milk cultures. He found that no other aldehydes or methyl ketones were produced. Day et al. (10) have reported the isolation of acetaldehyde and acetone from mixed-strain butter cultures. Chow (7, p. 126) has identified both acetaldehyde and acetone in cultured buttermilk.

Dimethyl sulfide has been isolated from bulk butter cultures by Day, Lindsay, and Forss (11). These workers have observed that

dimethyl sulfide has the capacity to smooth out the harsh flavor of diacetyl and acids associated with culture flavor.

Very recently, Lindsay and co-workers (33) have reported the identification of a comprehensive list of volatile compounds in butter cultures. Compounds identified include a wide range of aldehydes, ketones, alcohols, esters and sulfur compounds. Lindsay (30, p. 205-206) has found that not all these compounds were necessary to simulate natural butter culture flavor. This worker was able to prepare a synthetic butter culture with a flavor very like that of natural butter culture by adding 2.0 ppm of diacetyl, 0.5 ppm of acetaldehyde, 1250 ppm of acetic acid, 25.0 ppb of dimethyl sulfide, and a small amount of carbon dioxide to heated milk which had been acidified to pH 4.65 with delta-gluconolactone.

Green Flavor Defect of Butter Cultures

Mixed-strain butter cultures are known to develop a flavor defect described as green or yogurt-like under certain ill-defined conditions. In 1941, Hoecker and Hammer (24) reported that the aroma of ripened pure S. diacetilactis cultures resembled that of green butter cultures. These workers could not detect the diacetyl flavor organoleptically. However, analysis of the cultures showed that from 0.16 to 1.65 ppm of diacetyl was present. This same green culture aroma was observed by other workers in S. diacetilactis cultures

grown in citrated cottage cheese whey (34).

Badings and Galesloot (4, vol. B, p. 199-208) have demonstrated that butter cultures containing L. citrovorum and cultures containing both L. citrovorum and S. diacetilactis have a flavor in common but different from cultures containing only S. diacetilactis as aroma bacteria. By employing gas chromatographic analysis of butter culture and butter volatiles along with organoleptic comparisons with yogurt it was shown that actaldehyde was responsible for the green or yogurt-like flavor defect. Acetaldehyde has been shown to be responsible for the characteristic flavor of yogurt (48). It was noted that not all S. diacetilactis butters were downgraded and suggested that manufacturing procedures and conditions of culture propagation influence the final flavor of the butter (4, vol. 8, p. 199-208). Seitz (56, p. 60) has manufactured desirably flavored cultured butter using single-strain S. diacetilactis cultures; suggesting that the green flavor defect becomes apparent only when the effect of acetaldehyde on the flavor becomes too pronounced.

Badings and Galesloot (4, vol 8, p. 191-208) found that cultures containing L. citrovorum gave negative Schulz and Hingst reactions (55) for acetaldehyde but that mixed-strain butter cultures containing only S. diacetilactis as the aroma bacteria as well as non-citrate fermenting lactic streptococci gave positive reactions for acetaldehyde. From this these authors concluded that L. citrovorum attacked

acetaldehyde present in the culture. Lindsay, Day, and Sandine (32) have demonstrated the ability of a single-strain L. citrovorum culture to remove added acetaldehyde under several different incubation conditions. These workers have also shown the ability of L. citrovorum to lower the acetaldehyde concentration in mixed-strain cultures.

Lindsay, Day, and Sandine (32) found that a green flavor can result from excess numbers of S. lactis or S. diacetilactis in relation to the L. citrovorum population in mixed-strain starters. These authors have shown that acetaldehyde stimulates the growth of L. citrovorum in whole milk medium.

Lindsay (30, p. 175) has stated that the absolute quantities of acetaldehyde and diacetyl determine the intensity, but the relative amounts which affect the flavor balance are of greater importance. Cultures which contained diacetyl to acetaldehyde ratios from 13:1 to 5.5:1 were described as having harsh flavors, while those having ratios of from 4.4:1 to 3.2:1 had desirable flavors. A green flavor was apparent when the ratio dropped below 3.2:1. It was suggested that a small amount of acetaldehyde is necessary to impart a balanced flavor to the culture.

Lindsay (30, p. 187) has shown that the green flavor defect can result from overripening of mixed-strain butter cultures. This worker has shown that if the acidity of a mother culture was allowed to exceed

0.78 percent, the culture gave a green flavor on subsequent transfers.

Bacteriology of Butter Cultures

Taxonomy and Classification of Butter Culture Bacteria.

The microorganisms common to commercial butter cultures are also common to cultures used for making cottage and cheddar cheese, buttermilk, and sour cream. These cultures are added for either or both two purposes; to produce lactic acid or to produce a desirable aroma.

The taxonomy of lactic acid bacteria has been discussed in detail in the reviews of Hammer and Babel (19), Collins (9), Galesloot (15, vol. D, p. 143-158), and Reiter and Møller-Madsen (51). The classification and nomenclature of some of the microorganisms involved is still a controversial topic. There are three species of lactic streptococci which are generally recognized as being common to mixed-strain butter cultures. These are *S. cremoris*, *S. diacetilactis*, and *S. lactis* (51). Many criteria have been used to classify these bacteria, but reactions may vary within a species and lead to results which are difficult to interpret.

Sandine and co-workers (53) have made use of well-known and rapid methods to characterize starter culture bacteria. Lactic streptococci were differentiated from *Leuconostoc* organisms by the former's ability to produce sufficient acid in 48 hr at 30° C to coagulate and reduce litmus milk. *S. diacetilactis* and *S. lactis* were

differentiated from S. cremoris on the basis of the Niven et al. arginine hydrolysis test (41). S. diacetilactis was differentiated from S. lactis on the basis of the ability of S. diacetilactis to produce acetoin and diacetyl; these compounds being detected by the modified creatine test of King (28). The ability of S. diacetilactis to produce high levels of carbon dioxide from citrate was also used to differentiate it from S. lactis and S. cremoris, which lack the ability to ferment citrate.

Because of some instances where non-citrate fermenting strains of S. cremoris and S. lactis give a slightly positive creatine test, some doubt has been expressed as to the validity of this test to differentiate these organisms from S. diacetilactis (15, vol. D, p. 144). Sandine et al. (53) favor the classification of these organisms as S. lactis or S. cremoris var. aromaticus. However, Collins and Harvey (8) have shown that S. diacetilactis organisms may lose their ability to produce the citrate permease enzyme, and would thus give negative creatine tests.

The citrate fermenting aroma bacteria can be differentiated from the lactic streptococci in litmus milk incubated at 21° and 30° C (53). L. dextranicum can be differentiated from L. citrovorum by the former's ability to produce dextran (slimey colonies) when streaked on a sucrose enriched agar medium (53).

Collins (9) has discussed the many different names in use for

the genus Leuconostoc and the various species within this genus. Sandine et al. (53) indicated that much confusion has arisen regarding the classification of the Leuconostoc genus. These authors recommend the designation Leuconostoc mesenterioides for those microorganisms which do not produce diacetyl or acetoin when grown in non-fat milk but do elaborate dexran on sucrose medium. "Leuconostoc" organisms having neither of these properties should be placed in the genus Pediococcus (53).

Bacterial Composition of Mixed-Strain Butter Cultures. Galesloot (15, vol. D, p. 124) classifies mixed-strain butter cultures as to the types of aroma bacteria present. His classifications are: Cultures with only Leuconostoc sp. as aroma bacteria; those containing only S. diacetilactis as aroma bacteria; and cultures containing both of the above.

The earlier work on the bacterial composition of butter cultures has been summarized by Hammer and Babel (19). These authors stated that desirable butter cultures contain from one to ten million aroma bacteria per ml. They have cited work which demonstrated that a culture which failed to produce a desirable flavor and aroma contained less than 1000 aroma bacteria per ml, and that the lactic acid bacteria were present in numbers exceeding 100 million per ml.

Glenn and Prouty (16) have shown marked changes in the L. citrovorum population in a culture incubated at 22° C. They found that

about five percent of the total population was L. citrovorum through the first ten hr of incubation. After 16 hr the L. citrovorum population had decreased to about one percent of the total. After 25 hr of incubation the L. citrovorum population accounted for four percent of the total. Lindsay (30, p. 190) has shown that the L. citrovorum population of a desirable butter culture rises from about 2. 2 percent of the total in a culture that was not fully ripened to 3. 8 percent when this culture was subcultured and fully developed. This worker also noted a decrease in the S. diacetilactis population paralleling the increase in the L. citrovorum population.

Hammer and Babel (19) have cited work which showed that the aroma bacteria disappeared from cultures with successive transfers and a harsh acid or malt flavor became apparent. Recently however, Overcast and Skean (43) examined 72 lactic cultures formulated for various uses for citrate fermenting organisms. The cultures examined ranged in age from two days to over ten years and the numbers of citrate fermenting organisms varied from less than 10, 000 to 2. 1 billion organisms per ml. On the basis of a statistical analysis of their data, these authors concluded that the mean percentages of citrate fermenting bacteria in cultures were not significantly different according to their original source, the time they were maintained, or their particular use. These authors stated that their data did not support the idea that citrate fermenting organisms tend

to disappear with successive transfers.

De Mann and Galesloot (12) have shown that there are seasonal variations in the Leuconostoc population of butter cultures which can be prevented by the addition of 0.25 ppm of manganese. They attributed this fluctuation in the numbers of Leuconostoc sp. to seasonal variations in the manganese content of milk. These researchers have shown that the addition of manganese simulated the Leuconostoc organisms only when the cells were actively growing. The addition of manganese had no influence on the lactic streptococci. Reiter and Møller-Madsen (51) have cited work which demonstrates wide fluctuations in the manganese content of milk. Milk produced in the spring months is low in manganese, while milk produced during the fall months has the highest manganese levels. Work by Jensen and Møller-Madsen (25) has shown that the addition of manganese to culture milk when it is low in this mineral maintains a desirable type starter when Leuconostoc sp. are the aroma bacteria. These workers believe that maintaining the Leuconostoc population is important because of the better keeping quality of butter made with this type starter.

Galesloot (15, vol. D, p. 146) has reported that both the percentage of inoculum and the incubation temperature have an effect on the population of butter cultures. A small inoculum and high incubation temperatures increased the proportion of S. diacetilactis

organisms in cultures containing only these organisms as aroma bacteria. A large inoculum and lower incubation temperatures favored an increase in the Leuconostoc population in cultures containing these organisms as aroma bacteria. Cultures containing both Leuconostoc sp. and S. diacetilactis are apparently affected even more by the percent of inoculum and incubation temperature.

Galesloot (15, vol. D, p. 143) has shown that the rate of acid production by S. diacetilactis is usually slower than that of S. cremoris and S. lactis. Anderson and Leesment (3, vol. B, p. 217-224) have reported an incident in two-strain cultures where S. diacetilactis supplemented the S. cremoris or S. lactis strains to such an extent that the rate of acid production was lowered. Vedamuthu and co-workers (61) have shown that the addition of 0. 2 percent or more of citrate caused a dominance of a strain of S. diacetilactis over strains of S. cremoris or S. lactis in two-strain combinations. These authors felt that this strain dominance was due to the ability of S. diacetilactis to ferment citrate.

Biochemistry of Butter Cultures

The metabolism of mixed-strain cultures is complex because it encompasses the production of many products from many metabolites. The mechanisms involved in the associative growth of organisms in a complex biological fluid such as milk are difficult to study.

Consequently many investigators have turned to synthetic media for their studies. In these cases, care must be taken in relating results obtained in these media to those observed in milk media because both the inducible and constitutive enzymes of the cell may be affected. However, much information has been gathered by employing both of these approaches. A discussion of the metabolism of both homofermentative and heterofermentative lactic acid bacteria is pertinent to this investigation.

Carbohydrate Metabolism by Homofermentative Lactic Streptococci

The homofermentative lactic streptococci are so named because they produce lactic acid as the principal catabolic product from glucose. These are distinguished from heterofermentative lactic acid bacteria by their production of L (+) - lactic acid (15, vol D, p. 144). Lactose, the principal carbohydrate in milk is hydrolyzed to glucose and galactose; glucose being metabolized via the well-known Embden-Meyerhof pathway. Kandler (27) reported that galactose is transformed into glucose-1-phosphate, which can enter the Embden-Meyerhof pathway, by a reaction series involving uridine diphosphoglucose.

Van Slyke and Bosworth (60) found that S. lactis grown in pasteurized and separated milk at 32. 2° C fermented only 20 percent of the lactose in 96 hr. The percentage of fermented lactose

converted to lactic acid varied from 70 - 90 percent. Homofermentative bacteria apparently have enzymes which oxidize and decarboxylate glucose-6-phosphate to ribulose-5-phosphate (27). This could account for the formation of compounds other than lactic acid by homofermentative microorganisms.

Several interesting observations have been made concerning the metabolism of lactic streptococci. Palladina (46) found that S. lactis decomposed ethanol but that S. cremoris did not. Harvey (20) reported that some strains of S. cremoris and S. lactis produce acetone when grown in milk media; however, the strain of S. diacetilactis studied did not produce acetone but removed all the acetone from milk. This indicated that S. diacetilactis in some way utilizes acetone.

Carbohydrate Utilization by Heterofermentative Lactic Bacteria

The microorganisms of the Leuconostoc genus are classified as heterofermentative because they produce several catabolic products from glucose. They produce small amounts of D (-)-lactic acid, which distinguishes them from the lactic streptococci (15, vol. D, p. 144). Kandler (27) has outlined the metabolic pathway used by the heterofermentative bacteria for the utilization of hexose sugars. This pathway (shown in Figure 1) partially involves the well-known hexose monophosphate shunt. Heterofermentative organisms utilize this pathway rather than the Embden-Meyerhof pathway because they lack

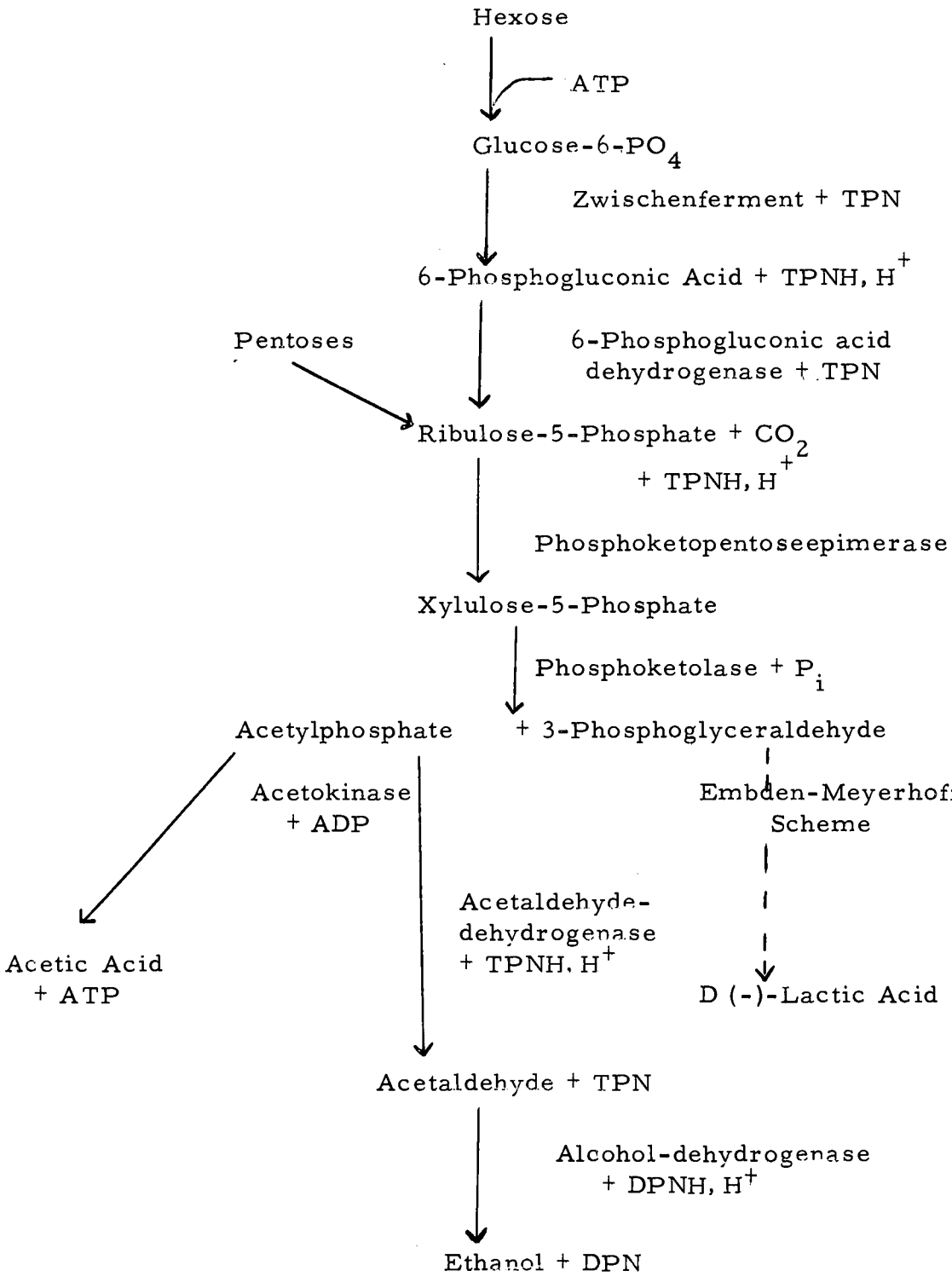


Figure 1. Carbohydrate metabolism of heterofermentative lactic acid bacteria. From Kandler (27, p. 524).

the enzyme aldolase, which catalyzes the conversion of fructose-1, 6-diphosphate to dihydroxyacetone phosphate and 3-phosphoglyceraldehyde (51).

Galesloot (15, vol. D, p. 149) has stated that the reduction of acetylphosphate to ethanol is a waste of considerable energy, but the Leuconostoc organisms must do this to consume the hydrogen released in the conversion of hexose to 6-phosphogluconic acid and the subsequent conversion of the latter compound to carbon dioxide and ribulose-5-phosphate. Under the proper conditions, the Leuconostocs convert acetylphosphate to acetic acid rather than ethanol.

Citric Acid Fermentation

Since diacetyl is one of the more important compounds contributing to the flavor of butter cultures and cultured butter, a discussion of its metabolic production is pertinent. Until recently citric acid fermentation by butter cultures has been the subject of much confusion, some of which still exists.

The pathways for enzymatic conversion of citric acid by S. diacetilactis have been summarized by Seitz (56, p. 91-96) and are shown in Figure 2. Galesloot (15, vol. D, 1. 153) has stated that the mechanisms for citrate utilization by the Leuconostoc sp. in butter cultures appear to be the same as those given in Figure 2. The most widely accepted beliefs concerning diacetyl and acetoin production are

Citric acid $\xrightarrow{1^a}$ Oxaloacetic acid + Acetic acid

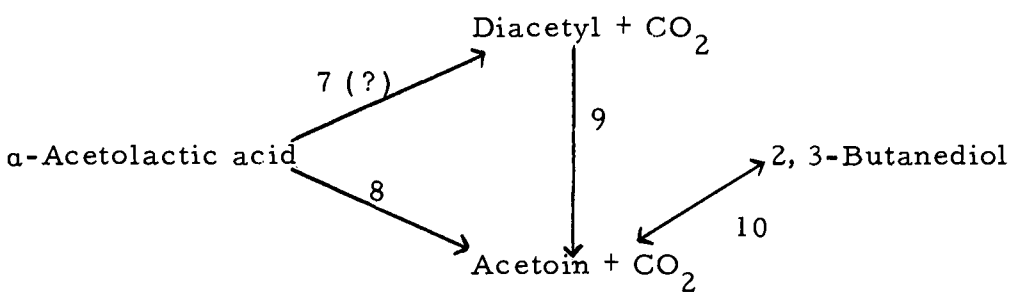
Oxaloacetic acid $\xleftrightarrow{2}$ Pyruvic acid + CO_2

2 Pyruvic acid + 2 TPP[b] $\xrightarrow{3}$ 2-Acetaldehyde · TPP + $2CO_2$

Acetaldehyde · TPP $\xrightarrow{4}$ Acetaldehyde + TPP

Acetaldehyde · TPP + Acetaldehyde $\xrightarrow{5}$ Acetoin + TPP

Acetaldehyde · TPP + Pyruvic acid $\xrightarrow{6}$ α - Acetolactic acid + TPP



[a]Enzymes catalyzing each reaction:

1. Citritase
2. Oxaloacetate decarboxylase
3. Pyruvate decarboxylase
4. Non-enzymatic
5. Acetoin synthetase
6. α -Acetolactate synthetase
7. α-Acetolactate oxidase
8. α-Acetolactate decarboxylase
9. Diacetyl reductase
10. 2, 3-butanediol dehydrogenase

[b]Thiamine pyrophospate

Figure 2. Pathways for enzymatic conversion of citric acid by S. diacetilactis. From Seitz (56, p. 95).

that no aroma is produced from sugar alone, but is produced from citrate alone and from a mixture of sugar and citrate. Harvey and Collins (21) have observed a citrate transport system in S. diacetilactis. These workers found that this transport system could be induced and permitted greatest entry of citrate into cells at pH values below 6.0. Marth (35) has summarized the ideas on the production of acetoin and diacetyl. Pyruvic acid is the key intermediate in the fermentation of lactose and citrate to acetoin and diacetyl. He has further stated that in the fermentation of lactose to pyruvate in the Embden-Meyerhof scheme, sufficient DPN is produced to reduce pyruvate to lactic acid, but that the fermentation of citrate results in the production of pyruvate without a simultaneous supply of reduced DPN and, thus, products other than lactic acid are formed. Galesloot (15, vol. D, p. 152-153) has pointed out that at pH values near 5.0, the sugar fermentation slows and when aroma production starts the pyruvic acid pool is fed mainly by citrate fermentation.

DeMann and Galesloot (12) have reported that active Leuconostoc starters produce little diacetyl aroma. These Leuconostocs tend to avoid the reduction of acetylphosphate to ethanol, but prefer to convert the former into acetic acid with the simultaneous gain of one ATP. The hydrogen thus generated during the sugar fermentation appears to be utilized in the reduction of diacetyl to acetoin and 2, 3-butanediol. It has been suggested that when the Leuconostocs cannot

complete their metabolic processes because of low numbers or a low pH, diacetyl tends to accumulate rather than being reduced (15, vol. D, p. 153).

Breakdown of Diacetyl

Michaelian and Hammer (36) have observed that diacetyl is not converted into volatile acids when it disappears from cultures of citric acid-fermenting streptococci. Stahly et al. (58) have added diacetyl and acetoin to cultures of citric acid-fermenting streptococci and noted an increase in 2, 3-butanediol as the diacetyl and acetoin contents decreased. Juni (26) has reported a pathway for the bacterial dissimilation of diacetyl. In this system acetylmethylcarbinol is formed when diacetyl is the substrate for pyruvic oxidase. Seitz et al. (57) have reported diacetyl reductase, an enzyme which catalyzes the irreversible reaction of diacetyl to acetoin, to be present in all strains of S. diacetilactis studied. These workers also found this enzyme in several of the Leuconostoc sp. studied.

EXPERIMENTAL

Cultures

Single-strain cultures, obtained from the Department of Microbiology, Oregon State University, were S. cremoris strains 459 and 799, S. diacetilactis strains DRC-1 and M 21-35, S. lactis strains L-2 and C2-F, L. citrovorum strain 91404, L. dextranicum strain 688, and L. mesenteroides strain P-60. A commercial mixed-strain butter culture obtained from Molkerei Laboratory, Niebuell, Schleswig, West Germany was selected for its previously demonstrated ability to produce a good cultured cream butter (30, p. 61).

Culturing Conditions

All single-strain cultures were maintained in reconstituted 11 percent solids non-fat milk medium. This medium was prepared by dissolving the non-fat milk solids in distilled water and autoclaving at 121°C for 10-12 min. The medium used to maintain cultures for gas-liquid chromatographic analysis was heated for one hour in a boiling water bath and cooled immediately to 5°C. All cultures were transferred every other day, using from one to three percent inoculum. All cultures were incubated at either 21 or 30°C, except as otherwise noted.

Raw whole milk (approximately 3. 8 percent milk fat and 8. 5 percent solids-not-fat) was used as the culturing medium for the commercial mixed-strain butter culture. Sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) was added to the extent of 0. 2 percent (approximately 0. 01 M calculated as citric acid) from a stock aqueous solution containing 0. 6 g per ml. The milk was then heated in a boiling water bath for one hour and cooled immediately to 5° C. The mother cultures were carried in duplicate in 300 ml of the whole milk medium. These were transferred every other day using from one to three percent inoculum and varying the incubation time at 21° C so that the titratable acidity of the cooled culture was from 0. 68 to 0. 78 percent expressed as lactic acid. When a flavor defect described as "green" was desired, the culture was ripened until the acidity exceeded 0. 80 percent. After this treatment subsequent transfers usually yielded a green-flavored culture.

Titratable acidities were determined in the customary manner for dairy products using nine gram samples according to the procedure outlined by Goss (17). All titratable acidities were calculated as percent lactic acid.

Measurement of pH

Measurements of pH were made with one of three different instruments; a Beckman Model G pH meter, a Beckman Zeromatic pH

meter, or a Beckman Expandomatic pH meter.

Determination of Microbial Population

All of the microbial numbers reported herein were determined by plating dilutions of the culture being studied on lactic agar (13). Buffered distilled water blanks, prepared in accordance with the procedure outlined in Standard Methods for the Examination of Dairy Products (1, p. 67), were used to make all dilutions. Plate counts are reported in accordance with standard methods (1, p. 75-79).

Methods for the Quantitative Determination of Acetaldehyde and Diacetyl in Culture

Determination of Diacetyl

Recently Pack et al. (45) have successfully adapted the Owades and Jakovac (44) method for the quantitative determination of diacetyl in mixed-strain starters. This method, with certain modifications, was used for all quantitative diacetyl determinations. Aliquots of the culture being tested were weighed rather than measured volumetrically, and instead of using dilutions of dimethyl glyoxime for the standard, diacetyl (gas chromatographically pure) was used to prepare the standard curve. All samples were read against a reagent blank at 530 mμ using a Beckman DU spectrophotometer.

Determination of Acetaldehyde

Acetaldehyde was quantitated by the 3-methyl-2-benzothiazalone hydrazone hydrochloride method described by Lindsay and Day (31). All samples were read against a reagent blank at 666 mμ using a Beckman DU spectrophotometer.

Determination of Acetaldehyde and Diacetyl Production by Single-Strain Lactic Streptococci

Single-strain lactic cultures were analyzed for acetaldehyde and diacetyl production, pH, and microbial population as a function of incubation time. Cultures studied were S. cremoris 459 and 799, S. diacetilactis DRC-1 and M 21-35, and S. lactis L-2 and C2-F. All of the data were collected at the initial time of inoculation and then after 3, 6, 8, 10, 12, and 14 hr incubation. The cultures were re-inoculated and all data were collected after 12 hr incubation and thereafter at two hour intervals to 18-24 hr.

Gas-Liquid Chromatographic Analysis

The volatile components from 14-16 hr cultures of each of the microorganisms studied for acetaldehyde and diacetyl production as a function of incubation time were analyzed by gas chromatography. The gas entrainment and on-column trapping technique described by Morgan and Day (39) was employed. Nitrogen was bubbled at a rate

of 10 ml per min over a period of five minutes through 2 ml of culture diluted to a total volume of 8 ml with distilled water and equilibrated at 60° C. A one-eighth inch by 12 ft column packed with 20 percent 1, 2, 3-tris(2-cyanoethoxy) propane on 60-80 mesh Celite 545 was used. The volatiles were entrained on this column and chromatographed at a column temperature of 50° C with an F and M Model 810 instrument. As a control, a sample of the heat-treated medium was chromatographed by the same procedure. Identity of the major components separated by this system was established by coincidence of retention times.

Studies on Acetaldehyde Production and Utilization in Lactic Cultures

Acetaldehyde Utilization by L. dextranicum 688

A culture of L. dextranicum 688 was incubated at 30° C for 16 hr and then divided into aliquots; one aliquot was not acidified (pH 4. 8) and the second was acidified to pH 3. 5 with sterile 20 percent phosphoric acid. Plate counts were made on an aliquot of the culture at this time. Acetaldehyde was added to each aliquot (2. 5 ppm) and incubation was continued for eight hours at 21° C. The acetaldehyde content was determined at two hour intervals as described. The diacetyl content was determined at the time of initiation of the experiment and after six hours of incubation as previously described.

The rate of acetaldehyde utilization by L. dextranicum 688 was determined on acidified and non-acidified cultures incubated at 30° C at two hour intervals up to eight hours. The amount of acetaldehyde added to the 30° C aliquots was 5. 8 - 6. 0 ppm.

Acetaldehyde Utilization by L. mesenteroides P-60

A culture of L. mesenteroides P-60 was incubated for 16 hr at 30° C and then divided into two aliquots; one aliquot was not acidified (pH 6. 5) and the second was acidified to pH 4. 5. Plate counts were made on an aliquot of the culture and then acetaldehyde (4. 3 ppm) was added to each aliquot and incubation was continued at 21° C for eight hours. This culture was analyzed in the same manner as the L. dextranicum culture previously described.

The rate of acetaldehyde utilization at 30° C by L. mesenteroides P-60 was determined by the same procedure as the rate at 21° C. The amount of acetaldehyde added to the 30° C aliquots was 6. 2-6. 3 ppm.

Acetaldehyde and Diacetyl Production by Single-Strain Mixtures

Several mixed-strain cultures containing a single-strain Streptococcus sp. in combination with a single-strain Leuconostoc sp. were prepared by inoculating sterile milk with appropriate single-strain cultures. The mixtures studied were:

S. cremoris 459	+	L. citrovorum 91404
S. cremoris 799	+	L. citrovorum 91404
S. cremoris 459	+	L. mesenteroides P-60
S. cremoris 799	+	L. mesenteroides P-60
S. cremoris 459	+	L. dextranicum 688
S. cremoris 799	+	L. dextranicum 688
S. diacetilactis DRC-1	+	L. dextranicum 688
S. diacetilactis M 21-35	+	L. dextranicum 688

The microbial population was determined on each single-strain culture before mixing. The resulting cultures were analyzed for acetaldehyde and diacetyl and the pH and flavor were determined after 12, 16, and either 21 or 24 hr of incubation at 21° C.

Determination of the Ratio of Different Lactic Organisms in a Selected Commercial Mixed-Strain Butter Culture

A commercial mixed-strain butter culture was analyzed for the ratio of lactic organisms after three successive transfers at 0.73-0.76 percent acidity, and again after a green flavor had been induced by ripening a subculture to 0.85 percent acidity and then transferring and ripening to 0.76 percent acidity. The simplified general classification method proposed by Sandine et al. (53) was used to characterize the individual microorganisms.

These cultures were analyzed for acetaldehyde and diacetyl content and were judged for flavor and aroma by three experienced

judges. Appropriate dilutions of the culture were plated in and on lactic agar (13), using both conventional pour plates and surface smear plates. These plates were incubated for 48 hr at 30° C. Approximately 100 colonies were picked at random from the smear plates into litmus milk and incubated for 48 hr at 30° C. These cultures were then transferred into sterile skim milk and incubated for 48 hr at 30° C. The King's Test (28) for diacetyl and acetoin was run on all cultures which coagulated and reduced litmus milk. Cultures which showed no acid production by coagulation were acidified to approximately pH 4. 5 with sterile 20 percent phosphoric acid and incubated for an additional six hours at 30° C before running the King's Test. The criteria used for classification were as follows:

Classification	King's Test	Acid Coagulation in 48 hr at 30° C
S. lactis or S. cremoris	negative	positive
S. lactis or S. cremoris var. aromaticus	slight positive	positive
S. diacetilactis	positive	positive
Leuconostoc sp.	positive after acidification	negative
Dead (or non-contributing organisms)	negative after acidification	negative

Organisms isolated from these cultures which were characterized as being either S. cremoris or S. lactis were differentiated

using the ability of the latter to decarboxylate arginine (41). The arginine hydrolysis broth medium developed by Mikolajcik (37) was used to differentiate these two Streptococci.

Green Flavor Removal

To determine the ability of L. citrovorum 91404 to remove acetaldehyde from a ripened culture, individual cultures of S. diacetilactis DRC-1 and M 21-35 were incubated for ten hours at 21° C, after which they were analyzed for diacetyl and acetaldehyde, pH, and microbial population. To each of these cultures was added two percent of an 18 hr L. citrovorum 91404 culture of known microbial population. Incubation was then continued at 21° C and each culture was analyzed for acetaldehyde and diacetyl content and the pH and flavor were determined after 6 and 12 hr of incubation.

On the basis of the results obtained from the above mentioned trials, it was decided to increase the amount of L. citrovorum 91404 added to find what levels were required to remove acetaldehyde from a ripened culture. A culture of S. diacetilactis DRC-1 was incubated for 12 hr at 21° C. This culture was then split into five equal aliquots. One of the aliquots was kept as a control. Of the other four aliquots, five percent of a 16 hr L. citrovorum 91404 culture was added to one, ten percent was added to another, 25 percent was added to another, and 50 percent was added to the fourth. All of the aliquots were

analyzed for acetaldehyde and diacetyl content at the initial time of addition of L. citrovorum 91404 and after 4, 8, and 12 hr of continued incubation at 21° C. The pH of the control was determined simultaneously with the above-mentioned analyses. Plate counts were made on the S. diacetilactis DRC-1 culture before it was split into aliquots and on the L. citrovorum 91404 culture. This entire procedure was repeated using a 12 hr S. cremoris 459 culture instead of the S. diacetilactis DRC-1 culture.

The ability of L. citrovorum 91404 to remove acetaldehyde from a ripened culture at 5° C was determined. A culture of S. diacetilactis DRC-1 was incubated at 21° C for 12 hr and then split into two aliquots. One aliquot was held as a control. To the other was added 50 percent of a 16 hr L. citrovorum 91404 culture. The two aliquots were analyzed for acetaldehyde and diacetyl content and then they were cooled and held at 5° C. Acetaldehyde and diacetyl concentrations were determined after 6, 12, 24, and 36 hr of incubation at 5° C. The pH of the control was determined simultaneously with the aformentioned analyses. Plate counts were made on the S. diacetilactis DRC-1 culture before it was split into aliquots and on the L. citrovorum 91404 culture. This entire procedure was repeated using a culture of S. cremoris 459 instead of the S. diacetilactis DRC-1 culture.

Acetaldehyde Removal in a Neutralized Culture

The effect of neutralizing a ripened lactic culture on the ability of added L. citrovorum 91404 to reduce the acetaldehyde concentration was determined. Three replicates of a culture of S. cremoris 459 were incubated for 12 hr at 21° C. After this time the pH of each replicate was adjusted to 6.5 with sterile 20 percent sodium hydroxide. Ten percent of a 16 hr culture of L. citrovorum 91404 was added to one replicate and 25 percent was added to a second replicate. The third replicate was held as a control. Each of the replicates was analyzed for acetaldehyde and diacetyl content and the pH was determined at the initial time of adding L. citrovorum 91404. These analyses were repeated after four and eight hours of continued incubation at 21° C. The number of microorganisms in the control culture and the L. citrovorum 91404 culture was determined by methods previously described.

RESULTS AND DISCUSSION

Production of Volatile Compounds by Single-Strain Lactic Cultures

The production of acetaldehyde and diacetyl over time by two strains of S. diacetilactis was studied. The data obtained are presented in Table 1. Table 2 shows the production of acetaldehyde over time by the two strains of S. cremoris employed. Table 3 shows the acetaldehyde production over time by the two strains of S. lactis studied. These tables also show the pH and the number of microorganisms at the time of each analysis. The amount of diacetyl produced by each of the strains of S. cremoris and S. lactis was found to be very low (less than 0. 1 ppm).

Each of the strain of S. diacetilactis accumulated diacetyl until the pH was lowered to about 5. 2, after which the concentration of this component decreased. This has been shown to be due to a diacetyl reductase enzyme (57). This enzyme catalyzes the irreversible reduction of diacetyl to acetoin. On continued incubation the diacetyl concentration began to increase again. This increase may be due to a further decrease in pH to a point where diacetyl reductase activity is inhibited. However, at no time did the ratio of diacetyl to acetaldehyde approach the 4:1 ratio reported for a good flavor balance in butter cultures (32). In fact, once the heated milk taste was masked,

Table 1. Acetaldehyde and diacetyl production by single-strain S. diacetilactis cultures.

Incubation time at 21°C (hr)	Acetaldehyde (ppm)	Diacetyl (ppm)	pH	Plate Count (X 10^7)
S. diacetilactis DRC-1				
0	0.16	0.36	6.59	3.2
3	0.76	1.18	6.58	21
6	1.31	2.68	6.28	99
8	5.06	3.50	5.84	270
10	9.00	2.00	5.35	280
12	9.25	2.55	5.10	310
14	9.62	1.73	4.70	270
24	10.88	1.82	4.45	---
12	9.38	1.66	5.10	219
14	9.44	0.84	4.95	200
16	9.12	1.00	4.80	232
18	9.38	1.05	4.70	195
20	10.00	1.32	4.70	200
S. diacetilactis M21 - 35				
0	0.11	0	6.50	5.3
3	0.72	0.32	6.25	12
6	1.72	0.59	6.05	53
8	2.16	0.77	5.80	120
10	4.38	0.83	5.45	260
12	5.40	0.78	5.15	370
14	6.13	0.46	4.95	290
12	4.63	0.73	5.35	270
14	5.75	0.59	5.10	310
16	6.25	0.64	4.90	320
18	6.50	0.73	4.80	370
20	6.63	0.82	4.70	340
22	6.38	0.91	4.70	310

Table 2. Acetaldehyde production by single-strain S. cremoris cultures

Incubation time at 21°C	Acetaldehyde (ppm)	pH	Plate Count (X 10^7)
S. cremoris 799			
0	0.10	6.35	2.9
3	0.66	6.23	12
6	2.19	5.69	31
8	4.06	5.30	62
10	5.62	4.82	150
12	5.81	4.56	170
14	5.00	4.45	190
24	3.50	4.22	180
12	5.00	4.70	170
14	4.63	4.65	200
16	4.50	4.55	210
18	4.00	4.50	180
20	3.63	4.45	190
22	3.38	4.40	190
24	3.13	4.35	210
S. cremoris 459			
0	0.09	6.43	2.4
3	0.41	6.35	6.1
6	0.88	6.17	20
8	1.66	5.83	51
10	3.63	5.46	90
12	6.50	4.70	160
14	7.88	4.43	150
12	7.25	4.70	160
14	7.31	4.65	170
16	7.06	4.50	210
18	6.88	4.40	210
20	6.50	4.40	120
22	6.13	4.35	190
24	6.00	4.30	190

Table 3. Acetaldehyde production by single-strain S. lactis cultures

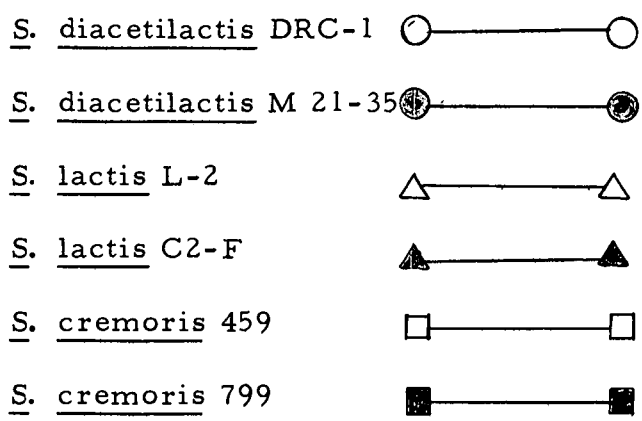
Incubation time at 21°C	Acetaldehyde (ppm)	pH	Plate Count (X 10^7)
S. lactis C2-F			
0	0.03	6.56	4.6
3	0.33	6.27	11
6	1.22	6.01	54
8	1.13	5.70	110
10	2.13	5.38	190
12	3.62	4.71	310
14	3.00	4.67	---
10	4.88	4.95	250
12	3.25	4.75	360
14	3.06	4.70	330
16	----	4.70	260
18	3.00	4.60	230
S. lactis L-2			
0	0.00	6.39	3
3	0.19	6.38	4
6	0.50	6.27	22
8	0.96	6.14	56
10	1.63	6.05	74
12	0.96	5.94	96
14	0.69	5.85	100
24	0.31	5.57	120
12	1.24	5.95	35
14	1.09	5.85	45
16	0.97	5.80	36
18	0.97	5.75	42
20	0.85	5.60	49

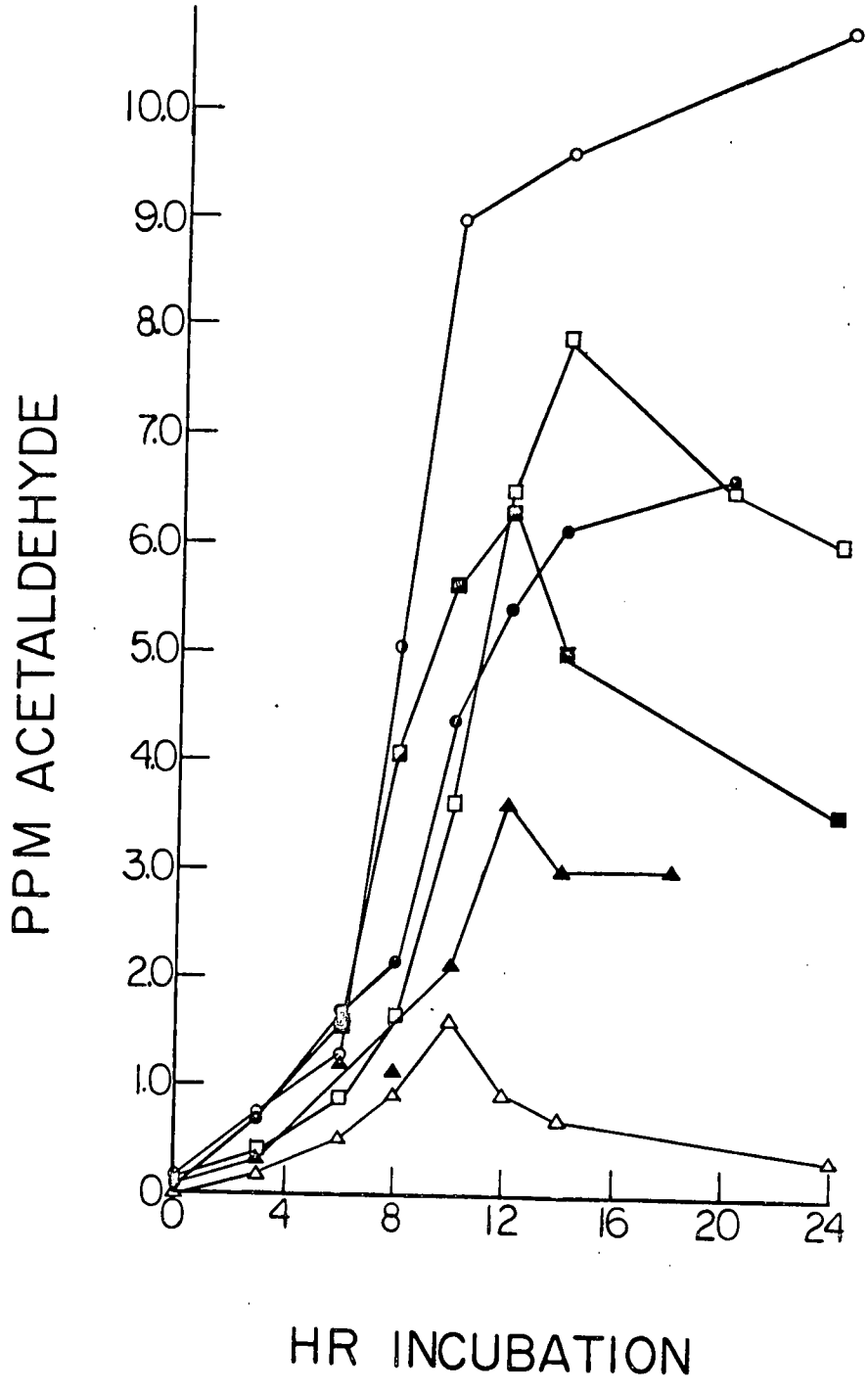
the flavor became distinctly green, increasing in intensity as incubation time increased.

Acetaldehyde production by all of the cultures studied appeared to parallel the increase in microbial population. This trend was confirmed by repeating all analyses on a new inoculum of culture. Production of this compound by the two strains of S. diacetilactis was slow for the first six hours of incubation, increased rapidly on continued incubation to about 12 hr, and then gradually leveled off. There was little or no decrease in the total acetaldehyde concentration on continued incubation to 20-24 hr. This is in contrast to the results obtained for acetaldehyde production by each of the two strains of S. cremoris and S. lactis employed in this study. As shown in Table 2, each of the strains of S. cremoris produced acetaldehyde continuously on incubation up to 12-14 hr, after which time the concentration of acetaldehyde decreased steadily. Each of the two strains of S. lactis (Table 3) produced acetaldehyde continuously on incubation up to about 10-12 hr, after which time the concentration of acetaldehyde decreased and then leveled off. Figure 3 graphically illustrates these trends.

To preclude the possibility that the decrease in acetaldehyde concentration was due to a pH dependent reaction with the milk proteins, sterile milk was acidified to varying levels (pH 4.0-6.5) with sterile phosphoric acid and a known amount of acetaldehyde was

Figure 3. Comparison of acetaldehyde production by single-strain cultures of lactic acid bateria incubated in sterile milk at 21°C.




PPM ACETALDEHYDE
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
0
4
8
12
16
20
24
HR INCUBATION

added. These mixtures were incubated at 21°C and analyzed at different intervals for acetaldehyde concentration. After eight hours at 21°C the acetaldehyde levels were only slightly lower for all samples than at the initiation of the trial. The rate of acetaldehyde loss was not affected by the pH of the milk medium and was assumed to be due to slow volatilization or reaction with the milk proteins.

In 1960, Harvey (20) reported that all strains of S. cremoris, S. lactis, and S. diacetilactis studied in his laboratory produced significant quantities of acetaldehyde when grown in milk media. He explained the accumulation of acetaldehyde as resulting from the decarboxylation of pyruvate, which is formed as a result of glucose metabolism. The results presented herein are in agreement with Harvey's (20) observations.

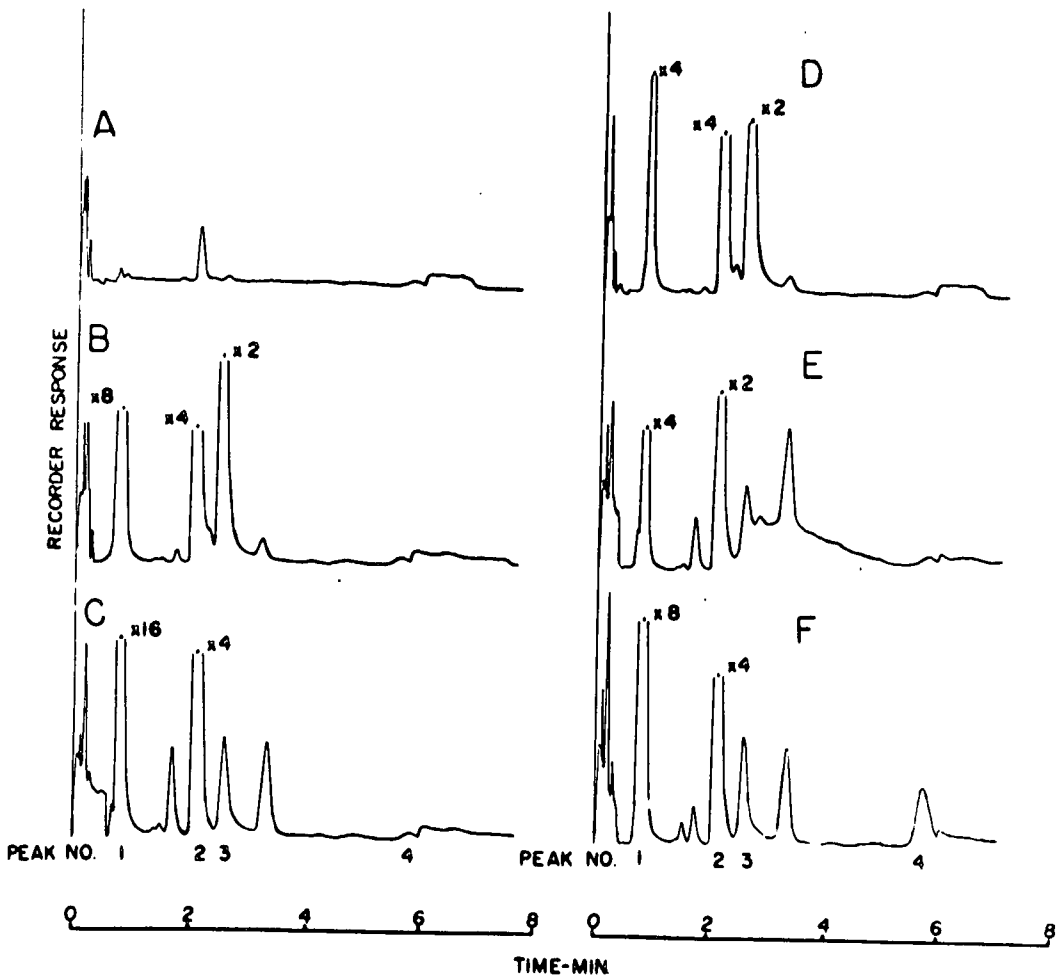
Gas chromatographic analysis of 14-16 hr cultures showed that S. cremoris 799 and S. lactis C2-F produced relatively high levels of ethanol. S. cremoris 459, S. lactis L-2, and both S. diacetilactis DRC-1 and M 21-35 produced relatively low levels of ethanol. Typical examples of the chromatograms obtained are shown in Figure 4. No attempt was made to identify all of the peaks obtained.

Ethanol has been reported to be a normal catabolic product of heterofermentative lactic acid bacteria, but not a usual product of homofermentative metabolism (35). Although Platt and Foster (49) have shown ethanol production by S. lactis and S. cremoris grown in

Figure 4. Chromatograms of the volatiles produced by single-strain cultures of lactic acid bacteria grown in boiled reconstituted milk at 21° C.

Chromatogram A is the heated milk control, B is S. cremoris 799, C is S. cremoris 459, D is S. lactis C2-F, E is S. lactis L-2, and F is S. diacetilactis M21-35.

Peak 1 is acetaldehyde, 2 is acetone, 3 is ethanol, and 4 is diacetyl.

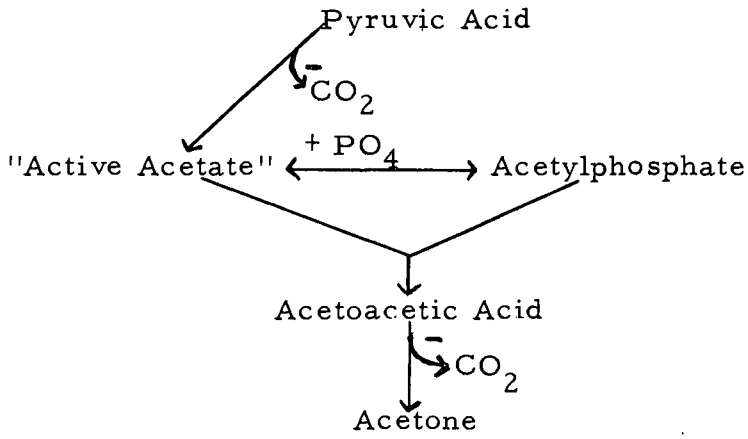

A
B
C
D
E
F
x8
x4
x2
x16
x4
x4
x2
x8
x4
RECORDER RESPONSE
PEAK NO. 1 2 3 4
PEAK NO. 1 2 3 4
0 2 4 6 8
0 2 4 6 8
TIME-MIN

non-milk media, it is strange to find two of the homofermentative species producing ethanol while the more heterofermentative S. diacetilactis cultures did not produce much ethanol under the same conditions. However, in unpublished results obtained in this laboratory, the ability of selected strains of S. diacetilactis to produce ethanol under different culturing conditions has been demonstrated (40).

It is possible that some of the homofermentative organisms which have the ability to attack acetaldehyde possess a mechanism to convert it to ethanol. However, if this is the case it is strange that only one of the strains of S. lactis and one of the strains of S. cremoris studied produced significant quantities of ethanol while the others did not. Free acetaldehyde, or acetaldehyde complexed with thiamine pyrophosphate can form a number of compounds, such as diacetyl or acetic acid. One or more of these mechanisms may be in operation in these lactic acid bacteria, hence acetaldehyde may not necessarily be reduced to ethanol.

Harvey and Collins (22) have concluded that pyruvate formed in excess of that needed for cell synthesis is converted to acetoin by S. diacetilactis as a detoxification mechanism. Thus the decarboxylation of pyruvate to form acetaldehyde may also be regarded as a detoxification mechanism. It is probable that some acetaldehyde is produced in this manner by S. diacetilactis, as well as from acetylphosphate, as indicated by Kandler (27).

All of the lactic streptococci studied produced significant quantities of acetone (Figure 4). The production of acetone by some strains of S. lactis and S. cremoris was reported by Harvey (20); however, this worker found that the strain of S. diacetilactis studied did not produce acetone but rather utilized acetone present in milk media. The results obtained in the present investigation show that acetone is produced by some strains of S. diacetilactis when grown in a boiled milk medium. There are no metabolic pathways in the literature to explain the production of acetone by lactic acid bacteria grown in milk media. Several organisms used industrially to produce butanol, notably Clostridium acetobutylicum and Clostridium butylicum, are known to produce acetone by the decarboxylation of acetoacetic acid (50, p. 340-344). It is well known that acetoacetic acid is a normal metabolite of some microorganisms, arising from a condensation of acetyl phosphate and "active acetate", these two compounds arising from pyruvate (42, p. 236-237). Some of the lactic streptococci may possess enzymes for the synthesis of acetoacetate and its subsequent decarboxylation to acetone. These enzymes may be constitutive or they may be inducible, being synthesized only when levels of pyruvate become high. This process may serve as a detoxification mechanism to drain the pyruvate pool. A possible scheme for acetone formation from pyruvate is:



Acetaldehyde and Diacetyl Production and Utilization in Lactic Cultures

Under certain ill-defined conditions, butter cultures develop a flavor defect described as green or yogurt-like. Badings and Galesloot (4, vol. B, p. 199-208), and Lindsay (8, p. 174-193) have shown that acetaldehyde is responsible for this defect. These workers have observed that L. citrovorum in some manner transforms acetaldehyde produced by lactic streptococci. Lindsay et al. (30) have found that a strain of L. citrovorum was capable of utilizing 100 percent of added acetaldehyde in both acidified (pH 4. 5) and non-acidified (pH 6. 5) milk media. During the course of the present investigation it was desirable to determine whether other Leuconostoc sp. were capable of utilizing acetaldehyde.

Results from initial studies on acetaldehyde utilization by L. dextranicum 688 at 21°C are shown in Table 4. All cultures were

Table 4. Acetaldehyde utilization by a 16 hr L. dextranicum 688[a, b] culture at 21°C

Time after acetaldehyde addition (hr)	Acetaldehyde (ppm)	Percent utilization of initial acetaldehyde
Trial 1 (non-acidified milk, pH 4.8)		
0	2.53	---
2	1.44	43.6
4	0.63	76.0
6[c]	0.25	91.2
8	0.08	98.2
Trial 2 (acidified milk, pH 3.5)[d]		
0	2.50	---
2	1.94	22.4
4	1.34	46.4
6[c]	1.00	60.0
8	0.94	62.4

[a] Total count of greater than 1000 x 10^6 organisms per ml.

[b] Diacetyl production was essentially zero.

[c] Negative to the King's Test.

[d] Acidified with sterile 20 percent phosphoric acid at the time of acetaldehyde addition.

incubated at 30° C for 16 hr before acetaldehyde was added. The L. dextranicum culture employed in this study produced sufficient acid to lower the pH of the milk medium to 4. 8, after which there was no further decrease in pH. Production of acid in milk media is normal behavior for this microorganism (6, p. 532). The previously demonstrated stability of acetaldehyde in acidified heated milk precluded the possibility that acetaldehyde removal was due to reaction with milk proteins (38). Almost complete acetaldehyde removal was evident in the non-acidified sample after eight hours of incubation. The acidified culture showed a reduction of 62 percent of the added acetaldehyde in a corresponding time. The King's Test (28) for diacetyl and acetoin was run on both the acidified and non-acidified cultures after six hours of incubation. Results of this test were negative, indicating that acetaldehyde was not shunted to acetoin.

The rate of acetaldehyde utilization by L. dextranicum 688 at 30° C is shown in Table 5. The rate of acetaldehyde utilization at 30° C was much slower than at 21° C. The acidified culture utilized only very little of the added acetaldehyde in eight hours, while the non-acidified sample utilized 36 percent in a corresponding time. However, the total amount of acetaldehyde utilized in the non-acidified cultures at both 21 and 30° C was about the same.

The rate of acetaldehyde utilization by a L. mesenteroides P-60 culture at 21° C is shown in Table 6. The cultures were

Table 5. Acetaldehyde utilization by a 16 hr L. dextranicum 688[a] culture at 30° C

Time after acetaldehyde addition (hr)	Acetaldehyde (ppm)	Percent utilization of initial acetaldehyde
Trial 1 (non-acidified milk, pH 4.8)		
0	5.75	---
2	5.25	8.1
4	4.84	15.8
6[b]	4.06	29.4
8	3.69	35.8
Trial 2 (acidified milk, pH 3.5)		
0	6.00	---
2	6.01	---
4	5.94	1.0
6[b]	5.80	3.3
8	5.81	3.2

[a] Total count of 150 x 10^7 organisms per ml.

[b] Negative to the King's Test.

[c] Acidified with sterile 20 percent phosphoric acid at the time of acetaldehyde addition.

Table 6. Acetaldehyde utilization by a 16 hr L. mesenteroides P-60[a, b] culture at 21°C

Time after acetaldehyde addition (hr)	Acetaldehyde (ppm)	Percent utilization of initial acetaldehyde
Trial 1 (non-acidified milk, pH 6.5)		
0	4.31	---
2	4.13	4.2
4	3.71	14.0
6	3.21	25.6
8	3.21	25.6
Trial 2 (acidified milk, pH 4.5)[c]		
0	4.31	---
2	4.32	---
4	4.32	---
6	4.19	2.8
8	4.13	4.2

[a] Total count of 25×10^7 organisms per ml.

[b] Diacetyl values were essentially zero.

[c] Acidified with sterile 20 percent phosphoric acid at the time of acetaldehyde addition.

incubated at 30° C for 16 hr before acetaldehyde was added. The non-acidified culture (pH 6. 5) utilized 26 percent of the added acetaldehyde in eight hours of incubation at 21° C, while the acidified culture (pH 4. 5) removed only 4. 2 percent in the same time.

The rate of acetaldehyde utilization at 30° C by a L. mesenteroides P-60 culture is shown in Table 7. The non-acidified replicate had utilized 48 percent of the added acetaldehyde after eight hours of incubation, while the acidified culture utilized only about ten percent in the same time. Results of the King's Test (28) performed on the cultures after six hours of incubation were negative. This indicated that acetaldehyde was not shunted to acetoin. From these results it can be seen that L. mesenteroides P-60 utilizes acetaldehyde more rapidly at 30° C than at 21° C, while the opposite is true for L. dextranicum 688.

The most probable reason for more rapid acetaldehyde utilization in non-acidified medium is that at a pH near neutrality Leuconostocs metabolize sugar with the subsequent production of reduced pyridine nucleotides. The addition of acetaldehyde to a culture of Leuconostocs supplies a hydrogen acceptor for the regeneration of reduced pyridine nucleotides without loss of energy (32). At pH values below 6. 0, the citrate permease transport system operates in these microorganisms (51). This enables the Leuconostocs to metabolize citrate, which does not result in the production of reduced

Table 7. Acetaldehyde utilization by a 16 hr L. mesenteroides P-60[a] culture at 30° C

Time after acetaldehyde addition (hr)	Acetaldehyde (ppm)	Percent utilization of initial acetaldehyde
Trial 1 (non-acidified milk, pH 6.5)		
0	6.23	---
2	5.75	7.7
4	4.38	30.0
6[b]	3.71	40.5
8	3.33	48.0
Trial 2 (acidified milk, pH 4.5)[c]		
0	6.31	---
2	6.19	1.9
4	5.75	8.9
6	5.65	10.5
8	5.69	9.8

[a] Total count of 35 x 10^7 organisms per ml.

[b] Negative to the King's Test.

[c] Acidified with sterile 20 percent phosphoric acid at the time of acetaldehyde addition.

pyridine nucleotides, hence the conditions for acetaldehyde reduction would not be favorable in acidified media.

Several experiments were conducted to determine the role of different culture bacteria in the production of a green flavor defect. It has been previously shown that high acetaldehyde production by S. lactis and S. diacetilactis in conjunction with a low L. citrovorum population can give rise to a green flavor defect (30, p. 192). Results of studies on acetaldehyde production by some selected single-strain mixtures are shown in Table 8. Samples one and two, which contained combinations of S. cremoris and L. citrovorum had a distinct green flavor after 12 and 16 hr of incubation at 21° C. After an additional five hours of incubation the green flavor was markedly less intense. During this period the Leuconostoc activity was sufficient to significantly lower the acetaldehyde produced by S. cremoris. As can be seen from the results, L. citrovorum 91404 was much more active in combination with S. cremoris 459 than with S. cremoris 799. This organism has previously been shown to utilize acetaldehyde when grown in combination with S. lactis or S. diacetilactis (30, p. 192). None of the mixtures containing either L. dextranicum 688 or L. mesenteroides P-60 in combination with a single-strain lactic streptococci were very effective in removing acetaldehyde. Some of the acetaldehyde produced in samples 3, 4, 5, and 6 appeared to be utilized, but it is difficult to interpret these results in view of the

Table 8. Acetaldehyde and diacetyl production by selected single-strain mixtures of lactic organisms.

Sample	Culture mixture	Plate count x 10^7 [a]	Incubation time at 21°C (hr)	pH	Diacetyl (ppm)	Acetaldehyde (ppm)	Flavor and aroma
1	2% S. cremoris 459	160	12	4.60	0.27	5.94	Green
	1% L. citrovorum 91404	2.1	16	4.59	0.64	4.75	Green
			21	4.38	1.96	1.56	Slightly green
2	2% S. cremoris 799	160	12	4.62	0.09	4.88	Green
	1% L. citrovorum 91404	2.1	16	4.60	0.25	4.13	Green
			21	4.37	0.50	2.39	Slightly green
3	2% S. cremoris 459	120	12	5.11	0	4.38	Green
	1% L. mesenteroides P-60	31	16	4.40	-	6.75	Green
			24	4.41	-	5.25	Green
4	2% S. cremoris 799	65	12	5.20	0	4.08	Green
	1% L. mesenteroides P-60	31	16	4.61	-	5.00	Green
			24	4.45	-	3.50	Green
5	2% S. cremoris 459	100	12	4.82	0	5.30	Green
	1% L. dextranicum 688	6.3	16	4.46	-	4.50	Green
			24	4.43	-	3.60	Green
6	2% S. cremoris 799	120	12	4.88	0	5.40	Green
	1% L. dextranicum 688	6.3	16	4.47	-	3.95	Green
			24	4.42	-	2.63	Slightly green
7	2% S. diacetilactis DRC-1	250	12	4.85	1.45	6.18	Green
	1% L. dextranicum 688	140	16	4.58	1.46	6.85	Green
			24	4.47	1.14	6.88	Green
8	2% S. diacetilactis M21-35	310	12	4.98	1.30	7.35	Green
	1% L. dextranicum 688	140	16	4.65	1.00	8.00	Green
			24	4.51	0.85	6.88	Green

[a]Plate counts made on cultures used to formulate mixture.

fact that the S. cremoris cultures used in these mixtures have been shown to be capable of utilizing acetaldehyde. Sample eight demonstrates that L. dextranicum 688 utilizes some of the acetaldehyde produced when grown in combination with S. diacetilactis M21-35 but does not utilize acetaldehyde when grown in combination with S. diacetilactis DRC-1 (sample 7).

Microbial Shift Occurring When a Commercial Mixed-Strain Butter Culture Develops a Green Flavor

The cause of the green flavor defect in mixed-strain butter cultures has been attributed primarily to an overgrowth of S. diacetilactis (15). It has been reported that the addition of 0. 2 percent citrate to skim milk causes a dominance of S. diacetilactis over S. cremoris and S. lactis (23, 61). This dominance was attributed to the ability of S. diacetilactis to utilize citrate as an additional carbon source and possibly involved antibiotic production and greater acid tolerance (23). DeMann and Galesloot (12) have shown that seasonal variations in the manganese content of milk may cause, in part, fluctuations in the numbers of Leuconostoc organisms. These workers were able to prevent variation in the numbers of Leuconostoc organisms by the addition of 0. 05 ppm of Mn^{++} to the medium.

According to Galesloot (15), a small percentage of inoculum and an increased incubation temperature coupled with transfers at

low acidity favor a shift in the aroma bacteria population of butter cultures toward S. diacetilactis, while the opposite method of propagation favors increases in the number of Leuconostoc organisms. However, Lindsay (20, p. 186-188) has shown that at least some commercial mixed-strain butter cultures containing S. diacetilactis must be transferred at low acidities to prevent the development of a green flavor.

Since the green flavor defect has been attributed to an overgrowth of S. diacetilactis, it was desirable to compare the numbers of various types of microorganisms present in a green culture with those found in a good flavored culture. The data obtained are presented in Table 9. The culture analyzed in series one was ripened to a titratable acidity of 0.76 percent. This culture was judged to have a good, well balanced flavor by three experienced judges. The culture represented in series two was subcultured from the one used in series one; the first subculture was ripened to a titratable acidity of 0.85 percent and then transferred and ripened to a titratable acidity of 0.76 percent. This culture was criticized as having a distinct green flavor by three experienced judges.

The most striking difference evident from the results obtained (Table 9) was the higher percentage of S. diacetilactis organisms and correspondingly lower percentage of S. cremoris in the green culture. From these results it appears that S. diacetilactis partially overgrows

Table 9. Percent distribution of lactic organisms in a good and green flavored commercial mixed-strain butter culture

Classification	Acid coagulation of milk in 48 hr	King's Test	Number of colonies	Percent of total colonies
Series 1[a]				
S. cremoris[b]	+	-	59	68.6
S. cremoris var. *aromaticus*	+	±	0	0
S. diacetilactis	+	+	26	30.2
Leuconostoc sp.[c]	-	+	1	1.2
		Total count = 59×10^7 per ml		
Series 2[d]				
S. cremoris	+	-	50	52.1
S. cremoris var. *aromaticus*	+	±	3	3.1
S. diacetilactis	+	+	42	43.8
Leuconostoc sp.	-	+	1	1.0
		Total count = 90×10^7 per ml		

[a]Culture had titratiable acidity of 0.76 percent, 6.73 ppm diacetyl and 1.28 ppm acetaldehyde.

[b]*S. cremoris* differentiated from *S. lactis* by the method of Mikolajcik (37).

[c]Positive King's Test after acidification of 48 hr culture with sterile 20 percent phosphoric acid to pH 4.5 and subsequent six hour incubation at 30° C.

[d]Culture had titratiable acidity of 0.76 percent, 2.09 ppm diacetyl and 4.60 ppm acetaldehyde.

S. cremoris. No other difference is sufficiently large to interpret with any degree of certainty. It is generally believed in the dairy industry that most of the homofermentative lactic streptococci found in a commercial mixed-strain butter culture are species of S. cremoris. In this study, all of the organisms classified as either S. lactis or S. cremoris were further characterized by the ability of the former to hydrolyze arginine (53). It was found that all of the homofermentative lactic streptococci in this culture were strains of S. cremoris.

Green Flavor Removal from a Ripened Culture

One of the major problems in the manufacture of cultured buttermilk is the development of a green flavor defect in this product. Once this defect develops, there is little that can be done for the product. The manufacture of cultured buttermilk involves adding from one to five percent of a lactic culture to pasteurized skim milk and incubating at 70° F (21° C) until the acidity develops to about 0. 75 percent (47, p. 58). A green flavor is not always perceptible until late in the culturing period.

It was desirable during the course of this investigation to determine if adding a Leuconostoc culture to a ripened lactic culture would remove some of the acetaldehyde causing the green flavor. After several trials where two percent of a L. citrovorum 91404 culture

was added to a 10-12 hr single-strain lactic culture with no success, it became obvious that a higher L. citrovorum inoculum was needed to effectively lower the acetaldehyde concentration. To determine what percentage of a culture of L. citrovorum must be added to effectively lower acetaldehyde concentration, single-strain cultures of S. cremoris 459 and S. diacetilactis DRC-1 were incubated for 12 hr at 21° C, after which each was split into five aliquots. One of these aliquots of each culture was held as a control. To the other four aliquots of each culture was added 5, 10, 25, and 50 percent respectively of a 16 hr L. citrovorum 91404 culture. These aliquots were then reincubated at 21° C, acetaldehyde and diacetyl concentrations were determined at four hour intervals. Results of this study are presented in Table 10. As can be seen from the data, an addition of 50 percent of L. citrovorum 91404 utilized almost all of the acetaldehyde produced by S. cremoris 459 and more than half of that produced by S. diacetilactis DRC-1. An addition of 25 percent of the L. citrovorum culture removed some of the acetaldehyde, being more effective in combination with S. cremoris 459 than with S. diacetilactis DRC-1. Using an inoculum of five or ten percent of the Leuconostoc culture removed only a small amount of acetaldehyde.

The ability of L. citrovorum 91404 to remove acetaldehyde from a ripened lactic culture under refrigeration conditions was determined. Two cultures, S. cremoris 459 and S. diacetilactis DRC-1

Table 10. Acetaldehyde removal from a 12 hr single-strain lactic culture by L. citrovorum 91404[a] at 21°C.

Culture	Incubation at 21°C (hr)	pH	0 percent		5 percent		10 percent		25 percent		50 percent	
			Acetaldehyde (ppm)	Diacetyl (ppm)	Acetaldehyde (ppm)	Diacetyl (ppm)	Acetaldehyde (ppm)	Diacetyl (ppm)	Acetaldehyde (ppm)	Diacetyl (ppm)	Acetaldehyde (ppm)	Diacetyl (ppm)
S. cremoris 459[c]	0	4.80	5.81	---	5.13	0	5.25	0	4.38	0	3.63	0
	4	4.55	6.38	---	5.63	0.10	6.13	0.10	5.43	0.18	4.35	0.18
	8	4.48	5.75	---	5.13	0.10	4.88	0.08	3.90	0.32	1.65	0.33
	12	4.30	5.50	---	4.63	0.21	4.50	0.23	2.70	0.68	0.33	0.91
S. diacetilactis DRC-1[d]	0	5.16	6.83	---	6.63	2.64	6.38	2.78	6.30	2.32	4.93	2.82
	4	4.80	7.38	---	6.95	2.46	6.50	2.00	6.08	1.96	4.13	1.46
	8	4.54	7.50	---	7.05	2.28	6.13	2.05	4.83	1.73	3.30	1.36
	12	4.52	7.30	---	6.70	2.36	6.00	2.14	4.88	2.55	2.13	1.55

[a]Total plate count = 30 x 10^6 organisms per ml for culture added to S. cremoris 459; total plate count = 14 x 10^5 per ml for culture added to S. diacetilactis DRC-1

[b]Incubation time after L. citrovorum 91404 was added

[c]Total plate count = 190 x 10^7 organisms per ml.

[d]Total plate count = 200 x 10^7 organisms per ml.

were incubated for 12 hr at 21° C, after which each was split into two aliquots. To one aliquot of each was added 50 percent of a 16 hr L. citrovorum 91404 culture. All aliquots were then refrigerated at 5° C. Acetaldehyde and diacetyl values were determined at the initiation of the trial and then after 6, 12, 24, and 36 hr of incubation at 5° C. Results from this study are presented in Table 11. The Leuconostoc organisms reduced the acetaldehyde concentration in the S. diacetilactis DRC-1 culture from 6. 08 ppm to 1. 75 ppm in 36 hr at 5° C. The Leuconostoc organisms were much less effective in removing acetaldehyde from the S. cremoris 459 culture, decreasing the acetaldehyde concentration from 6. 75 ppm to 4. 00 ppm in 36 hr.

The effect of neutralizing a ripened lactic culture before the addition of L. citrovorum 91404 to reduce the acetaldehyde concentration was determined. Results of this study (Table 12) indicate that neutralizing a ripened single-strain lactic culture does not decrease the levels of L. citrovorum needed to effectively reduce the acetaldehyde concentration. From the results presented in Table 12, it can be seen that the addition of 10 or 25 percent of a culture of L. citrovorum 91404 to a 12 hr S. cremoris 459 culture removed little of the acetaldehyde on continued incubation for eight hours.

On the basis of the results presented (Tables 10, 11, and 12) it can be concluded that it is possible to remove a green flavor from a ripened lactic culture. It is also obvious that it takes a large addition

Table 11. Acetaldehyde removal from a 12 hr single-strain lactic culture by L. citrovorum 91404[a] at 5° C

Culture	Incubation time at 5° C (hr)[b]	pH	Amount L. citrovorum added: 0 percent Acetaldehyde (ppm)	0 percent Diacetyl (ppm)	50 percent Acetaldehyde (ppm)	50 percent Diacetyl (ppm)
S. diacetilactis DRC-1[c]	0	4.84	8.00	1.00	6.08	2.82
	6	4.80	7.58	1.50	4.68	1.59
	12	4.75	7.55	1.34	3.93	1.36
	24	4.75	9.50	1.66	2.90	1.27
	36	4.75	9.50	1.14	1.75	1.16
S. cremoris 459[d]	0	4.70	6.93	---	6.75	0
	6	4.58	6.83	---	6.13	0.11
	12	4.51	6.40	---	5.30	0.12
	24	4.51	6.88	---	4.95	0.35
	36	4.51	6.35	---	4.00	0.55

[a] Total plate count = 44×10^6 organisms per ml.

[b] Incubation time after addition of L. citrovorum 91404.

[c] Total plate count = 300×10^7 organisms per ml.

[d] Total plate count = 200×10^7 organisms per ml.

Table 12. Acetaldehyde removal by L. citrovorum 91404[a] from a neutralized 12 hr S. cremoris 459[b, c] culture

Incubation time at 21°C (hr)[b]	pH	Amount of L. citrovorum 91404 added				
		0 percent	10 percent		25 percent	
		Acetaldehyde (ppm)	Acetaldehyde (ppm)	Diacetyl (ppm)	Acetaldehyde (ppm)	Diacetyl (ppm)
0	6.50[d]	6.30	7.88	0	5.65	0
4	4.71	7.88	8.58	0.04	7.20	0.06
8	4.56	7.75	7.45	0.05	5.93	0.10

[a] Total plate count = 56×10^6 organisms per ml.

[b] Total plate count of control = 120×10^7 organisms per ml.

[c] Culture pH adjusted to 6.5 with sterile 20 percent sodium hydroxide.

[d] pH before neutralization was 5.16.

of a Leuconostoc culture to effectively remove acetaldehyde from the ripened culture. The addition of small amounts of a culture of L. citrovorum to a lactic culture had little effect on the acetaldehyde content of the ripened culture. The most probable reason for this is that the L. citrovorum population is very low in relation to the population of lactic acid bacteria. These few Leuconostoc cells are probably rapidly overgrown by the Streptococci, which are actively metabolizing while the Leuconostocs must adapt to growth under these new conditions. Another plausible reason for this failure of low levels of L. citrovorum to remove acetaldehyde is that the lactic acid streptococci may produce inhibitory substances which curtail the growth and multiplication of Leuconostocs. It is well-known that some lactic streptococci do produce inhibitory substances. There may also be a question of strain compatibility, although incompatibility is generally believed to be due to inhibitory substances (51).

It would be impractical for a manufacturer to add high levels of a Leuconostoc culture to a vat of ripened buttermilk to remove a green flavor. However, it may be possible to add substances that are either simulatory to Leuconostocs or inhibitory to lactic acid streptococci to reduce the levels of Leuconostocs required to remove acetaldehyde.

SUMMARY AND CONCLUSIONS

The production of acetaldehyde by single-strain cultures of S. lactis, S. cremoris, and S. diacetilactis was found to parallel the increase in microbial population. S. lactis and S. cremoris were found to remove some of the acetaldehyde produced on continued incubation at 21° C. S. diacetilactis did not remove any of the acetaldehyde produced. The ratio of diacetyl to acetaldehyde in both strains of S. diacetilactis studied was found to be unfavorable for a desirable culture flavor at all times up to 22-24 hr incubation. All of the cultures studied produced a distinct green flavor when grown in a milk medium.

All strains of the microorganisms studied were found to produce ethanol when grown in a boiled milk medium. The amount of ethanol produced varied widely among different strains. All strains of the microorganisms studied produced significant quantities of acetone when grown in a boiled milk medium. The strains of S. diacetilactis employed showed no evidence of acetone utilization when grown in this medium.

Single-strain cultures of L. dextranicum and L. mesenteroides were shown to be capable of utilizing added acetaldehyde. The amount of acetaldehyde utilized varied widely depending on the culturing conditions.

Several two-strain mixtures, containing one strain of a Leuconostoc sp. and one strain of a lactic streptococci, were formulated to study acetaldehyde and diacetyl production at 21° C. The production and utilization of these compounds varied widely between different mixtures.

The ratio of different lactic organisms in a good-flavored commercial butter culture was determined using well-known methods to classify individual microorganisms. The microbial shift occurring when this culture developed a green flavor was found to be a partial overgrowth of the homofermentative lactic streptococci by the S. diacetilactis population.

Some success was achieved in removing acetaldehyde from a ripened single-strain lactic culture by adding a large inoculum of a single-strain L. citrovorum culture and continuing incubation. This L. citrovorum culture was capable of decreasing the acetaldehyde concentration in a ripened lactic culture held at either 5 or 21° C. Neutralizing the ripened lactic culture before the addition of L. citrovorum did not significantly increase the rate of acetaldehyde utilization.

The following conclusions were drawn from the findings of this investigation:

1. The production of acetaldehyde by the lactic acid bacteria studied paralleled the increase in microbial population.

2. Some homofermentative lactic streptococci are capable of utilizing some of the acetaldehyde they produce.
3. All of the lactic acid bacteria studied produced both acetone and ethanol, the amount of the latter compound varied widely in different strains of a species.
4. L. dextranicum and L. mesenteroides have the ability to utilize acetaldehyde, but neither utilizes it as rapidly as L. citrovorum.
5. One cause of a green flavor defect in mixed-strain butter cultures is a partial overgrowth of the homofermentative lactic streptococci population by S. diacetilactis.
6. High levels of L. citrovorum remove acetaldehyde from a ripened single-strain lactic culture at both 5 and 21° C.

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