AN ABSTRACT OF THE THESIS OF

Masahiko Yamada for the degree of Master of Science in Microbiology presented on January 27, 1989.

Title: Studies on Roles of Lactic Acid Bacteria and Yeast in the Flavor of Bakery Products.

Redacted for Privacy

Abstract approved: _____
W. E. Sandine

Roles of lactic acid bacteria in flour pre-ferment and white bread were investigated. Volatile compounds produced by the organisms were identified using gas-liquid chromatography (GLC).

Lactic acid bacteria were isolated from domestic commercial compressed yeast and active dry yeast. Numbers per gram of sample were 10^8 to 10^9 in compressed yeast and 10^4 in active dry yeast. These lactic acid bacteria were identified by physiological and fermentation characteristics. Commercial yeast preparations were found to contain both homo- and heterofermentative lactobacilli, and Leuconostoc mesenteroides. Breads with or without lactic acid bacteria were prepared and analyzed by GLC for volatile compounds present. Lactic acid bacteria in compressed yeast were found to contribute at least to the production of acetic acid.

Dough like-preparations for conversion to pre-ferments were inoculated with combinations of yeast and different types of lactic acid bacteria to investigate the behaviors of these organisms. The

pre-ferments were considered useful flavor enhancers for bakery products. From results of GLC analyses, it was found that by adding certain lactic acid bacteria to the pre-ferment with yeast, the content of volatile compounds produced was changed dramatically. Pre-ferments inoculated with different lactic acid bacteria but without yeast also were examined for volatile compounds produced. Each strain produced characteristic metabolites in the pre-ferment. Lactococcus diacetylactis 18-16, which produced an elevated amount of diacetyl in the pre-ferment without yeast, did not produce a significant amount of the compound in pre-ferment with yeast. Cells of the bacterium added directly to sponge dough of bread increased the concentrations of acetoin and acetic acid present, but not diacetyl.

Since the column packing material used in this study was found to be very suitable for the analysis of volatile compounds, direct injection of cultures on GLC column was carried out to determine the compounds produced by Leuconostoc strains. Most citrate-utilizing strains of Leuconostoc did not produce diacetyl or acetoin in modified MRS or acidified milk cultures, but did in citrate solutions. When the citrate-utilizing strains did produce diacetyl and acetoin, the amounts of ethanol produced by them were always small.

Studies on Roles of Lactic Acid Bacteria and Yeast in the Flavor of Bakery Products

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed January 27, 1989

Commencement June 1989

APPROVED:

Redacted for Privacy

Professor of Microbiology in charge of major

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Date thesis is presented January 27, 1989

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ACKNOWLEDGEMENTS

I wish to express appreciation to my major professor, Dr. W. E. Sandine, for his generous guidance and assistance throughout this study.

I am grateful to Dr. T. F. Sugihara of Western Regional Research Center, U. S. Department of Agriculture, Berkeley, CA, who introduced Dr. W. E. Sandine to me and gave me the topic of Chapter 1 of this thesis.

I wish to thank my employer, Shikishima Baking Co., Ltd., for financial support.

Finally, I thank all my friends of Dairy Microbiology Laboratory who always helped me and let me enjoy studying here.

To my wife, Satoko, whose
love, generosity and assistance
let me achieve this study.

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INTRODUCTION

There were three main objectives to the research reported in this thesis and each is considered in a separate chapter. These objectives were as follows:

Chapter 1. Isolation and identification of lactic acid bacteria contaminating commercial baker's yeast preparations and investigation of their role on volatile compounds in bread.

Chapter 2. Investigation of microbiological changes and volatile compounds found in dough-like pre-ferments inoculated with yeasts and lactic acid bacteria.

Chapter 3. Detection of volatile compounds produced by Leuconostoc using gas-liquid chromatography.

In this text, the following abbreviations are used:

Lb. = Lactobacillus

Lc. = Lactococcus

Lu. = Leuconostoc

DMF = dimethyl fumarate

GLC = gas-liquid chromatography

CHAPTER 1

IDENTIFICATION OF LACTIC ACID BACTERIA ISOLATED FROM COMMERCIAL YEAST PREPARATIONS AND THEIR ROLE ON VOLATILE COMPOUNDS IN BREAD

ABSTRACT

Lactic acid bacteria were isolated from two brands of compressed yeast and one brand of active dry yeast. Numbers per gram of sample were from 10^8 to 10^9 in compressed yeast and 10^4 in active dry yeast. These lactic acid bacteria were identified by physiological and fermentation characteristics. It was found that commercial baker's yeast contained different types of Lactobacillus, both homo- and heterofermentative, and Leuconostoc. Leuconostoc(Lu.) mesenteroides strains were found in all samples.

Bread experiments showed that lactic acid bacteria in compressed yeast lowered the pH of bread dough. Gas-liquid chromatography (GLC) detection of volatile compounds in breads indicated that these bacteria contributed at least to the production of acetic acid.

INTRODUCTION

In yeast manufacture, baker's yeast is propagated with primary raw material, molasses and certain nutritive chemicals. Molasses and nutritive chemicals are fed into a fermenter at a controlled rate with aeration so that the sugar is consumed for propagation of new cells instead of being underutilized by fermentation.

Before propagation, molasses is sterilized by heating, and air is filtered or heated to prevent contamination from either source. However, baker's yeast preparations contain a large number of bacteria. Carlin (1958) reported that compressed yeast contained bacteria at 2 to 3 x 10^9 cells/g. Robinson et al. (1958) analyzed bacterial counts of a pre-ferment with and without compressed yeast and found that compressed yeast carried most of the bacteria in the pre-ferment. These bacteria were mostly lactic acid bacteria of the Lactobacillus and Leuconostoc genera. Some coliform bacteria, usually Enterobacter aerogenes and occasionally Escherichia coli, also were found (Reed and Peppler, 1973). Fowell (1967) reported on a method to detect bacteria in compressed yeast for use in quality control. In his method, lactic acid bacteria were enumerated using MRS agar as plating medium. His results indicated that compressed yeast contained lactic acid bacteria, mainly of the Lactobacillus genus. Takeda et al. (1984) isolated lactic acid bacteria from compressed yeast and active dry yeast in Japan and identified them at the species level. They also found that most were lactic acid bacteria belonging to the Lactobacillus genus.

In the United States, the species of lactic acid bacteria found in commercial baker's yeast preparations have never been described.

Although lactic acid bacteria in baker's yeast preparations are contaminants, they may be beneficial because of their potential to suppress the activities of gram-negative bacteria and also to contribute to bread flavors. The contribution of lactic acid bacteria in compressed yeast to bread flavor has been considered in published reports but still is unclear. Carlin (1958) determined the flavor scores of breads made using laboratory derived pure cultures of yeasts with or without lactic acid bacteria and compared the scores to bread made using commercial compressed yeast preparation as a control. He found that adding lactic acid bacteria to pure yeast cultures elevated flavor scores of bread as compared to the product made with only pure yeast. However, scores for bread samples made using pure yeasts plus lactic acid bacteria were lower than the control. He concluded that lactic acid bacteria in compressed yeast were desirable for the flavor of white bread. According to Sugihara (1985), lactic acid bacteria in compressed yeast contribute to much of the flavor of white bread. Kohn et al. (1961) analyzed carbonyl compounds present in dough which was fermented by yeasts containing very low (2.0×10^1/g) numbers of bacteria. He found that even with low bacterial counts, the amounts of carbonyl compounds present were not different from those found in dough fermented with commercial yeast preparations with high (1.3×10^6) numbers of bacteria present. GLC detection of volatile compounds in bread have been carried out by many investigators (Maga, 1974) to analyze bread flavors, and over 100 flavor and

aroma compounds have been detected. Those found have included organic acids, esters, aldehydes, ketones, and alcohols.

In the present study, domestic yeast preparations were examined to determine the number and types of lactic acid bacteria present. GLC analyses also were conducted to determine possible roles for these bacteria in contributing to volatile compounds in bread.

MATERIALS AND METHODS

Samples

Samples of commercial products were compressed yeast and active dry yeast. Compressed yeast samples were one-pound packages obtained from local bakeries. Two brands of compressed yeast were used. Samples of Fleischmann's compressed yeast were from two packages, each obtained at different times, and that of Budweiser compressed yeast was from one package. The samples of active dry yeast also were the Fleischmann brand and were obtained from a local supermarket.

Number of yeast and lactic acid bacteria in commercial baker's yeast

Yeast cells per gram of sample were detected by serial dilution using spread plates employing Bacto YM (Difco) agar incubated aerobically at 30°C. The medium for isolation of lactic acid bacteria was MRS (Difco) agar supplemented with 0.5% $CaCO_3$ and 500 ppm dimethyl fumarate (DMF), adjusted to pH 5.8. DMF was used as a component of the agar medium because laboratory data indicated that baker's yeast (Saccharomyces cerevisiae) was inhibited by this compound. Pour plates were incubated aerobically at 30°C for 3 days, and the number of colonies surrounded by clear zones were counted as lactic acid bacteria.

Identification of lactic acid bacteria

Strains of lactic acid bacteria from commercial baker's yeast were selected from different colonies surrounded with clear zones on the supplemented MRS agar plates of each sample. Strains were characterized by gram stain, shape, catalase test, production of gas from glucose, growth at 15°C and 45°C, production of dextran from sucrose, isomer of lactic acid produced and fermentation of carbohydrates.

Gas production was observed by transferring the strains into MRS broth containing durham tubes with an overlay of vaspar (vaseline:parafin = 1:1) and incubating at 30°C for 3 days. Growth at 15°C and 45°C was observed in MRS broth by incubating for 6 days. Sucrose agar plates were used for the observation of dextran formation. This agar contained, per liter: 10 g of tryptone, 5 g of yeast extract, 100 g of sucrose, 2.5 g of gelatine, 5 g of glucose, and 15 g of agar. The strains were streaked on the plates and incubated aerobically at 30°C for 4 days.

The amount and isomer of lactic acid produced were determined by lactic acid enzyme kit (Boehringer Mannheim Biochemicals). Strains were transferred into MRS broth and incubated at 30°C for 24 hr. Supernatants were obtained by centrifugation, and following membrane filtration, they were analyzed. Fermentation of carbohydrates was determined by API Rapid CH. Cells from MRS cultures were harvested by centrifugation, washed and resuspended in the medium for this test. Reactions were observed up to 48 hr.

Identification of the lactic acid bacteria was achieved by comparing the results with the information from Rogosa (1974) and Mitsuoka (1969).

Preparation of organisms for bread

Yeast used for bread manufacture was isolated from Fleischmann's compressed yeast. The method of propagation of the yeasts was based on the experiments by Hino et al. (1987). The medium for preculture contained, per liter: 100 ml of molasses (Grandma's molasses; Mott's USA), 2.8 g of $CO(NH_2)_2$, 1.0 g of $(NH_4)_2SO_4$, 0.4 g of KH_2PO_4, and 0.3 g of $MgSO_4 \cdot H_2O$. The medium (1,000 ml) was inoculated with the yeast and incubated at 30°C for 22 hr with shaking at 200 rpm. After propagation, the preculture was centrifuged, and the pellets used as the inoculum for bulk culture propagation. The bulk culture medium was prepared by dissolving 0.3 g of $MgSO_4 \cdot 7H_2O$ in 1,300 ml of water. Bulk propagation was carried out in a 2-L fermenter (Multigen Model F-2000; New Brunswick Scientific) at 30°C for 12 hr with the agitation at 250 rpm along with aeration. The pH of the medium was kept at 5.2 by adding 0.1 N NaOH automatically. During propagation, nutrient supplements (16% sugar) were added to the medium every 30 min. The nutrient supplements were prepared by mixing 10.7 g of $CO(NH_2)_2$, 3.9 g of $Na_2HPO_4 \cdot 12H_2O$, 333 ml of molasses (see above) and 666 ml of water. The volumes (v) of the nutrient supplements were determined from the formula $v = rA$. For r, the value of 0.16 g sugar/g wet yeast/hr was used. For A, which was the weight (g) of

yeast present in the medium at any given time, the information of White (1954) was used. For the last one hour, yeast propagation was carried out without adding the nutrient supplement. After growth, yeast cells were harvested by centrifugation, washed with water, and blotted dry with filter paper. The moisture content of the yeasts was 71.5%, measured by heating the yeast cells at 105°C for 4 hr and determining the dry weight by weighing. Yeasts were stored at 4°C for two days before bread experiments were begun. When lactic acid bacteria isolated from compressed yeast were added to bread, they were grown in MRS broth (Difco) at 30°C for 20 hr. Those used were Lactobacillus(Lb.) plantarum F7, Lb. casei F8 and Lb. fermentum B1.

Preparation of bread

The formulation for bread sponge dough was as follows: 350 g of flour, 0.25 g of NH_4Cl, 0.05 g of $CaCO_3$, 10 g of yeast, and 200 ml of water. When lactic acid bacteria were added to the sponge dough, cells from MRS cultures were harvested by centrifugation at 3,020 x g for 10 min, washed with water, and resuspended in water with yeast. The volumes of MRS cultures of lactic acid bacteria used were 3.5 ml for F7 and F8, and 17 ml for B1. The sponge dough was mixed using a Kitchen Aid mixer (Hobart). The mixing temperature was 25°C. Fermentation of the sponge dough was carried out in an incubator at 28°C for 4.5 hr. The additional ingredients for making the final dough were as follows: 150 g of flour, 25 g of sugar, 15 g of shortening, 10 g of salt, and 120 ml of water. The fermented sponge

dough and additional ingredients were mixed using the same mixer as for the sponge dough. The mixing temperature was 28°C. The final dough was kept at room temperature for 20 min, then cut into 390-g sections, rounded, and kept at room temperature for 10 min. After that, the rounded dough was sheeted and molded. The dough then was panned in an aluminum bread pan (10 cm x 20 cm x 6 cm) and proofed at 38°C for 50 min. After baking at 200°C for 25 min, the bread was cooled by placing at room temperature (25°C) for 4.5 hr.

Yeast and bacterial counts of bread

Yeast counts were detected using spread plates of Bacto YM agar. Bacterial counts were determined using spread plates of MRS agar supplemented with 500 ppm DMF. The agar plates were incubated aerobically at 30°C.

Preparation of samples of bread for gas-liquid chromatography

Bread was kept at -20°C for 15 min after cooling at room temperature, and the crust part was removed to a depth of a half inch from surface. A cubic bread crumb (30 g) was excised, placed into a Stomacher bag, and mixed by hand with 60 ml of cooled (4°C), double-distilled water to make a slurry-like mixture and with care to minimize aeration. The mixture then was centrifuged at 5,090 x g for 15 min and successively filtered with the Acrodisc 1.2 μm and 0.45 μm average pore diameter size filters (Gelman Science). The

filtrate was kept at -80°C and thawed when used as the sample for GLC.

Gas-liquid chromatographic analyses

Volatile compounds were detected by a GLC instrument (Model 5710A; Hewlett Packard) equipped with a flame-ionization detector. A reporting integrator (Model 3390A; Hewlett Packard) was connected to the chromatograph. The glass column (Supelco) used was 6 feet long, 1/4 inch OD and 2 mm ID. The packing used was 6.6% Carbowax 20M/ 80/120 Carbopack B (Supelco) (Di Corcia et al., 1980). A PureCol liner (Supelco) was inserted in the column inlet to avoid deterioration of the packing by nonvolatiles. The injector temperature was 170°C, the detector temperature 200°C, and the oven temperature programmed to increase from 90 to 130°C at 2°C/min. As the carrier gas, N_2 was used with a flow rate of 20 ml/min at 69 lb/in^2. H_2 at 22 lb/in^2 and air at 26 lb/in^2 were used as the combustible mixture in the flame ionization detector.

The standard solution was prepared by dissolving the volatile compounds in double distilled water; sec-butanol was used as the internal standard. Concentrations of volatile compounds present in the samples were measured by the Internal Standard calculation procedure for the integrator used. A 0.5 µl sample containing the internal standard at 49.5 ppm was injected directly into the gas chromatograph.

RESULTS

Identification of lactic acid bacteria from baker's yeast

Counts of yeasts and lactic acid bacteria in baker's yeast preparations are shown in Table 1.1. Fleischmann's compressed yeast contained lactic acid bacteria at 10^8 cells/g, and Budweiser at 10^9 cells/g. Fleischmann's active dry yeast contained lactic acid bacteria at 10^4 cells/g. Colonies surrounded with clear zones on MRS agar plates of each sample were selected and examined for amount and isomer of lactic acid produced so as to make sure that the strains were lactic acid bacteria. All strains produced lactic acid in MRS broth and the isomers produced varied by strains (Table 1.2). Strains then were examined for physiological and fermentation characteristics. All strains were gram-positive and catalase-negative. It was found that many strains were the same or very similar. Strains were divided into 6 types, each having identifying keys at the species level. The results of identification and carbohydrate reactions are shown in Table 1.3 and Tables 1.4 through 1.8 respectively.

Type I strains were identified as Lb. fermentum. Type II strains which produced dextran from sucrose were identified as Lu. mesenteroides. Type III strains resembled Lb. casei; however, they fermented xylose and other carbohydrates such that they were considered Lb. xylosus. Gilliland and Speck (1977) reported that sterile mineral oil which was used in the API Rapid CH method caused some results to be different from those of conventional

tubed media, and, therefore, it was not a reliable way to identify the strains. Consequently, this strain type was identified only as a Streptobacterium of the Lactobacillus genus.

The type IV strain was initially identified as Lb. plantarum. However, fewer carbohydrates were fermented so it seemed to be an atypical Streptobacterium producing DL-lactic acid. Therefore, this strain was considered either Lb. coryniformis subsp. coryniformis or Lb. curvatus.

Type V isolate was Lb. plantarum. Type VI was identified as Lb. casei, although the strain of type VI did not metabolize lactose in the API method and therefore seemed to be Lb. casei subsp. alactosus. As mentioned above, it is not reliable to identify a strain to the level of subspecies based on only the results of the API method. Therefore, the subspecies of this strain was not determined. These six or closely related species were distributed in the commercial yeast preparations as shown in Table 1.9.

Bread experiments

Breads were examined for pH, yeast counts, bacterial counts and volatile compounds produced to determine possible roles for lactic acid bacteria present in commercial compressed yeast on bread properties. Three breads were baked, one made with Fleischmann's compressed yeast, a second with only pure yeast which contained no lactic acid bacteria, and a third with the pure yeast and lactobacilli consisting of Lb. plantarum F7, Lb. casei F8

and Lb. fermentum B1 previously isolated from commercial compressed yeast.

The pH of bread made with only pure yeast cultures was higher than the other two breads at every stage (Table 1.10). Although bread with pure yeast and lactobacilli showed 10 times the initial bacterial counts and 5 times the final counts of those in bread made with Fleischmann's compressed yeast, the pH of both breads decreased to the same final level (pH 5.05 and 5.02 respectively).

Although all breads revealed stable or slightly increasing yeast counts at every stage, they had different bacterial counts (Table 1.10). The bread with only the pure yeast culture contained bacteria at no more than 10^3 cells/g. Bread made with Fleischmann's compressed yeast initially contained 4.4 x 10^6 bacterial cells/g and these came from the lactic acid bacteria contaminating the yeast preparation. The number of bacteria in the bread increased in the sponge dough but did not increase further after the final dough was mixed. Bread made with the pure yeast and three strains of Lactobacillus initially contained 3.6 x 10^7 bacterial cells/g; these decreased in the sponge dough and increased after the final dough was mixed. It also was noted that the three bacterial strains revealed different colony appearances on MRS agar plates: Lb. plantarum F7 colonies were yellow to orange and smooth, Lb. casei F8 colonies were jagged and Lb. fermentum B1 colonies were white and smooth. Therefore, approximate counts of each strain on the MRS agar plates could be obtained. In this regard, it was found that Lb. fermentum B1 cells decreased down to

<1.0 x 10^6/g at the final stage; on the other hand, Lb. plantarum F7 and Lb. casei F8 cells increased in number to the final stage (1.7 x 10^7 and 2.4 x 10^7, respectively).

Volatile compounds present in crumbs of the three bread types were analyzed by GLC (Table 1.11). Those detected in bread made with only the pure yeast were considered to have been produced by yeast and therefore also expected to be present in the other two bread types. Bread made with Fleischmann's compressed yeast contained diacetyl and, compared to bread made with only the pure yeast, almost the same concentrations of ethanol and propanol, less acetaldehyde and acetoin, and more isobutanol, isoamyl alcohol and acetic acid. Concentrations of all compounds, except acetoin and acetic acid, found in bread made with the pure yeast culture and lactobacilli were lower than those found in the bread made with only pure yeast.

DISCUSSION

It was found that commercial compressed yeast contained lactic acid bacteria at 10^8 to 10^9 cells/g. These counts were comparable to those reported by Carlin (1958) and Takeda et al. (1984). In the samples of Fleischmann's compressed yeast, different cell counts of lactic acid bacteria were seen batch-by-batch, but the relative order of the counts was the same. Budweiser compressed yeast contained more lactic acid bacteria than that of the Fleischmann's yeast. What factors caused these differences in cell counts were not determined. The Fleischmann's active dry yeast sample revealed higher yeast counts and lower lactic acid bacterial counts than the compressed yeast. Therefore, during the drying process, most lactic acid bacteria must have died.

From the identification results for lactic acid bacteria isolated from commercial yeast preparations, the following were noted:

1. Commercial yeast preparations contained a large number (10^8 to 10^9/g) of both homo and heterofermentative Lactobacillus, and also Leuconostoc.
2. Lactobacilli isolated did not include Thermobacteria.
3. Compressed yeast contained four different types of lactobacilli; Fleischmann's compressed yeast contained four while Budweiser yeast contained two types.
4. Type II, identified as Lu. mesenteroides, was found in all samples. Type III, Streptobacterium of the Lactobacillus genus, was found in three of four samples.

The source of these Lactobacillus and Leuconostoc contaminants of commercial yeast preparations has not been determined. It is possible that they came from molasses, other ingredients or contaminated equipment. Leuconostoc mesenteroides is often found in sugar cane and as a result it causes slime production in sugar factories (Imrie and Tilbury, 1972). Therefore, it is possible that this bacterium came from the molasses used in propagating the yeast in the manufacturing plant. Although contaminants, lactic acid bacteria in yeast preparations may be beneficial because of their potential to suppress the activities of gram-negative bacteria and also to contribute to bread flavors. In this regard, there are reports on the ability of lactobacilli to inhibit spoilage organisms, especially gram-negative bacteria (Gilliland, 1985).

To investigate the roles of lactic acid bacteria on volatile compounds in bread, bread made with Fleischmann's compressed yeast, made with only pure yeast or made with pure yeast and lactobacilli were examined. The bread with only pure yeast showed $<1.0 \times 10^3$ bacterial cells/g and higher pH values than the other two breads. From these results, the lactic acid bacteria in compressed yeast were considered responsible for lowering the pH of bread dough. The approximate cell numbers of each Lactobacillus strain in bread with pure yeast plus lactobacilli were obtained by observing the shape and color of their colonies on MRS agar plates. These observations revealed that the cells of Lb. fermentum B1 kept decreasing while those for the other two strains were increasing. Many strains of Lactobacillus have been reported to produce

products antagonistic for gram-positive and gram-negative bacteria (Gilliland, 1985). Lactobacillus plantarum F7 and/or Lb. casei F8 demonstrated this property by inhibiting the growth of Lb. fermentum B1.

Volatile compounds in crumbs of the three breads were analyzed by GLC. Since the column packing material used was developed for the analysis of alcoholic beverages, the volatiles were extracted with water. Although bread is known to contain over 100 flavor compounds, volatiles found in this study were the ones present in major amounts (Maga, 1974). Three samples contained the same compounds, except for diacetyl. These compounds also were found in bread crumbs analyzed by Hironaka (1985 a,b). They also found aldehydes other than acetaldehyde; however, in the present study, water was used for extraction and it may not have dissolved the aldehydes. Even if extracted, a wide peak of water would have interfered with the detection of these compounds. However, even if breads in this study contained aldehydes other than acetaldehyde, their concentrations must have been very low (Maga, 1974).

Among breads made with Fleischmann's compressed yeast and made with only pure yeast, the former contained higher concentrations of acetic acid and isobutanol. The higher concentration of acetic acid was considered due to the presence of lactic acid bacteria in the compressed yeast. Isobutanol is known to be produced from α-keto acid which is formed from sugar or amino acid by yeast, and therefore its higher concentration in the former sample was believed to be due to the presence of yeast but not

lactic acid bacteria. The former sample contained diacetyl, but it was not determined whether or not this compound was produced by lactic acid bacteria present in the compressed yeast. The reason why the former sample contained almost half the concentration of acetaldehyde compared to the later might relate to the reducing activity of heterofermentative lactic acid bacteria present in the compressed yeast. These bacteria were reported to reduce acetaldehyde to ethanol (Keenan, 1968).

Among bread samples made with only pure yeast and bread made with pure yeast and lactobacilli, the latter contained much lower concentrations of volatile compounds except for acetoin and acetic acid. These lower concentrations were considered due to the reduced metabolic activities of yeast. It seemed that the lactobacilli had an inhibitory action against yeast, perhaps by competing for an energy source or by producing antagonistic compounds. Even with the reduced yeast metabolic activities, bread made with these two organisms contained higher concentrations of acetoin and acetic acid than bread made with only the pure yeast culture. Therefore, lactobacilli present in the bread were responsible for production of these compounds.

From the results obtained with the three types of breads, it was apparent that the lactic acid bacteria present in compressed yeast contributed at least to the production of acetic acid. In bread made with the pure yeast and lactobacilli, the growth of Lb. fermentum B1, which had the ability to produce acetic acid, was inhibited by the other lactobacilli. Had this not occurred, this bacterium would have produced more acetic acid.

Since all samples of commercial yeast preparations were found to contain Lu. mesenteroides and one, Budweiser compressed yeast, to contain Lb. fermentum, these heterofermentative lactic acid bacteria likely produced the acetic acid which was potential to contribute to bread flavor. The roles of homofermentative lactic acid bacteria were less clear. Schulz (1966) reported that during the sour rye fermentation, heterofermentative lactic acid producers played more important roles than homofermentative species. According to Sugihara (1985), heterofermentative lactobacilli are of major importance in the fermentation of many types of bakery goods. In the manufacture of white bread, it is likely that suitable manipulation of the ratio between homofermentative and heterofermentative lactic acid bacteria could be utilized to enhance and make more uniform desirable product flavor.

Table 1.1. Numbers of yeasts and lactic acid bacteria found in commercial baker's yeast when aliquots were plated on YM and MRS agar respectively, and incubated at 30°C for 3 days.

Sample	Yeast (CFU[a]/g)	Lactic bacteria (CFU/g)
Fleischmann's compressed yeast 1st sample	1.6×10^{10}	6.0×10^{8}
Fleischmann's compressed yeast 2nd sample	2.0×10^{10}	3.2×10^{8}
Budweiser compressed yeast	2.1×10^{10}	2.0×10^{9}
Fleischmann's active dry yeast	5.2×10^{10}	1.5×10^{4}

[a] Colony Forming Unit

Table 1.2. Average amount[a] of lactic acid produced by lactic acid bacterial isolates grown in MRS broth at 30°C for 24 hr.

Source	Strain	L-lactate (g/l)	D-lactate (g/l)	Total lactate (g/l)
Fleischmann's compressed yeast 1st sample	F3	6.53	0.00	6.53
	F4	2.18	7.01	9.19
	F6	0.00	7.86	7.86
	F7	3.49	7.63	11.12
	F8	10.25	0.00	10.25
Fleischmann's compressed yeast 2nd sample	F11	0.00	5.57	5.57
	F12	7.14	0.00	7.14
	F13	0.00	7.07	7.07
Budweiser compressed yeast	B1	2.91	4.32	7.23
	B4	6.69	0.00	6.69
	B5	0.00	7.76	7.76
	B6	3.59	3.80	7.39
	B8	3.04	4.29	7.33
Fleischmann's active dry yeast	FADY3	0.00	5.47	5.47
	FADY4	0.00	6.78	6.78

[a] Data were from three trials.

Table 1.3. Identification of lactic acid bacteria isolated from commercial yeast preparations.

Type	Strain	Characteristics	Identity
I	B1 B6 B8	Rod Heterofermentative Growth at 15°C:-, 45°C:+ Isomer of lactic acid: DL	Lactobacillus fermentum
II	F6 F11 F13 B5 FADY3 FADY4	Cocci or coccoidal rod Heterofermentative Production of dextran Growth at 15°C:+, 45°C:- Isomer of lactic acid: D	Leuconostoc mesenteroides
III	F3 F12 B4	Rod Homofermentative Growth at 15°C:+, 45°C:- Isomer of lactic acid: L	Streptobacterium of Lactobacillus genus
IV	F4	Rod Homofermentative Growth at 15°C:+, 45°C:- Isomer of lactic acid: DL	Lactobacillus coryniformis subsp. coryniformis or Lactobacillus curvatus
V	F7	Rod Homofermentative Growth at 15°C:+, 45°C:- Isomer of lactic acid: DL	Lactobacillus plantarum
VI	F8	Rod Homofermentative Growth at 15°C:+, 45°C:- Isomer of lactic acid: L	Lactobacillus casei

Table 1.4. Carbohydrate reaction of Type I strains determined by API method.

Carbohydrate	Lb. fermentum[a]	Type I strains		
		B1	B8	B6
Gluconate	+	+	+	+
Arabinose	d	+	+	+
Xylose	d	-	-	-
Rhamnose	-	-	-	-
Sorbose	-	-	-	-
Ribose	+	+	+	+
Glucose	+	+	+	+
Mannose	w[b]	+	+	+
Fructose	+	+	+	+
Galactose	+	+	+	+
Sucrose	+	+	+	+
Maltose	+	+	+	+
Cellobiose	-	-	-	-
Lactose	+	-	-	+
Trehalose	d[c]	+	+	+
Melibiose	+	-	-	+
Raffinose	+	-	-	+
Melezitose	-	-	-	-
Starch	-	-	-	-
Mannitol	-	-	-	-
Sorbitol	-	-	-	-
Esculin	-	-	-	-
Salicin	-	-	-	-
Amygdalin	-	-	-	-

[a] From Rogosa (1974). [b] Weak reaction. [c] Some strains +, others -.

Table 1.5. Carbohydrate reaction of Type II strains determined by API method.

Carbohydrate	Lu. mesenter.[a]	Type II strains					
		F11	FADY3	F13	FADY4	B5	F6
Gluconate		-	-	-	+	+	-
Arabinose	+	-	-	-	-	-	-
Xylose	d[b]	+	+	+	+	+	+
Rhamnose		-	-	-	-	-	-
Sorbose		-	-	-	-	-	-
Ribose	+	-	-	+	-	+	+
Glucose	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+
Galactose	+	-	-	-	+	+	+
Sucrose	+	+	+	+	+	+	+
Maltose	+	-	-	+	+	+	+
Cellobiose	d	-	-	-	-	+	+
Lactose	d	-	-	-	+	-	-
Trehalose	d	+	+	+	+	+	+
Melibiose	d	+	+	+	+	+	+
Raffinose	d	+	+	+	+	+	+
Melezitose		-	-	-	-	-	-
Starch		-	-	-	-	-	-
Mannitol	d	-	-	-	-	-	-
Sorbitol		-	-	-	-	-	-
Esculin	+	-	-	+	-	+	+
Salicin	d	-	-	-	-	-	-
Amygdalin	d	-	-	-	-	-	-

[a] From Rogosa (1974). [b] Some strains +, others -.

Table 1.6. Carbohydrate reaction of Type III strains determined by API method.

Carbohydrate	Lb. xylosus[a]	Type III strains		
		B4	F12	F3
Gluconate	+	+	+	-
Arabinose	-	+	+	-
Xylose	+	+	+	+
Rhamnose	-	-	-	-
Sorbose	-	-	-	-
Ribose	+	+	+	+
Glucose	+	+	+	+
Mannose	+	+	+	+
Fructose	+	+	+	+
Galactose	+	+	+	+
Sucrose	+	-	+	-
Maltose	+	+	+	+
Cellobiose	+	+	+	+
Lactose	-	+	-	+
Trehalose	+	+	+	+
Melibiose	-	-	-	-
Raffinose	-	-	-	-
Melezitose	-	-	-	-
Starch	+	+	+	+
Mannitol	+	+	+	+
Sorbitol	-	-	-	-
Esculin	-	+	+	+
Salicin	+	+	+	+
Amygdalin	+	+	+	+

[a] From Rogosa (1974).

Table 1.7. Carbohydrate reaction of Type IV strain determined by API method.

Carbohydrate	Lb. curvatus[a]	Type IV strain F4	Lb. coryniformis[a]
Gluconate	+	-	+
Arabinose	-	-	-
Xylose	-	-	-
Rhamnose	-	-	+
Sorbose		-	
Ribose	+	+	-
Glucose	+	+	+
Mannose	+	+	+
Fructose	+	+	+
Galactose	+	-	+
Sucrose	-	-	+
Maltose	+	+	+
Cellobiose	+	-	-
Lactose	d[b]	-	-
Trehalose	-	-	-
Melibiose	-	-	d
Raffinose	-	-	d
Melezitose	-	-	-
Starch		-	
Mannitol	-	+	+
Sorbitol	-	-	d
Esculin	+	-	d
Salicin	+	-	d
Amygdalin	-	-	-

[a] From Rogosa (1974). [b] Some strains +, others -.

Table 1.8. Carbohydrate reaction of Type V and VI strains determined by API method.

Carbohydrate	Type V		Type VI	
	Lb. plantarum[a]	F7	Lb. casei[a,b]	F8
Gluconate	+	+	+	+
Arabinose	d[c]	+	-	-
Xylose	d	-	-	-
Rhamnose	-	-	-	-
Sorbose	-	-	+	+
Ribose	+	+	+	+
Glucose	+	+	+	+
Mannose	+	+	+	+
Fructose	+	+	+	+
Galactose	+	+	+	+
Sucrose	+	+	d	+
Maltose	+	+	d	+
Cellobiose	+	+	+	+
Lactose	+	+	-	-
Trehalose	+	+	+	+
Melibiose	+	+	-	-
Raffinose	+	+	-	-
Melezitose	d	+	+	+
Starch	d	+	-	-
Mannitol	+	+	+	+
Sorbitol	+	+	+	+
Esculin	+	+	+	+
Salicin	+	+	+	+
Amygdalin	+	+	+	+

[a] From Rogosa (1974). [b] Lb. casei subsp. alactosus.
[c] Some strains +, others -.

Table 1.9. Species identification of lactic acid bacteria isolated from commercial baker's yeast preparations.

Sample	Type	Species
Fleischmann's compressed yeast 1st sample	II	Leuconostoc mesenteroides
	III	Lactobacillus sp. (Streptobacterium)
	IV	Lactobacillus coryniformis subsp. coryniformis[a]
	V	Lactobacillus plantarum
	VI	Lactobacillus casei
Fleischmann's compressed yeast 2nd sample	II	Leuconostoc mesenteroides
	III	Lactobacillus sp. (Streptobacterium)
Budweiser compressed yeast	I	Lactobacillus fermentum
	II	Leuconostoc mesenteroides
	III	Lactobacillus sp. (Streptobacterium)
Fleischmann's active dry yeast	II	Leuconostoc mesenteroides

[a] Or Lactobacillus curvatus.

Table 1.10. Temperature, pH, yeast and bacterial counts of breads made with different combinations of yeast and lactobacilli.

Test mode	Make stage and time (hours)		
	Sponge dough (0)	Sponge dough (4.5)	End ferment. (6.5)
Made with Fleischmann's compressed yeast			
Temperature (°C)	24.5	30.0	31.0
pH	5.76	5.15	5.02
Yeast counts (CFU[a]/g)	2.1×10^8	2.3×10^8	1.5×10^8
Bact. counts (CFU/g)	4.4×10^6	1.2×10^7	8.3×10^6
Made with only pure yeast culture			
Temperature	25.0	29.5	33.0
pH	5.61	5.44	5.30
Yeast counts	1.8×10^8	2.1×10^8	2.3×10^8
Bact. counts	$<1.0 \times 10^3$	$<1.0 \times 10^3$	$<1.0 \times 10^3$
Made with pure yeast plus lactobacilli			
Temperature	25.0	29.0	32.0
pH	5.67	5.28	5.05
Yeast counts	1.5×10^8	2.0×10^8	2.1×10^8
Bact. counts	3.6×10^7	2.3×10^7	4.1×10^7
Lb. plantarum F7	9.0×10^6	1.0×10^7	1.7×10^7
Lb. casei F8	1.7×10^7	1.0×10^7	2.4×10^7
Lb. fermentum B1	1.1×10^7	3.0×10^6	$<1.0 \times 10^6$

[a] Colony Forming Unit.

Table 1.11. Concentration[a] (w/w) of volatile compounds found in bread made with different combinations of yeast and lactobacilli.

Bread	Acet-ald. (ppm)	Ethyl alc. (%)	Dia-cetyl (ppm)	Prop. alc. (ppm)	I.but. alc. (ppm)	Acet-oin (ppm)	I.amyl alc. (ppm)	Acetic acid (ppm)
Made with Fleischmann's compressed yeast								
	5.8	0.56	1.0	6.6	31.2	53.8	7.9	188.9
Made with only pure yeast culture								
	10.1	0.61	ND	5.6	7.5	79.0	4.9	105.7
Made with pure yeast plus lactobacilli[b]								
	2.4	0.38	ND	3.8	4.2	111.4	1.3	138.9

[a] Average of three injections. [b] Lactobacillus plantarum F7, Lactobacillus casei F8 and Lactobacillus fermentum B1.
Abbrevation: Acetald. = acetaldehyde; Ethyl alc. = ethanol; Prop. alc. = propanol; I. but. alc. = isobutanol; I. amyl alc. = isoamyl alcohol; ND = not detected.

REFERENCES

Carlin, G. T. 1958. The fundamental chemistry of bread making I. Proc. Am. Soc. Bakery Engr.:56-63.

Di Corcia, A., R. Samperi, and C. Severini. 1980. Gas Chromatographic column for the rapid determination of congeners in potable spirits. J. Chromatogr. 198:347-353.

Fowell, M. S. 1967. Infection control in yeast factories and breweries. Process Biochem. 2:11-15.

Gilliland, S. E. and M. L. Speck. 1977. Use of Minitek system for characterizing lactobacilli. Appl. Environ. Microbiol. 33:1289-1292.

Gilliland, S. E. 1985. Role of starter culture bacteria in food preservation, p.175-185. In S. E. Gilliland (ed.), Bacterial Starter Cultures for Foods. CRC Press, Inc., Boca Raton, Fla.

Hino, A., H. Takano., and Y. Tanaka. 1987. New freeze-tolerant yeast for frozen dough preparations. Cereal Chem. 64:269-275.

Hironaka, Y. 1985a. Effect of fermentation conditions on flavor substances in French bread produced by the straight dough method. Nippon Shokuhin Kogyo Gakkaishi 32:486-492.

Hironaka, Y. 1985b. Effect of fermentation conditions on flavor substances in sweet baked goods produced by the sugar containing sponge dough method. Nippon Shokuhin Kogyo Gakkaishi 32:592-596.

Imrie, F. K. E. and R. H. Tilbury. 1972. Polysaccharides in sugar cane and its products. Sugar Technol. Rev. 1:291-361.

Keenan, T. W. 1968. Production of acetic acid and other volatile compounds by Leuconostoc citrovorum and Leuconostoc dextranicum. Appl. Microbiol. 16:1881-1885.

Kohn, F. E., L. Wiseblatt., and L. S. Fosdick. 1961. Some volatile carbonyl compounds arising during panary fermentation. Cereal Chem. 38:165-169.

Maga, J. A. 1974. Bread flavor. CRC Crit. Rev. Food Technol. 5:55-142.

Mitsuoka, T. 1969. Recent trends in the taxonomy of lactobacilli. J. of the Food Hygienic Society of Japan 10:147-156.

Reed, G. and H. J. Peppler. 1973. Yeast Technology. AVI Publishing Co., Westport, Conn.

Robinson, R. J., T. H. Lord, J. A. Johnson, and B. S. Miller. 1958. The aerobic microbiological population of pre-ferments and the use of selected bacteria for flavor production. Cereal Chem. 35:295-305.

Rogosa, M. 1974. Lactobacillus, p.576-593. In R. E. Buchanan and N. E. Gibbons (ed.), Bergey's Manual of Determinative Bacteriology, 8th ed. The Williams and Wilkins Co., Baltimore.

Schulz, A. 1966. Fundamentals of rye bread production. Baker's Digest 40(4):77-80.

Sugihara, T. F. 1985. The lactobacilli and streptococci: bakery products, p.119-125. In S. E. Gillilanbd (ed.), Bacterial Starter Cultures for Foods. CRC Press, Inc., Boca Raton, Fla.

Takeda, T., S. Okada, and M. Kozaki. 1984. Characteristics of lactic acid bacteria isolated from bread-dough, compressed-yeast and active dry-yeast. Nippon Shokuhin Kogyo Gakkaishi 31:642-648.

White, J. 1954. Yeast Technology. Champan and Hall, London.

CHAPTER 2

MICROBIOLOGICAL CHANGES AND VOLATILE COMPOUNDS FOUND IN DOUGH-LIKE PRE-FERMENTS INOCULATED WITH YEASTS AND LACTIC ACID BACTERIA

ABSTRACT

Dough-like preparations for conversion to pre-ferments were inoculated with combinations of yeast and different types of lactic acid bacteria and examined for pH, yeast counts, bacterial counts and volatile compounds produced. The pre-ferments were considered useful flavor enhancers for bakery products. From results of gas-liquid chromatography (GLC) analyses, it was found that by adding certain lactic acid bacteria to the pre-ferment with yeast, the content of volatile compounds produced was changed dramatically. Lactococcus(Lc.) diacetylactis 18-16, which produced an elevated amount of diacetyl in the pre-ferment without yeast, did not produce a significant amount of the compound in pre-ferment with yeast. Heterofermentative lactic acid bacteria mainly increased the concentrations of ethanol and/or acetic acid in the pre-ferments with yeast. Pre-ferments inoculated with different lactic acid bacteria but without yeast also were examined for volatile compounds produced. Each strain produced characteristic metabolites in the pre-ferment. Cells of Lc. diacetylactis 18-16 were added directly to sponge dough of bread to determine the behavior of the strain in bread. This organism increased the concentrations of acetoin and acetic acid in bread, but not diacetyl.

INTRODUCTION

Flavor intensity of bread is complex and is influenced by four factors: (a) ingredients, (b) yeast and bacterial fermentation products, (c) mechanical and/or biochemical degradations, and (d) thermal reaction products (Jackel, 1969). The flavor compounds of bread have been isolated and identified mainly to make synthetic fresh bread flavors. Because staling of bread is accompanied by the loss of fresh odor, synthetic bread flavors were expected to complement or compensate for the loss of odor. The production of synthetic bread flavor mixture, however, is difficult since identity of all the compounds making up the flavor have not yet been identified (Maga, 1974).

In the 1960s, development of short fermentation continuous-mixing processes stimulated intensive research on dough pre-ferments (Johnson and Miller, 1957). The pre-ferments are the mixtures of yeast, water, yeast foods, sugar, salt, and, in some case, nonfat dry milk or flour. The mixtures are allowed to ferment for a several hours and then added to dough. Although continuous-mixing processes have been phased out, pre-ferments sometimes have been added directly to conventional dough to eliminate the sponge process or used to supplement the deficiency of fermentation flavors of chemically leavened dough products (Jackel, 1963; Sharpell, 1985).

Many investigators have attempted to enhance bread flavor by combining pre-ferments with yeasts and selected microorganisms. Carlin (1958, 1959) isolated lactobacilli and

Leuconostoc species from compressed yeast and fermented them in "brew". He added the brew to dough and succeeded in achieving higher bread flavor scores as compared to controls not containing the brew. Robinson et al. (1958) evaluated the odor of bread made with pre-ferments containing yeasts and selected microorganisms which were isolated from pre-ferment, sponge dough and dairy cultures. The best flavor score was given to bread made with pre-ferments of Lactobacillus(Lb.) bulgaricus or Lb. bulgaricus plus buttermilk cultures.

Linko et al. (1960) reported the effect of several different bacteria on the amount of carbonyl compounds in pre-ferments. Results showed little effect on the amount of carbonyl compounds by any of the bacteria, except that Pediococcus cerevisiae increased the amount of propionaldehyde-acetone. Bundus et al. (1969) patented a method to produce synthetic bread flavor in cultured whey. They cultured yeast with non-toxic bacteria, such as group N streptococci (Lactococcus) or lactobacilli, in whey. It was claimed that specific combinations of these microorganisms promoted a synergistic effect to enhance bread flavor as compared to culture made with yeast alone. The cultured whey was dried and added to bread dough.

Diacetyl is an important flavor compound found in bread and it is believed to be produced by microorganisms in the dough. This compound is very desirable for many dairy products, such as cultured buttermilk, cottage cheese and sour cream (Sandine and Elliker, 1970). It is now known to be produced by citrate fermenting strains of Lactococcus lactis (formerly known as

Streptococcus diacetylactis and referred to in this thesis as Lactococcus diacetylactis), Leuconostoc species and some lactobacilli; some yeasts also have the ability to produce diacetyl. Visser't Hooft and deLeeuw (1935) suggested that diacetyl was largely responsible for bread flavor. They demonstrated that acetoin in bread dough was slowly oxidized to diacetyl, but because of its high volatility, little diacetyl accumulated. Baker (1957) ranked compounds for pleasant bread flavor in order of their probable importance and placed diacetyl as the top compound. Wiseblatt and Kohn (1960) made a synthetic bread flavor by mixing selected compounds found in fresh bread and examined the flavor fortification of chemically leavened bread. Diacetyl was selected as one of the important flavor compounds added to the synthetic bread flavor. On the other hand, Thomas and Rothe (1957) reported that acetoin and diacetyl were not important for bread flavor. The reason was that in their experiments, even though the content of acetoin and diacetyl increased during storage, it did not enhance bread flavor. Although the question about whether or not diacetyl is desirable for bread flavor has not been resolved, it at least seems that diacetyl has potential to be a flavor enhancer for bread and other bakery products.

In this research, pre-ferments formulated by combining yeasts and different types of lactic acid bacteria were examined for bacterial counts and volatile compounds produced. Because of its ability to produce diacetyl, Lc. diacetylactis was especially studied as a pre-ferment component. Also, the effect of Lc. diacetylactis directly added to bread dough was studied.

MATERIALS AND METHODS

Organisms

The strains used in this study are shown in Table 2.1. For the pre-ferment experiments, the yeast was grown in a 2-L fermenter (Multigen Model F-2000; New Brunswick Scientific). The medium for propagation of the yeast contained 40 g of YM broth (Difco) and 20 g of glucose dissolved in 1,300 ml of distilled water. The medium was inoculated with 5 ml from a YM broth culture of the yeast and fermented at 30°C for 20 hr with agitation at 170 rpm along with aeration. The pH of the medium was kept at 5.0 by adding 0.1N NaOH automatically. After propagation, the yeast cells were harvested by centrifugation and blotted dry with filter paper. The yeast cells were stored at 4°C.

Strains of lactic acid bacteria for the pre-ferment were grown in MRS broth (Difco) from a 1% inoculum at 30°C for 20 hr. Cells of each strain were harvested from the culture by centrifugation and added to the pre-ferment. Since each strain had a different growth rate, in order to equalize the initial bacterial counts to 10^8 cells/ml in the sample, the volume of each culture to be centrifuged for the pre-ferment was as follows: 25 ml for Lc. diacetylactis 18-16, Lc. cremoris, Lb. fermentum B1, Leuconostoc(Lu.) dextranicum 181, and Lu. mesenteroides cj-1, 5 ml for Lb. plantarum F7, and 2 ml for each culture of mixed strains. For pre-ferment experiments with the lactic acid bacteria alone, the volume of each culture centrifuged

was the same as above, except 5 ml was used for Lu. dextranicum 181 and Lu. mesenteroides cj-1.

For bread experiments, Lc. diacetylactis 18-16 was grown in MRS broth under same conditions as above. For this culture, 100 ml were centrifuged and the pellet added to the sponge dough.

Preparation of pre-ferment and bread

Ingredients for the pre-ferment were 50 g of flour, 7.5 g of nonfat dry milk, 2.5 g of glucose, 0.5 g of salt, 0.25 g of sodium citrate, 0.05 g of NH_4Cl, 0.01 g of $MnSO_4 \cdot H_2O$, 1 g (or 0 g) of yeast and/or harvested lactic acid bacteria, and 150 ml of distilled water. The ingredients, except yeast, lactic acid bacteria and water, were placed in a Stomacher bag. Yeast and harvested lactic acid bacteria were resuspended in water, added to the ingredients in the Stomacher bag and mixed by stomaching for 20 sec. The pre-ferments were incubated at 30°C for 8 hr.

The formulation for bread sponge dough was as follows: 280 g of flour, 16 g of nonfat dry milk, 1 g of sodium citrate, 0.2 g of NH_4Cl, 0.04 g of $MnSO_4 \cdot H_2O$, 8 g of Fleischmann's compressed yeast, and 160 ml of water. Cells of Lc. diacetylactis 18-16 were collected by centrifugation and resuspended in water with the yeast. The sponge dough was mixed using a KitchenAid mixer (Hobart). The mixing temperature was 24.5°C. Fermentation of the sponge dough was carried out in the incubator at 28°C for 4.5 hr. Additional ingredients for making the final dough were as follows: 120 g of flour, 20 g of sugar, 16 g of shortening, 8 g of salt, and 96 ml of

water. The fermented sponge dough and additional ingredients were mixed using the same mixer as for the sponge dough. The mixing temperature was 27.5°C. The final dough was kept at room temperature for 30 min, then cut into 390-g sections, rounded, and kept at room temperature for 10 min. After that, the rounded dough was sheeted and molded. The dough then was panned in an aluminum bread pan (10 cm x 20 cm x 6 cm) and proofed at 38°C for 50 min. After baking at 200°C for 25 min, the bread was cooled at room temperature for 3 hr.

Bacterial counts

The number of yeast cells in the pre-ferment and bread was determined by using spread plates of YM agar incubated aerobically at 30°C. Counts of lactic acid bacteria in pre-ferment were detected on spread plates of MRS agar supplemented with 500 ppm dimethyl fumarate and incubated aerobically at 30°C. For the pre-ferment with Lc. diacetylactis 18-16 and Lc. cremoris, KM agar plates were used to distinguish between the two species (Kempler and McKay, 1980). The cells of yeast and lactic acid bacteria in bread were enumerated in the same manner as stated above.

Preparation of samples for gas-liquid chromatography

After the fermentation, the pre-ferment in the Stomacher bag was cooled at -20°C for 30 min, centrifuged at 5,090 x g for 10 min and filtered with 0.45 μm Acrodisc filter (Gelman Science). The

supernatant was used as the sample. For the bread dough sample just before baking, 30 g of proofed dough was cut and put into the Stomacher bag. The bread dough was mixed with 60 ml of cooled (4°C) double-distilled water, by hand smashing the dough in the water in a manner not to aerate it. The mixture then was centrifuged at 5,090 x g for 15 min and successively filtered with the Acrodisc 1.2 μm and 0.45 μm filters. The filtrate was used as the sample. These procedures were carried out at a low temperature with cooled equipment to avoid evaporation of volatile compounds. For the bread after baking, a sample was kept at -20°C for 15 min after cooling at room temperature for 3 hr, and the crust part was removed a half inch inside the surface. Bread crumb (30 g) was cut in a cubic shape, placed into the Stomacher bag, and mixed by hand with 60 ml of cooled (4°C) double-distilled water to make a slurry-like mixture so as to minimize aeration. From this mixture, sample was prepared in the same manner as for the bread dough sample.

The samples for GLC were kept at -80°C, and, when analyzed, were thawed in cooled water and kept in ice before the analysis so as to avoid evaporation of volatile compounds and to minimize influences of enzymes present.

Gas-liquid chromatographic analyses

Volatile compounds were detected by a gas chromatograph (Model 5710A; Hewlett Packard) equipped with a flame-ionization detector. A reporting integrator (Model 3390A; Hewlett Packard)

was connected to the chromatograph. The glass column (Supelco) used was 6 feet long, 1/4 inch OD and 2 mm ID. The packing used was 6.6% Carbowax 20M/ 80/120 Carbopack B (Supelco) (Di Corcia et al., 1980). A PureCol liner (Supelco) was inserted in the column inlet to avoid deterioration of the packing by non-volatiles. The injector temperature was 170°C, the detector temperature 200°C, and the oven temperature programmed to increase from 90 to 130°C at 2°C/min. As the carrier gas, N_2 was used with a flow rate of 20 ml/min at 69 lb/in^2. H_2 was used at 22 lb/in^2, and air at 26 lb/in^2.

The standard solution was prepared by dissolving the volatile compounds in double-distilled water; sec-butanol was used as the internal standard. Concentrations of volatile compounds present in the samples were measured by the Internal Standard calculation procedure for the integrator used. A 0.5 µl sample containing the internal standard at 49.5 ppm was injected directly into the gas chromatograph.

RESULTS

The pre-ferments in combination with yeast and lactic acid bacteria were examined for pH, yeast counts, bacterial counts and volatile compounds produced. The combinations of yeast and lactic acid bacteria used are shown in Table 2.2. The pre-ferments inoculated with only lactic acid bacteria also were examined for pH and volatile compounds to study their effects in the pre-ferments. Strains of lactic acid bacteria were added to the pre-ferments as soon as they were harvested from broth cultures. Preliminary experiments showed that in the pre-ferments, numbers of cells of yeast and most lactic acid bacteria used became maximized in 8 hr when added at the initial count levels used in this study. Therefore, the pre-ferments were examined during 8 hr.

pH of pre-ferments

pH values of the pre-ferments in combination with yeast and lactic acid bacteria are shown in Table 2.3. The pH when only yeast was present decreased from 6.30 to 5.59 at 4 hr but did not decrease further at 8 hr. When lactic acid bacteria were present, the pH at 8 hr was lower, decreasing to less than 5.0. Since the two Leuconostoc strains had much higher initial counts compared to those of other lactic acid bacteria, they showed the lowest pH at 8 hr. Without nonfat dry milk, the pre-ferment with yeast and Lc. diacetylactis 18-16 showed a much lower pH than that with nonfat dry milk. The pre-ferments inoculated with lactic acid bacteria

alone had almost the same pH as those with yeast and lactic acid bacteria (Table 2.7); an exception was with the two Leuconostoc strains which showed higher pH values because volumes of these cultures added to the pre-ferment were only 1/5 of those for the pre-ferment with yeast plus the bacterial strains.

Yeast and bacterial counts of pre-ferments

The three pre-ferments containing yeast and Lc. diacetylactis 18-16 had 3 times the yeast count at 8 hr compared to those at 0 hr (Table 2.4). Also, the yeast count of the pre-ferments with only yeast increased by the same magnitude. With Lactobacillus and Leuconostoc strains, however, the yeasts did not grow to 10^8 cells/ml. On the other hand, the lactic acid bacteria, except for Lu. dextranicum 181 in the pre-ferment with yeast, kept growing for 8 hr (Table 2.5). Without nonfat dry milk, cells of Lc. diacetylactis 18-16 did not increase after 4 hr. In the pre-ferment with both of Lc. diacetylactis 18-16 and Lc. cremoris, Lc. diacetylactis 18-16 kept growing and Lc. cremoris started to grow after 4 hr. Lactobacillus plantarum F7 doubled its cell count every 4 hr, but Lb. fermentum B1 ceased to multiply after 4 hr. Leuconostoc mesenteroides cj-1 doubled its cell count every 4 hr, even after the cell count reached 10^9/ml. On the contrary, Lu. dextranicum 181 did not multiply at all.

Volatile compounds in pre-ferments with yeast and lactic acid bacteria

By GLC, the amounts of volatile compounds produced in the pre-ferments inoculated with the combinations of yeast and lactic acid bacteria were examined to find the effects of adding the different lactic acid bacteria with yeast. The chromatogram of standard solution containing the internal standard is shown in Fig. 2.1. All compounds detected in these experiments were found in the pre-ferment with only yeast (Table 2.6). The predominant compounds were ethanol and acetic acid. By adding Lc. diacetylactis 18-16, the amounts of acetaldehyde, acetoin and acetic acid were dramatically increased. The amounts of acetoin and acetic acid in the pre-ferment with Lc. diacetylactis 18-16, but without nonfat dry milk, were almost a half of those with nonfat dry milk. With both Lc. diacetylactis 18-16 and Lc. cremoris, the amounts of these three compounds were not changed much, but more diacetyl was detected compared to that without Lc. cremoris (Fig. 2.2). By adding Lb. plantarum F7, the amounts of acetoin and acetic acid were increased, but not to the same magnitude as occurred when Lc. diacetylactis 18-16 was added. The pre-ferment with Lb. fermentum B1 showed a greatly increased amount of ethyl acetate and acetic acid (Fig. 2.3). The large amount of ethyl acetate was produced only when this strain was added. No diacetyl or acetoin was detected in this sample. Samples with the two Leuconostoc strains produced higher concentrations of ethanol and acetic acid

than that with only yeast, but did not contain any detectable amount diacetyl or acetoin.

Volatile compounds in pre-ferments with only lactic acid bacteria

Pre-ferments inoculated with only lactic acid bacteria were also examined by GLC to find what volatile compounds they produced (Table 2.7). The GLC procedures used were the same as for the pre-ferments with the combinations of yeast and lactic acid bacteria. The pre-ferments with only lactic acid bacteria did not produce any detectable propanol, isobutanol or isoamyl alcohol, except that the pre-ferment with mixed strains produced a trace amount of propanol. Lactococcus diacetylactis 18-16 in the pre-ferment without yeast produced 3.2 ppm of diacetyl and two times the concentration of acetoin as that of the pre-ferment with yeast and this bacterium (Fig. 2.4). Pre-ferment inoculated with Lb. fermentum B1 did not contain the high concentration of ethyl acetate found in the pre-ferment with yeast and this Lactobacillus.

Bread experiments

Breads were baked by the sponge dough method to determine the effect of adding cells of Lc. diacetylactis 18-16 directly to the dough. Breads with and without this bacterium were baked as well as bread with only Lc. diacetylactis 18-16 to observe its behavior without yeast. Results of pH, temperature, yeast and bacterial counts in each bread are shown in Table 2.8. The bread with only

yeast showed a higher pH than the bread with yeast and Lc. diacetylactis 18-16 at every stage. The yeast counts of both breads were the same. Since commercial compressed yeast was found to contain lactic acid bacteria at 10^8 to 10^9 cells/g, the bread with only yeast contained bacteria at 4.8 x 10^6 cells/g initially and these bacteria multiplied in the sponge dough. Cells of Lc. diacetylactis 18-16 also increased in the sponge dough from 10^8 to 10^9 cells/g and decreased the pH. After the final dough was mixed, neither bread showed the increased bacterial counts.

Volatile compounds produced in the bread samples were analyzed by GLC. When cells of Lc. diacetylactis 18-16 were added directly to the sponge dough with yeast, the concentrations of acetoin and acetic acid before baking were increased greatly compared to bread with only yeast at the same stage (Table 2.9). After baking, the concentrations of acetoin and acetic acid in both breads were increased compared to the samples before baking. Before baking, the concentration of diacetyl in bread with yeast and Lc. diacetylactis 18-16 was higher than bread with yeast alone, but after baking, both breads contained almost the same concentration of diacetyl. The concentrations of alcohols in both breads were the same before and after baking. Bread with Lc. diacetylactis 18-16, but without yeast, was examined for volatile compounds produced. Since no chemical leavening agent was added in place of yeast, the dough did not expand, and, after baking, the crumb of the bread was dense and wet, and did not contain any gas holes. This bread contained acetaldehyde, ethanol, diacetyl, acetoin, and acetic acid.

After baking, the concentration of diacetyl was increased slightly, and those of acetoin and acetic acid were decreased.

DISCUSSION

The pre-ferments inoculated with selected lactic acid bacteria were examined microbiologically and for volatile compounds influencing flavor and/or aroma. Yeast cells in the pre-ferments did not increase when co-cultured with lactic acid bacteria except with Lc. diacetylactis 18-16. On the contrary, strains of lactic acid bacteria kept multiplying for 8 hr. As an energy source for organisms, the pre-ferments contained glucose, maltose from the flour starch and lactose from nonfat dry milk. Since baker's yeast do not utilize lactose, when lactic acid bacteria either unable or weakly able to utilize lactose were added, the yeast and lactics competed with each other for glucose and probably also maltose. Lactococcus diacetylactis 18-16 readily utilized lactose and grew without competing with yeast; other lactics preferred carbohydrates other than lactose in the pre-ferments and so competed with yeast. Yeast cells are much layer than lactic acid bacteria and therefore they may utilize more carbohydrate substrate per cell during growth than the later. That may be a reason why lactic acid bacteria but not yeast could grow in the pre-ferments containing a limited amount of available carbohydrate. Another possible reason was that the strains of lactic acid bacteria used in the pre-ferments produced an inhibitory substance(s) toward baker's yeast. Many types of lactic acid bacteria are now known to have antagonistic actions against gram-positive and gram-negative bacteria, yeasts and molds (Gilliland 1985). Those inhibitory activities may be due to antibiotics, bacteriocins, acids or other metabolites. According to

Sugihara et al. (1970), acids in sour dough are more inhibitory for the baker's yeast fermentation than are the sour dough yeasts. The inhibitory activity of these acids is largely due to non-dissociated acetic acid which can enter the yeast cells and inhibit their growth. In this regard, Samson et al. (1955) reported that acetic acid inhibited glucose fermentation and phosphate uptake.

In the 1960's, pre-ferments were used to replace sponge dough fermentations in conventional and continuous-mixing procedure so as to save processing time, space and labor. Since the main effect of pre-ferments was to add flavor, the flavor compounds produced in the pre-ferments have been analyzed by many researchers. Smith and Coffman (1960) reported on the analysis of the flavor compounds of pre-ferments by GLC. They detected twenty-seven compounds. In the present study, compounds detected in the pre-ferments produced with yeasts and lactic acid bacteria were acetaldehyde, ethanol, ethyl acetate, diacetyl, propanol, isobutanol, acetoin, isoamyl alcohol and acetic acid.

The column packing material used in this study was found to be very suitable for the analysis of volatile compounds produced in the pre-ferments. Direct injection of samples into the gas chromatograph was possible without extraction procedures being necessary. However, a few problems remain. First, for samples of the pre-ferments containing nonfat dry milk and lactic acid bacteria, the gas chromatograms sometimes presented interfering ghost peaks. The ghost peaks overlapped many peaks of volatile compounds and interfered with their resolution. The reason for the appearance of the ghost peaks was not found. A second problem

was that this packing material tended to absorb some portion of acetic acid so that it was difficult to detect accurately the amount in the sample. It is believed that this problem is due to insufficient deactivation of the Carbopack surface (Di Corcia et al., 1979). However, since acetic acid came out as a sharp, symmetrical peak, the elution data still could be used. The third problem concerned quantifying the amount diacetyl. Diacetyl gives a very strong odor even at small concentrations and even trace amounts therefore are important in fermented food flavors. Consequently, it was desired to detect even small concentrations of diacetyl. The column packing material used under the conditions of this study sometimes did not detect diacetyl at less than 1 ppm, and, even when detected, the data were variable. Therefore, detection of diacetyl at less than 1 ppm was less accurate than detection at higher levels.

Commercial compressed baker's yeast was found to contain a large number of lactic acid bacteria; therefore, pure yeast cells were cultured and used to inoculate the pre-ferments in this study. Since the pre-ferment inoculated with only yeast contained bacteria at only 1.0×10^3 cells/ml at 8 hr, the volatile compounds detected in the sample were considered to be primarily yeast metabolites. In addition to ethanol, alcohols found in the pre-ferments in this study were propanol, isobutanol and isoamyl alcohol. These alcohols also are found in wine and beer as fusel oils along with active amyl alcohol (2-methyl-1-butanol) (Reed and Peppler, 1973). Also, chromatogram of the pre-ferments revealed a peak just before isoamyl alcohol which seemed to be active amyl alcohol, but it was not analyzed.

By adding Lc. diacetylactis 18-16, concentrations of acetaldehyde, acetoin and acetic acid in the pre-ferment were increased dramatically (from 7.0 to 25.9 ppm, 4.3 to 197.2 ppm and 89.6 to 400.5 ppm, respectively). The pre-ferment contained citrate, so the increased acetoin and acetic acid were considered to be produced by Lc. diacetylactis through citrate metabolism. Acetaldehyde concentration was increased as an intermediate in diacetyl synthesis which is formed by condensation of "active acetaldehyde" and acetyl CoA. According to Harvey and Collins (1962), citrate is transported into cells of Lc. diacetylactis by citrate permease. This enzyme is induced at pH values lower than 6.0 and has an optimum pH around 5.0. Citrate inside the cell is degraded to oxaloacetate and acetate by citritase whose optimum pH is 7.4 to 7.6 (Harvey and Collins, 1961). Oxaloacetate is converted to pyruvate by oxaloacetate decarboxylase. Through further citrate intermediate metabolism, diacetyl, acetoin, and CO_2 are formed from pyruvate. Two pathways, through acetolactate to acetoin and through the condensation of acetaldehyde-TPP and acetyl-CoA to diacetyl, have been proposed for conversion of pyruvate to the citrate metabolic end-products (Kempler and McKay, 1981).

For Lc. diacetylactis 18-16, nonfat milk was necessary to promote growth in the pre-ferment. Nonfat milk provided lactose and a source of nitrogen for this bacterium. Without nonfat milk, the pre-ferment with yeast and Lc. diacetylactis did not contain the same amount of acetoin and acetic acid as that with nonfat milk because the bacterium could not keep growing.

When Lc. diacetylactis is used as a flavor-producing organism in dairy product starter cultures, Lc. cremoris and/or Lc. lactis is added as an acid-producing organism to achieve a low enough pH for Lc. diacetylactis to metabolize citrate. For this reason, Lc. cremoris was added to the pre-ferment with yeast and Lc. diacetylactis 18-16 in order to see the effect of additional acid production. Results of the experiment showed that the amount of volatile compounds were the same or slightly decreased compared to that without Lc. cremoris, except that the concentration of diacetyl was increased from 0.8 to 1.1 ppm. Thus the additional acid produced by Lc. cremoris apparently did enhance diacetyl productions somewhat.

Pre-ferment inoculated with Lc. diacetylactis 18-16, but without yeast, contained acetaldehyde, diacetyl, acetoin, and acetic acid, and had greatly increased concentrations of diacetyl and acetoin compared to the pre-ferment with yeast and this bacterium. Since the pH of the former was almost the same as the later, the number of cells of Lc. diacetylactis was considered to be essentially the same. Therefore, the increased diacetyl and acetoin apparently was not caused by increased cell growth. Although acetoin concentration was doubled, the concentration of diacetyl was more than doubled. From these results, it was concluded that the increased acetoin and diacetyl was due to increased availability of carbohydrates which activated the citrate metabolism of Lc. diacetylactis (Collins, 1972). Also, it is possible that some part of the increased diacetyl was due to the absence of diacetyl reduction by yeast. Yeast cells are known to reduce diacetyl to acetoin and 2,3-

butanediol (Reed and Peppler, 1973). According to a patent by Liebs et al. (1969), baker's yeast has the potential to remove diacetyl from beer. In pre-ferments with the combination of yeast and Lc. diacetylactis, it is likely that yeast cells reduced the diacetyl produced by Lc. diacetylactis to acetoin and 2,3-butanediol. Consequently, almost the same concentration of diacetyl as was present in the pre-ferment with only yeast was found in the pre-ferment made with both organisms.

Diacetyl contributes a buttery flavor and therefore is a very important compound in some dairy products. Therefore it would be useful to add the pre-ferments containing diacetyl to breads or other baking products in which buttery flavor is desirable as a flavor enhancer. At a concentration of 3 to 5 ppm, diacetyl gives very pleasant odor (Vedamuthu, 1982); however, in this study, the pre-ferment with yeast and Lc. diacetylactis did not contain that concentration of diacetyl.

In the pre-ferment prepared with yeast and Lb. plantarum F7, yeast growth was inhibited and therefore the amount of ethyl alcohol was decreased compared to that prepared with only yeast. This sample however contained more acetoin and acetic acid, which also was true for pre-ferment made with yeast and Lc. diacetylactis. Pre-ferment made with only Lb. plantarum F7 also contained almost the same amounts of those compounds. In this regard, Montivile et al. (1987) reported on the production of diacetyl-acetoin by Lb. plantarum strains. They found that citrate stimulated diacetyl-acetoin synthesis. Lactobacillus plantarum is the predominant organism in sponges and doughs used for soda cracker production

(Sugihara, 1978). It seems likely therefore that compounds produced by Lb. plantarum F7 in this study also are found in soda cracker doughs.

Lactobacillus fermentum B1 was heterofermentative lactic acid bacterium and therefore would be expected to produce ethyl alcohol, acetic acid and CO_2 in addition to lactic acid. Pre-ferment made with yeast and Lb. fermentum B1 contained greatly increased acetic acid and ethyl acetate compared to that made with only yeast (from 89.6 to 1,204.6 ppm and 11.8 to 85.8 ppm, respectively), but ethanol did not increase. The amount of ethanol produced by yeast was very large, so the effect of Lb. fermentum B1 on an amount of ethanol was not detectable. Since pre-ferment with only Lb. fermentum B1 contained just a trace of ethyl acetate, the greatly increased amount of ethyl acetate apparently resulted from the combined metabolism of the yeast and Lb. fermentum. Interestedly, other pre-ferments which contained almost the same amount of ethyl alcohol and acetic acid as that made with yeast and L. fermentum B1 did not show this amount of ethyl acetate. An view of this, the combined growth of these organisms needs to be studied further because ethyl acetate offers a fruity odor to foods. High concentrations of ethyl acetate also are found in some wines (Reed and Peppler, 1973).

The mixed strains used for the pre-ferments in this study consisted of five strains of Lactobacillus, one of which was heterofermentative, and one strain of Leuconostoc. Since all the six strains were isolated from commercial compressed baker's yeast, the pre-ferment with yeast and the mixed strains was expected to

give a flavor similar to that of bread dough. Although Lb. plantarum F7 and Lb. fermentum B1 were present in this pre-ferment, they did not seem sufficiently active to produce acetoin or form ethyl acetate, respectively. The yeast cells used for the three pre-ferments, which were inoculated with the mixed strains, Lu. dextranicum 181, or Lu. mesenteroides cj-1, were cultured in different batch from that for yeast cells used for other pre-ferments, and, in addition, were stored at 4°C for a shorter period (5 days shorter). Consequently, those yeast cells seemed to be more active in production of isobutanol in the pre-ferments.

The behavior of Lc. diacetylactis 18-16 added directly to the bread sponge dough was studied also. One of the reasons for adding Lc. diacetylactis to bread was to see if the bread contained more diacetyl than when made without this organism. Also, nonfat dry milk was added to the sponge dough because it was found to stimulate Lc. diacetylactis 18-16 to produce acetoin and probably diacetyl also. In the conventional sponge dough method, nonfat dry milk is added to final dough. As a control, bread with only yeast was used. The control had a higher sponge dough pH (5.72) than that of conventional sponge dough (around 5.20) because of the buffering effect of the nonfat dry milk. Bread with Lc. diacetylactis 18-16 but without yeast contained the end products of citrate metabolism by this bacterium, such as diacetyl, acetoin and acetic acid. Therefore, it was expected that Lc. diacetylactis in the bread dough along with yeast would have produced diacetyl and acetoin, but during baking half of the diacetyl was evaporated while most of the acetoin remained. This apparently was the reason why the

bread made with yeast and Lc. diacetylactis 18-16 did not contain increased diacetyl after baking. Since the Lc. diacetylactis cells present in the bread dough in this study were not sufficiently active in citrate metabolism to produce a significant amount of diacetyl, it was not determined whether or not the yeast in the bread dough reduced the diacetyl produced by Lc. diacetylactis 18-16. The reason why the concentration of acetoin after baking was increased was not found, but it was likely due to chemical reaction induced by heating.

One of the strategies to get more diacetyl in bread made with yeast and Lc. diacetylactis 18-16 was to lower the pH of the sponge dough. However, in this experiment the pH was higher than 5.0, actually 5.39, which was not optimum to produce diacetyl and acetoin. To lower the pH in the sponge dough, nonfat dry milk was replaced by whey or lactose, the later being the main component of nonfat dry milk which stimulates the citrate metabolism of Lc. diacetylactis. As second strategy, more citrate could be added to bread in order to get more end products of citrate metabolism (Vedamuthu, 1982; Kaneko et al., 1987). As third strategy, Lc. diacetylactis pre-ferment cultures containing diacetyl can be added to the final dough, thus avoiding any inhibitions by yeast.

In the food industry, the demand for "natural flavors" is increasing because of "natural" or "healthy" concerns of consumers. Many flavor and fragrance companies are developing biotechnological methods to produce natural flavors, and some of them have already succeeded (Dziezak, 1986). In the baking industry, chemically leavened dough products were developed but

they lacked fermentation flavors. To overcome flavor deficiency, bread flavor products are desired (Sharpell, 1985). Actually, some companies now are producing flavor products for bakery products. Therefore, the pre-ferments used in this study may be useful for many bakery products as flavor enhancers. Also, it is advantageous to utilize flavor producing organisms by adding them directly or as pre-ferments to bakery products.

In this study, it was found that each strain of lactic acid bacteria produced its characteristic volatile compounds in the pre-ferment. Therefore, it is possible to select certain strains of lactic acid bacteria to produce the desired flavors depending on the product. For bread dough products, it may be necessary to add both yeast and lactic acid bacteria to pre-ferments because yeast also can contribute to fermentation flavors of the bread. In pre-ferment or bread with yeast, adding cells of Lc. diacetylactis did not contribute to increased diacetyl. Lactococcus diacetylactis, however, has the potential to produce more diacetyl, and by changing the method or formulation of bread pre-ferments, more of this flavor compound likely can be produced in the final product.

Table 2.1. Organisms used in this study.

Organism	Source
Yeast F	Fleischmann's compressed yeast
Lactococcus diacetylactis 18-16	This laboratory
Lactococcus cremoris	This laboratory
Lactobacillus plantarum F7	Fleischmann's compressed yeast
Lactobacillus fermentum B1	Budweiser compressed yeast
Leuconostoc dextranicum 181	This laboratory
Leuconostoc mesenteroides cj-1	This laboratory
Lactobacillus sp. F3 (Streptobacterium)	Fleischmann's compressed yeast
Lactobacillus sp. F4 (Atypical Streptobacterium)	Fleischmann's compressed yeast
Lactobacillus casei F8	Fleischmann's compressed yeast
Leuconostoc mesenteroides F13	Fleischmann's compressed yeast

Table 2.2. Combinations of yeast and lactic acid bacteria used for pre- ferments.

Combination
Yeast F + No bacteria
Yeast F + Lactococcus diacetylactis 18-16 (without N.F.D.M.)
Yeast F + Lactococcus diacetylactis 18-16
Yeast F + Lactococcus diacetylactis 18-16 + Lactococcus cremoris
Yeast F + Lactobacillus plantarum F7
Yeast F + Lactobacillus fermentum B1
Yeast F + Leuconostoc dextranicum 181
Yeast F + Leuconostoc mesenteroides cj-1
Yeast F + mixed strains Lactobacillus sp. F3 Lactobacillus sp. F4 Lactobacillus plantarum F7 Lactobacillus casei F8 Lactobacillus fermentum B1 Leuconostoc mesenteroides F13

Table 2.3. pH of the pre-ferments made with combinations of yeast and lactic acid bacteria and incubated at 30°C.

Combination of organisms	0 hr	4 hr	8 hr
Yeast F only	6.30	5.59	5.56
Yeast F + Lc. diacetylactis 18-16 (Without N.F.D.M.)	5.82	4.80	4.21
Yeast F + Lc. diacetylactis 18-16	6.35	5.24	4.73
Yeast F + Lc. diacetylactis 18-16 + Lc. cremoris	6.34	5.15	4.51
Yeast F + Lb. plantarum F7	6.36	5.24	4.53
Yeast F + Lb. fermentum B1	6.35	5.13	4.76
Yeast F + mixed strains	6.39	5.20	4.48
Yeast F + Lu. dextranicum 181	6.36	4.77	4.26
Yeast F + Lu. mesenteroides cj-1	6.38	4.71	4.07

Abbreviation: Lc. = Lactococcus ; Lb. = Lactobacillus ; Lu. = Leuconostoc.

Table 2.4. Yeast counts (CFU[a]/ml sample) of the pre-ferments made with combinations of yeast and lactic acid bacteria and incubated at 30°C.

Combination of organisms	0 hr	4 hr	8 hr
Yeast F only	5.1×10^7	9.1×10^7	1.6×10^8
Yeast F + Lc. diacetylactis 18-16 (Without N.F.D.M.)	5.2×10^7	9.3×10^7	1.2×10^8
Yeast F + Lc. diacetylactis 18-16	5.0×10^7	8.4×10^7	1.4×10^8
Yeast F + Lc. diacetylactis 18-16 + Lc. cremoris	5.4×10^7	9.8×10^7	1.2×10^8
Yeast F + Lb. plantarum F7	6.1×10^7	9.2×10^7	8.7×10^7
Yeast F + Lb. fermentum B1	6.1×10^7	7.0×10^7	9.7×10^7
Yeast F + mixed strains	6.0×10^7	6.9×10^7	8.3×10^7
Yeast F + Lu. dextranicum 181	6.1×10^7	7.3×10^7	6.8×10^7
Yeast F + Lu. mesenteroides cj-1	6.8×10^7	7.0×10^7	6.8×10^7

[a] Colony Forming Unit.
Abbreviation: Lc. = Lactococcus ; Lb. = Lactobacillus ; Lu. = Leuconostoc.

Table 2.5. Bacterial counts (CFU[a]/ml sample) of the pre-ferments made with combinations of yeast and lactic acid bacteria and incubated at 30°C.

Combination of organisms	0 hr	4 hr	8 hr
Yeast F only	$<1.0 \times 10^3$	$<1.0 \times 10^3$	1.0×10^3
Yeast F + Lc. diacetylactis 18-16 (Without N.F.D.M.)	1.3×10^8	3.7×10^8	3.4×10^8
Yeast F + Lc. diacetylactis 18-16	1.2×10^8	4.0×10^8	6.1×10^8
Yeast F + Lc. diacetylactis 18-16 + Lc. cremoris	2.1×10^8	3.3×10^8	5.4×10^8
Lc. diacetylactis 18-16	1.5×10^8	2.6×10^8	4.0×10^8
Lc. cremoris	6.0×10^7	7.7×10^7	1.4×10^8
Yeast F + Lb. plantarum F7	1.1×10^8	2.1×10^8	4.8×10^8
Yeast F + Lb. fermentum B1	1.4×10^8	3.0×10^8	2.0×10^8
Yeast F + mixed strains	1.8×10^8	3.9×10^8	5.0×10^8
Yeast F + Lu. dextranicum 181	4.9×10^8	3.6×10^8	5.3×10^8
Yeast F + Lu. mesenteroides cj-1	6.1×10^8	1.2×10^9	2.6×10^9

[a] Colony Forming Unit.
Abbreviation: Lc. = Lactococcus ; Lb. = Lactobacillus ; Lu. = Leuconostoc.

Table 2.6. Concentration[a](v/v) of volatile compounds produced in the pre-ferments made with combinations of yeast and lactic acid bacteria and incubated at 30°C for 8 hr.

Combination	Acetald. (ppm)	Ethyl alc. (%)	Ethyl acet. (ppm)	Diacetyl (ppm)	Prop. alc. (ppm)	I.but. alc. (ppm)	Acetoin (ppm)	I.amyl alc. (ppm)	Acetic acid (ppm)
Yeast F									
+ No bacteria	7.0	1.12	11.8	0.7	8.4	11.8	4.3	9.6	89.6
+ Lc. d. 18-16 (No N.F.D.M.)	9.3	0.98	1.6	0.7	5.0	9.8	92.5	6.2	237.8
+ Lc. d. 18-16	25.9	1.01	4.4	0.8	5.2	9.7	197.2	13.7	400.5
+ Lc. d. 18-16 + Lc. c.	24.2	0.82	2.9	1.1	4.5	7.4	181.7	9.6	376.9
+ Lb. pl. F7	9.0	0.71	0.7	0.6	7.4	6.4	37.1	8.3	163.5
+ Lb. ferm. B1	1.3	0.76	85.8	ND	5.7	6.3	ND	4.6	1204.6
+ Mixed strains	1.6	1.08	5.4	ND	13.0	21.1	2.3	10.3	658.8
+ Lu. dex. 181	ND	1.38	10.5	ND	8.8	33.9	ND	9.8	901.9
+ Lu. mes. cj-1	ND	1.40	27.7	ND	5.3	30.1	ND	10.0	989.1

[a] Average of three injections.

Abbreviation: Acetald. = Acetaldehyde; Ethyl alc. = ethanol; Ethyl acet. = ethyl acetate; Prop. alc. = propanol; I. but. alc. = isobutanol; I. amyl alc. = isoamyl alcohol; Lc. d. 18-16 = Lactococcus diacetylactis 18-16; Lc. c. = Lactococcus cremoris; Lb. pl. F7 = Lactobacillus plantarum F7; Lb. ferm. B1 = Lactobacillus fermentum B1; Lu. dex. 181 = Leuconostoc dextranicum 181; Lu. mes. cj-1 = Leuconostoc mesenteroides cj-1; ND = not detected.

Table 2.7. Concentration[a](v/v) of volatile compounds produced in the pre-ferments with only lactic acid bacteria when incubated at 30°C for 8 hr.

Strain	pH	Acet-ald. (ppm)	Ethyl alc. (%)	Diace-tyl (ppm)	Acet-oin (ppm)	Acetic acid (ppm)	Other comp. (ppm)
Lc. diacetylactis 18-16	4.67	5.5	ND	3.2	360.7	389.0	
Lb. plantarum F7	4.54	1.6	ND	ND	36.6	119.1	
Lb. fermentum B1	4.96	ND	0.13	ND	ND	879.3	(Ethylace.) 1.0
Mixed strains	4.48	ND	0.04	ND	ND	512.1	(Prop.al.) 0.7
Lu. dextranicum 181	4.81	ND	0.22	ND	ND	152.4	
Lu. mesenteroides cj-1	4.52	ND	0.31	ND	ND	136.9	

[a] Average of three injections.

Abbreviation: Acetald. = acetaldehyde; Ethyl alc. = ethanol; Other comp. = other compounds; Ethylace. = ethyl acetate; Prop.al. = propanol; Lc. = Lactococcus ; Lb. = Lactobacillus ; Lu. = Leuconostoc; ND = not detected.

Table 2.8. Temperature, pH, yeast and bacterial counts of breads.

Stage Total hr	Sponge dough Initial point 0hr	Sponge dough After 4.5 hr 4.5hr	Final ferment. After 50 min 6.5hr
Bread with yeast but without Lc. diacetylactis 18-16			
Temperature (°C)	24.5	27	31
pH	6.04	5.72	5.35
Yeast counts (CFU/g)	2.6×10^8	2.5×10^8	1.7×10^8
Bact. counts (CFU/g)	4.8×10^6	1.4×10^7	7.6×10^6
Bread with yeast and Lc. diacetylactis 18-16			
Temperature	24.5	28	31
pH	6.03	5.39	5.12
Yeast counts	2.4×10^8	2.5×10^8	1.6×10^8
Bact. counts	4.0×10^8	8.9×10^8	4.5×10^8
Bread without yeast but with Lc. diacetylactis 18-16			
Temperature	25.5	28	30
pH	6.29	5.47	5.27

Abbreviation: Lc. = Lactococcus ; CFU = Colony Forming Unit.

Table 2.9. Concentration[a](w/w) of volatile compounds found in bread crumbs.

Combination	Acetald. (ppm)	Ethyl alc. (%)	Ethyl acet. (ppm)	Diacetyl (ppm)	Prop. alc. (ppm)	I.but. alc. (ppm)	Acetoin (ppm)	I.amyl alc. (ppm)	Acetic acid (ppm)
Bread with yeast but without Lc. diacetylactis 18-16									
Before baking	87.6	1.31	3.4	1.2	19.4	30.0	47.2	19.1	188.0
After baking	12.9	0.64	ND	1.0	7.6	18.5	78.0	4.9	220.1
Bread with yeast and Lc. diacetylactis 18-16									
Before baking	91.7	1.36	6.3	1.7	17.8	36.9	161.7	10.8	424.7
After baking	17.3	0.70	ND	0.8	7.4	20.2	187.8	3.7	513.0
Bread without yeast but with Lc. diacetylactis 18-16									
Before baking	7.1	0.0009	ND	0.9	ND	ND	283.1	ND	319.9
After baking	5.8	0.0006	ND	1.0	ND	ND	191.8	ND	129.4

[a] Average of three injections.

Abbrevation: Acetald. = acetaldehyde; Ethyl alc. = ethanol; Prop. alc. = propanol; I. but. alc. = isobutanol; I. amyl alc. = isoamyl alcohol; Lc. = Lactococcus; ND = not detected.

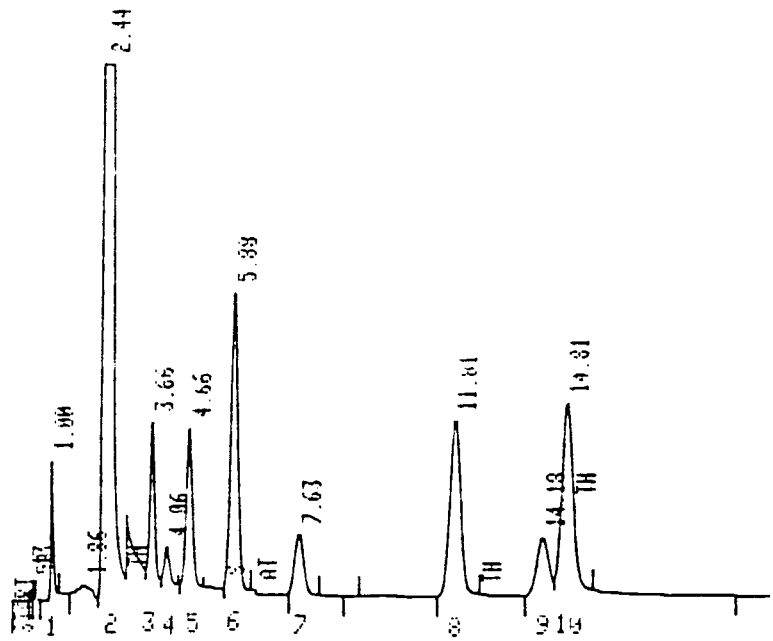

Fig. 2.1. Chromatogram of standard solution.
Peaks: 1 = acetaldehyde (49.5 ppm); 2 = ethanol (0.95%); 3 = ethyl acetate (25 ppm); 4= diacetyl (5 ppm); 5 = propanol (25 ppm); 6 = sec-butanol as Internal Standard (49.5 ppm); 7 = isobutanol (25 ppm); 8 = acetoin (92.5 ppm); 9 = isoamyl alcohol (25 ppm); 10 = acetic acid (200 ppm).

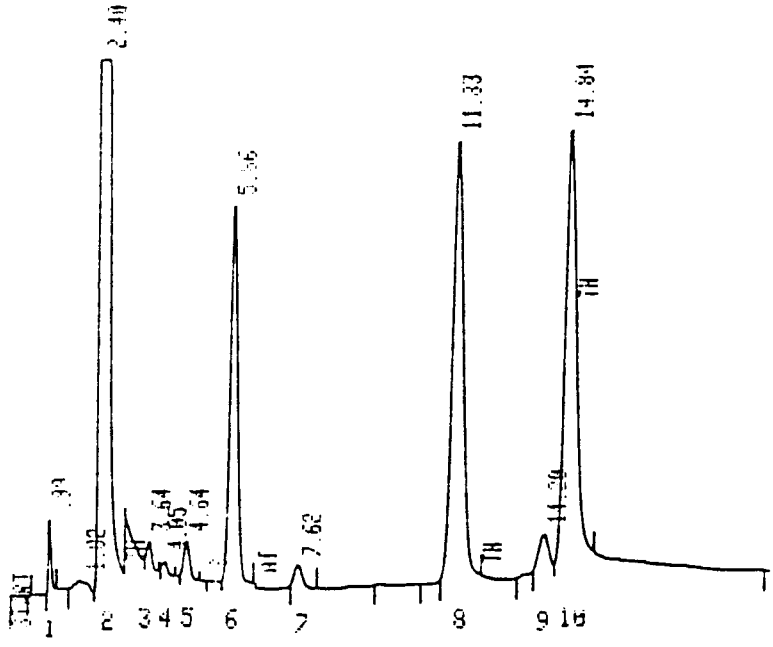

Fig. 2.2. Chromatogram of pre-ferment inoculated with yeast, Lactococcus diacetylactis 18-16, and Lactococcus cremoris .
Peaks: 1 = acetaldehyde; 2 = ethanol; 3 = ethyl acetate; 4= diacetyl; 5 = propanol; 6 = sec-butanol as Internal Standard; 7 = isobutanol; 8 = acetoin; 9 = isoamyl alcohol; 10 = acetic acid.

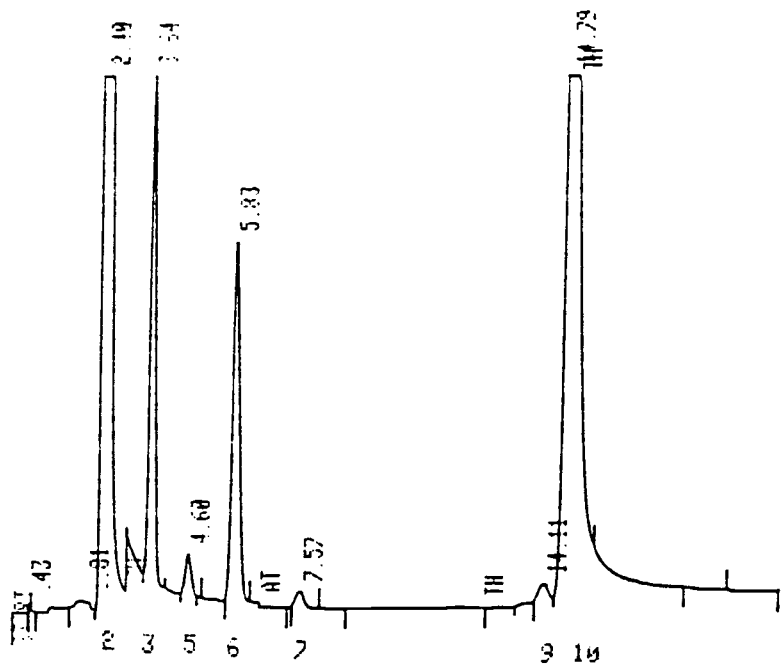

Fig. 2.3. Chromatogram of pre-ferment inoculated with yeast and Lactobacillus fermentum B1.
Peaks: 2 = ethanol; 3 = ethyl acetate; 5 = propanol; 6 = sec-butanol as Internal Standard; 7 = isobutanol; 9 = isoamyl alcohol; 10 = acetic acid.

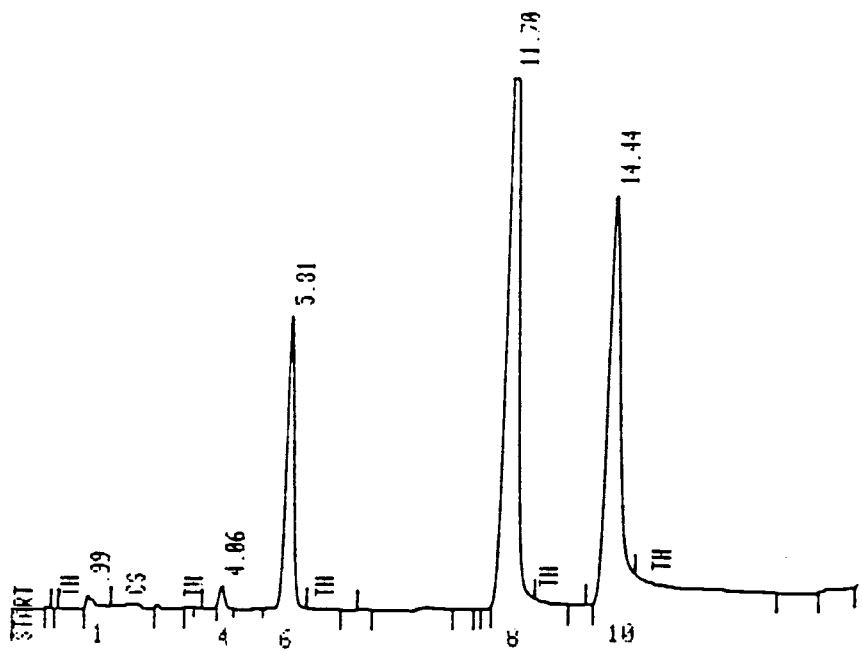

Fig. 2.4. Chromatogram of pre-ferment inoculated with Lactococcus diacetylactis 18-16 alone.
Peaks: 1 = acetaldehyde; 4= diacetyl; 6 = sec-butanol as Internal Standard; 8 = acetoin; 10 = acetic acid.

REFERENCES

Baker, J. C. 1957. The effects of yeast on bread flavor. Bakers Digest 31(5):64-68, 79.

Bundus, R. H. and A. J. Luksas. 1969. U.S. Patent 3,466,174.

Carlin, G. T. 1958. The fundamental chemistry of bread making I. Proc. Am. Soc. Bakery Engr.:56-63.

Carlin, G. T. 1959. The fundamental chemistry of bread making II. Proc. Am. Soc. Bakery Engr.:136-147.

Collins, E. B. 1972. Biosynthesis of flavor compounds by microorganisms. J. Dairy Sci. 55:1022-1028.

Di Corcia, A., R. Samperi, and C. Severini. 1979. Modified graphitized carbon black for the gas chromatographic analysis of alcoholic beverages. J. Chromatogr. 170:245-248.

Di Corcia, A., R. Samperi, and C. Severini. 1980. Gas chromatographic column for the rapid determination of congeners in potable spirits. J. Chromatogr. 198:347-353.

Dziezak, J. D. 1986. Biotechnology and flavor development; an industrial research perspective. Food Technol. 40(4):108-113, 120.

Gilliland, S. E. 1985. Role of starter culture bacteria in food preservation, p.175-185. In S. E. Gilliland (ed.), Bacterial Starter Cultures for Foods. CRC Press, Inc., Boca Raton, Fla.

Harvey, R. J. and E. B. Collins. 1961. Role of citritase in acetoin formation by Streptococcus diacetylactis and Leuconostoc citrovorum. J. Bacteriol. 82:954-959.

Harvey, R. J. and E. B. Collins. 1962. Citrate transport system of Streptococcus diacetylactis. J. Bacteriol. 83:1005-1009.

Jackel, S. S. 1963. U.S. Patent 3,102,033.

Jackel, S. S. 1969. Fermentation flavors of white breads. Bakers digest 43(5):24-28, 64.

Johnson, J. A. and B. S. Miller. 1957. Pre-ferments. Bakers Digest 31(3):30-35, 76-77.

Kaneko, T., H. Suzuki, and T. Takahashi. 1987. The effects of metal ions on diacetyl production by Streptococcus lactis subsp. diacetylactis 3022. Agric. Biol. Chem. 51:2315-2320.

Kempler, G. M. and L. L. McKay. 1980. Improved medium for detection of citrate-fermenting Streptococcus lactis subsp. diacetylactis. Appl. Environ. Microbiol. 39:926-927.

Kempler, G. M. and L. L. McKay. 1981. Biochemistry and genetics of citrate utilization in Streptococcus lactis subsp. diacetylactis. J. Dairy Sci. 64:1527-1539.

Liebs, P., M. Krueger, and H. C. Wolter. 1969. Removal of diacetyl from beer. German (East) Patent 1,801,509. Nov. 6.

Linko, Y., B. S. Miller, and J. A. Johnson. 1962. Quantitative determination of certain carbonyl compounds in pre-ferments. Cereal Chem. 39:263-272.

Maga, J. A. 1974. Bread flavor. CRC Crit. Rev. Food Technol. 5:55-142.

Montville, T. J., M. E. Meyer, and A. H.-M. Hsu. 1987. Influence of carbon substrates on lactic acid, cell mass and diacetyl-acetoin production in Lactobacillus plantarum. J. Food Protect. 50:42-46.

Reed, G. and H. J. Peppler. 1973. Yeast Technology. AVI Publishing Company, Inc., Westport, Connecticut.

Robinson, R. J., T. H. Lord, J. A. Johnson, and B. S. Miller. 1958. The aerobic microbiological population of pre-ferments and the use of selected bacteria for flavor production. Cereal Chem. 35:295-305.

Samson, F. E., A. M. Katz, and D. L. Harris. 1955. Effects of acetate and other short-chain fatty acids on yeast metabolism. Archives of Biochemistry and Biophysics 54:406-423.

Sandine, W. E. and P. R. Elliker. 1970. Microbially induced flavors in fermented foods. Agri. and Food Chem. 18:557-562.

Sharpell, F. H. 1985. Microbial flavors and fragrances, p.965-981. In M. Moo-Young et al. (ed.), Comprehensive Biotechnology, Vol. 3. Pergamon Press.

Smith, D. E. and J. R. Coffman. 1960. Analysis of food flavors by gas-liquid chromatography. Analytical Chemistry 32:1733-1737.

Sugihara, T. F., L. Kline, and L. B. Mcgready. 1970. Nature of the San Francisco sour dough French Bread process. II. Microbiological aspects. Bakers Digest 44(2):51-57.

Sugihara, T. F. 1978. Microbiology of the soda cracker process. J. Food Protect. 41:977-979.

Thomas, B. and M. Rothe. 1957. Zur frage der beteiligung von acetoin and diacetyl am aroma des weiβbrotes. Ernahrungsforschung 2:751-757.

Vedamuthu, E. R. 1982. Fermented milks, p.199-226. In A. H. Rose (ed.), Fermented Foods. Academic Press.

Visser't Hooft, F. and F. J. G. deLeeuw. 1935. The occurrence of acetylmethylcarbinol in bread and its relation to bread flavor. Cereal Chem. 12:213-229.

Wiseblatt, L. and F. E. Kohn. 1960. Some volatile aromatic compounds in fresh bread. Cereal Chem. 37:55-66.

CHAPTER 3

GAS-LIQUID CHROMATOGRAPHIC ANALYSIS OF VOLATILE COMPOUNDS PRODUCED BY LEUCONOSTOC

ABSTRACT

Volatile compounds produced by Leuconostoc strains in modified MRS broth, milk acidified by growth of Lactococcus(Lc.) cremoris WG2, milk acidified with H_3PO_4, and citrate solutions were determined by gas-liquid chromatography (GLC). Column packing material used was 6.6% Carbowax 20M/ 80/120 Carbopack B. Most citrate-utilizing strains of Leuconostoc did not produce diacetyl or acetoin in the MRS or acidified milk cultures, but did in the citrate solutions. Leuconostoc(Lu.) cremoris CAF7 produced diacetyl and acetoin in the milk culture acidified with H_3PO_4. When the citrate-utilizing strains did produce diacetyl and acetoin, the amounts of ethanol produced by them were always small. Results of this study confirmed the inhibitory effect of glucose metabolism on diacetyl and acetoin production by Leuconostoc. Gas-liquid chromatographic detection of diacetyl and acetoin produced in the citrate solutions was found to be a simple and exact method to analyze the ability of Leuconostoc strains to produce those compounds.

INTRODUCTION

Lactic acid bacteria are widely-used in the production of various fermented foods, including dairy products, vegetables, sour dough bread and meat products. Microorganisms of this type play important roles in producing flavors and various acids in the foods. Diacetyl is an important and well-known flavor compound produced by some lactic acid bacteria and is desirable to have present in fermented foods. This compound is now recognized to be produced through citrate metabolism of diacetyl-producing bacteria. Therefore, it is important to be able to detect diacetyl in cultures or fermented foods in order to determine whether or not the bacteria used have the ability to produce this compound.

Many methods to detect diacetyl and acetoin have been developed. The well-known method designed by Westerfeld (1945) has been used widely by many researchers. His method was originally used to detect acetoin in blood. In the presence of α-naphthol and alkali, acetoin is slowly oxidized to diacetyl which reacts with creatine to form a red color. The intensity of the color is measured spectrophotometrically. However, this method can not distinguish between diacetyl and acetoin. Hill et al. (1954) modified the Westerfeld method to detect diacetyl in citrus juices, but this method was still unsatisfactory. Methods of salting-out chromatography and ion-exchange chromatography have been used to separate diacetyl and acetoin which then can be quantified by the Westerfeld method (Speckman and Collins,1968; Keen and Walker, 1973). These methods require considerable time and a large

number of manipulative steps. Pack et al. (1964) designed an apparatus which has proven satisfactory for diacetyl determination in dairy products; it was a modification of a method originally designed by Owades and Jacovac (1963) to determine diacetyl in beer. This method provides reliable quantification of diacetyl but also is time and labor consuming. Walsh and Cogan (1974a,b) separated diacetyl and acetoin by steam distillation and then were able to detect each compound separately. However, recent experiments have shown that steam distillation causes the conversion of α-acetolactate to diacetyl and acetoin (Jordan and Cogan, 1988).

Many investigators have attempted to detect diacetyl and acetoin separately by GLC. Such a method would be expected to detect these compounds more accurately and in less time than other methods. In this regard, Doelle (1969) reported a method to detect diacetyl, acetone and alcohols by GLC. However, his method examined only standard solutions, and diacetyl eluted as a slightly tailing peak. Mckay and Baldwin (1974) detected diacetyl and acetoin in bacterial cultures by GLC. Although this method emphasized the usefulness of GLC to detect the two compounds, an extraction process using dichloromethane was needed. Lee and Drucker (1975) also detected diacetyl and acetoin in bacterial culture supernatants by GLC. However, the supernatants were extracted with diethyl ether, and then acetoin in the extracts was converted to diacetyl with ferric chloride before analysis. Montville et al. (1987a) reported the detection of diacetyl and acetoin in bacterial cultures by GLC. The method required head space or

methylene chloride extraction and did not detect acetoin at concentrations lower than about 150 ppm.

Diacetyl is a highly volatile compound and usually produced in trace amounts; acetoin, usually produced in much larger quantities, undergoes a slow oxidation to diacetyl. In order to easily detect these compounds and avoid interference from other compounds, direct injection of sample to GLC columns is desired. Palo and Ilkova (1970) determined lower alcohols, acetaldehyde, acetone and diacetyl in milk products by direct injection of samples onto GLC columns. Although this method seemed useful because of no sample preparation requirement before analysis, diacetyl eluted 30 min after sample injection, and acetoin was not detected. However, this method provided a basis for further research. Thornhill and Cogan (1984) described GLC analysis of the end products of lactic acid bacteria without an extraction process. Samples were prepared by filtration or centrifugation of cultures. They were able to detect diacetyl, acetoin, 2,3-butylene glycol, ethanol and acetic acid by direct injection of the samples. In this method, diacetyl were detected but not quantified because of the trace amounts present.

In the present study, direct injection of cultures on GLC column was carried out to determine volatile compounds produced by lactic acid bacteria, with special attention to diacetyl and acetoin. Leuconostoc strains from the Department of Microbiology culture collection were examined to determine which produced diacetyl and acetoin. Since cultural conditions to optimize diacetyl production by Leuconostoc have not yet been established, several different types of growth supernatants were tested.

MATERIALS AND METHODS

Organisms

Bacterial strains used in this study are shown in Table 3.1. They were propagated at 30°C in MRS broth (Difco) containing 0.1% sodium citrate. Lactococcus cremoris WG2 for milk culture experiments was grown in modified M17 broth (with glucose in place of lactose) at 30°C (Terzaghi and Sandine, 1975). Gas production from glucose was observed by transferring the strains into tubes of MRS broth containing a durham tube and an overlay of vaspar (vaseline:parafin = 1:1) and incubating at 30°C. To test for dextran production from sucrose, strains were streaked on sucrose agar plates containing, per liter: 10 g of tryptone, 5 g of yeast extract, 100 g of sucrose, 2.5 g of gelatin, 5 g of glucose, and 15 g of agar. The agar plates were incubated aerobically at 30°C.

Sample preparation

For analysis of MRS cultures, MRS broth (de Man et al., 1960), modified by omission of Tween 80 and sodium acetate and addition of 0.1% sodium citrate, was used (Thornhill and Cogan, 1984). When 0.5 mM of $CuSO_4 \cdot H_2O$ and 5 mM of sodium ascorbate were added, $CuSO_4 \cdot H_2O$ was added directly to the MRS broth before sterilization, and sodium ascorbate solution (5%) was filtered and added to the sterilized broth to give a final concentration of 5 mM. Bacterial strains were grown in the broth from one loop inoculum with incubation at 30°C for 20 hr, except 43 hr for Lu. cremoris CAF7.

After incubation, the cultures were cooled at 4°C for 20 min and centrifuged at 5,900 x g for 15 min. Supernatants were used as samples.

For analysis of milk culture acidified with Lc. cremoris WG2, 11% nonfat dry milk solution with 0.2% sodium citrate and 0.1% yeast extract was used. The 0.5 mM of $CuSO_4 \cdot H_2O$ and 5 mM of sodium ascorbate were added in the same manner as for the MRS broth noted above. Milk solutions were given 1% inoculum from a 24-hr culture of Leuconostoc strain and 1% of Lc. cremoris WG2, and incubated at 30°C for 24 hr. After incubation, samples were prepared in the same manner as for the MRS cultures noted above, except filtration was carried out with a 0.45 μm Acrodisc filter (Gelman Science).

For samples of milk cultures acidified with H_3PO_4, 11% nonfat dry milk solutions with 0.2% sodium citrate were provided a 1% inoculum of 24-hr cultures of Leuconostoc strains, except a 72-hr old culture was used in the case of strain CAF7. After 24 hr of incubation at 30°C, the milk cultures were acidified to pH 4.5 by adding 2N H_3PO_4; incubation was continued for 9 more hr at 30°C. Samples were prepared in the same manner as for the MRS broth cultures noted above, except filtration was through a 0.45 μm Acrodisc filter.

For experiments with non-growing cells of Leuconostoc strains, citrate solutions containing 50 mM acetic acid, 30 mM citric acid and 1 mM glucose, adjusted to pH 5.0, were prepared using a modified method of Jordan and Cogan (1988). Cells grown at 30°C for 16 hr (72 hr for strain CAF7) in MRS broth modified by omission of

Tween 80 and sodium acetate were harvested by centrifugation at 3,440 x g for 10 min, washed once with 10 mM H_3PO_4 solution, adjusted to pH 5.0, and resuspended in the citrate solution at twice the volume of modified MRS broth used for their propagation. Mixtures with the cells were incubated at 30°C for 4 hr. After incubation, samples were prepared in the same manner as for the MRS culture experiments noted above.

Gas-liquid chromatographic analysis

A Model 5710A gas chromatograph (Hewlett Packard) equipped with a flame-ionization detector was used. A Model 3390A reporting integrator (Hewlett Packard) was connected to the chromatograph. The glass column (Supelco) used was 6 feet long, 1/4 inch OD and 2 mm ID. A PureCol linear (Supelco) was inserted in the column inlet. The packing used was 6.6% Carbowax 20M/ 80/120 Carbopack B (Supelco) (Di Corcia et al., 1980). The temperatures of the injector and the detector were 150°C. The oven temperature was programmed to increase from 90 to 130°C at 2°C/min. N_2 was used as carrier gas at a flow rate of 20 ml/min at 69 lb/in^2. H_2 was used at 22 lb/in^2, and air at 26 lb/in^2.

The volatile compound-containing standard solution was prepared by dissolving the volatiles in double-distilled water. The amount of each compound in the standard was determined based on possible concentrations present in samples. Sec-butanol was used as the internal standard. Concentrations of volatile compounds present in the samples were determined by the Internal Standard

calculation procedure for the integrator used. In this procedure , it was assumed that each compound gave a linear standard curve which went through the origin. The concentration of each compound was calculated by comparing the ratios of two peak areas for each compound in the standard solution and sample to those for sec-butanol. A 0.5 µl sample containing the internal standard at 49.5 ppm was injected directly into the gas chromatograph. The injection was duplicated for each sample. Reproducibility of volatile compound peaks by the gas chromatograph was checked to see if components in the medium interfered with the analysis. For modified MRS medium, uninoculated broth containing known amount of volatile compounds was analyzed. For milk cultures acidified with Lc. cremoris WG2, known amounts of volatile compounds were added to the supernatant of uninoculated milk containing 0.2% sodium citrate and 0.1% yeast extract acidified by lactic acid to pH 4.5 and then analyzed. For milk cultures acidified to pH 4.5 with H_3PO_4, the supernatant of uninoculated milk, also containing 0.2% sodium citrate, was examined in this same manner.

RESULTS

Characteristics of strains

Gas or dextran producing abilities of Leuconostoc strains and Lactococcus diacetylactis (Lactococcus lactis subsp. lactis biovar. diacetylactis, formerly known as Streptococcus diacetylactis) 18-16 are shown in Table 3.1. These results contradicted identification of some strains with respect to their identity as specified on culture labels. Leuconostoc strain 14AM produced only a few bubbles of gas from glucose in the MRS broth. Also, while Leuconostoc cremoris is known as a non-dextran producer on sucrose agar, strain 104 had this property.

Packing material for gas-liquid chromatography

In GLC analysis, the packing material used gave symmetric and sharp peaks of volatile compounds present in samples (Fig. 3.1-3.5). Although this packing was found useful, it and a PureCol linear tended to absorb some portion of acetic acid and the absorbed portion of the compound eluted at subsequent sample injections. Diacetyl and acetoin also showed this same tendency, but not to the extent of acetic acid. To resolve these problems, 0.5 µl of double distilled water or 0.1% ethanol solution was injected one or more times between sample injections; this stabilized and cleaned up the column packing and PureCol linear.

Reproducibility of GLC peak heights with known amounts of volatile compounds added to the uninoculated modified MRS broth

and milk acidified with lactic acid or H_3PO_4 was determined. Results are shown in Table 3.2. The same quantity of each compound as used for the standard curve solution was added to the three types of uninoculated medium, and reproduction was determined by the Internal Standard calculation procedure. Each compound showed a little higher or lower average concentration in the three uninoculated media than in the standard solution. Acetoin in the uninoculated modified MRS broth and milk acidified with lactate showed distinctly less responses than it did in the standard solution. This appeared to be due to certain components of yeast extract which suppressed its expected elution peak. In the uninoculated milk acidified with H_3PO_4, this compound had the opposite tendency. Concentrations of volatile compounds found in samples were corrected to account for these effects in each medium, respectively. Reproduction of quantification in citrate solutions was found the same as in the standard solution. Acetic acid in modified MRS broth showed more-tailing peaks and therefore a more inaccurate response than in the standard solution, and, in addition, the broth was found to contain a certain amount of acetic acid. Therefore, the Internal Standard calculation procedure was not appropriate for the quantification of acetic acid in MRS cultures. Standard curve for acetic acid in the uninoculated modified MRS broth was obtained separately by plotting amounts of acetic acid added and responses. Although this curve was not linear, it was used for the quantification of acetic acid in MRS cultures.

MRS cultures

The pH and concentration of volatile compounds produced in MRS cultures inoculated with various strains are shown in Table 3.3. Lactococcus diacetylactis 18-16 produced acetaldehyde, diacetyl, acetoin and acetic acid. These compounds are believed to be produced from citrate metabolism. Kaneko et al. (1986) reported that sodium ascorbate or cysteine, which lowered the Eh in the medium, stimulated Lc. diacetylactis to produce diacetyl. Also, they found that the addition of metal ions, such as Cu^{2+}, Mo^{6+}, Fe^{2+} or Fe^{3+}, to the medium stimulated diacetyl production (Kaneko et al., 1987). To analyze possible stimulatory effects of these factors on Lc. diacetylactis 18-16, the modified MRS medium with 0.5 mM of $CuSO_4 \cdot H_2O$ and 5 mM of sodium ascorbate was inoculated and analyzed for volatile compounds produced. Results showed that the culture contained 3 times the amount of diacetyl as that found without the additives; supplemented culture also showed a higher pH. The higher pH was considered a reflection of suppressed growth by Lc. diacetylactis 18-16, and this result agreed with that of Kaneko et al. (1987).

Leuconostoc strains, except for 14AM, in the MRS medium produced mainly ethanol and acetic acid; some strains produced acetaldehyde and trace amounts of propanol, but non-detectable amounts of diacetyl or acetoin. Strain 14AM produced a trace amount of ethanol though it seemed to grow as well as the other strains. Strain CAF7 revealed a much higher pH and did not produce as much ethanol as the other Leuconostoc strains.

Milk cultures acidified with Lc. cremoris WG2

The pH and concentrations of volatile compounds produced in milk cultures acidified with Lc. cremoris WG2 are shown in Table 3.4. The uninoculated milk control contained 4.7 ppm of ethanol and 9.3 ppm of acetic acid, so the data for these compounds were corrected accordingly. The milk culture with Lc. cremoris WG2 alone contained ethanol, acetoin and acetic acid. In the sample with Lc. cremoris WG2 and Lc. diacetylactis 18-16, diacetyl and greatly increased acetoin were detected. When the milk culture contained 0.5 mM of $CuSO_4 \cdot H_2O$, the concentration of diacetyl was increased almost 2.5 times compared to that without $CuSO_4 \cdot H_2O$. Adding 5 mM of sodium ascorbate besides $CuSO_4 \cdot H_2O$ did not increase the concentration of diacetyl but did elevate those of acetoin and acetic acid. The strains of Leuconostoc in milk cultures with Lc. cremoris WG2 produced large amounts of ethanol and acetic acid; however, strain 14AM produced a slight amount of ethanol and large amounts of acetoin and acetic acid. Samples with Leuconostoc strains contained less ethanol and acetic acid compared to those in the MRS cultures. Samples with strains 16-9 or CAF7 only contained acetoin.

Milk cultures acidified with H_3PO_4

It has been reported that citrate utilization of Leuconostoc strains is stimulated at lower than neutral pH values in the presence of glucose or lactose; it was also noted that 10 mM glucose or lactose totally inhibited acetoin production from citrate (Cogan, 1975; Cogan

et al., 1981; Jordan and Cogan, 1988). The inhibitory effect of glucose or lactose on acetoin production was likely due to the presence of intermediates of glucose metabolism (Cogan et al., 1981). This likely was the reason why Leuconostoc strains failed to produce diacetyl and acetoin in the MRS medium or milk cultures with Lc. cremoris WG2 in the present study. Therefore, milk cultures with Leuconostoc strains and sodium citrate but without Lc. cremoris WG2 or yeast extract were acidified with H_3PO_4 after 24 hr of incubation at 30°C and then examined for volatile compounds produced after an additional 9 hr of incubation. The reason why these experiments were carried out was that since Leuconostoc can not grow in milk rapidly because of their weak proteolytic activity, they were expected to produce less inhibitory intermediates from lactose in milk. In these experiments, Lc. diacetylactis 18-16, Leuconostoc strain 14AM and Leuconostoc mesenteroides cj-1 were not examined. The uninoculated milk acidified with H_3PO_4 contained 25.9 ppm acetic acid so quantitative data for this compound were corrected accordingly. In milk cultures acidified with H_3PO_4, strain CAF7 only produced diacetyl and acetoin (Table 3.5). All strains produced ethanol and acetic acid, but CAF7 produced only an extremely small amount of ethanol compared to other strains. Strains 181 and 180 produced much less acetic acid than other strains; this also was true when they were grown in MRS media or milk cultures with Lc. cremoris WG2.

Citrate solutions

Since Leuconostoc strains, other than CAF7, did not produce detectable diacetyl and acetoin in milk cultures acidified by H_3PO_4, non-growing cells of strains of Leuconostoc and Lc. diacetylactis 18-16 were examined for diacetyl and acetoin production; the modified method of Jordan and Cogan (1988) was used for the analyses. The cells were washed in H_3PO_4 solutions and resuspended in the citrate solutions containing 50 mM of acetic acid, 30 mM of citric acid and 1 mM of glucose, adjusted to pH 5.0. After 4 hr of incubation at 30°C, strains other than 180, 181 and cj-1 produced diacetyl and acetoin in these solutions (Table 3.6). Lactococcus diacetylactis 18-16 and Leuconostoc strain 14AM produced the most diacetyl; strains 14AM and 104 produced the most acetoin. Strain CAF7 produced much less acetoin than other strains producing this compound. Strains 180, 181 and cj-1 produced much more ethanol than strains producing diacetyl and acetoin. Some strains apparently utilized the acetic acid initially present in the solutions. Also the amounts of acetic acid in all samples were not increased from that in control.

DISCUSSION

From the negative results for gas and dextran production, and the production of diacetyl and acetoin but almost no ethanol in MRS culture, strain 14AM was considered as Lc. diacetylactis. Orberg and Sandine (1984) reported that this strain has a minimum inhibitory concentration for vancomycin of <10 μg/ml, which was not common for Leuconostoc; also it contained a 5.5 Md plasmid. Kempler and McKay (1981) found that all strains of Lc. diacetylactis in their study contained 5.5 Md plasmids which encoded for citrate permease activity. Strain 104, which was labeled as Lu. cremoris, produced dextran on sucrose agar plate. So, this strain should be reclassified as dextran-producing Leuconostoc, such as Lu. dextranicum or Lu. mesenteroides.

The GLC column packing material used in this study was useful for analysis of volatile compounds produced in the different types of cultures. However, the packing and linear insert did cause some problems, such as absorption of some portion of diacetyl, acetoin and acetic acid. Jordan and Cogan (1988) reported that the decarboxylation of α-acetolactate to diacetyl and acetoin occurred spontaneously, and that high temperatures stimulated the conversion. This conversion might have been involved in analysis problems for diacetyl and acetoin in the present study. Also, in the analysis of milk cultures acidified with Lc. cremoris WG2, the packing material tended to be unstable and sometimes caused ghost peaks after many serial injections of samples. These problems could be resolved by injections of 0.1% ethanol solution and then holding

at 90°C to clean up and stabilize the packing material and linear insert.

Lactococcus diacetylactis 18-16 revealed its ability to produce diacetyl and acetoin from citrate in MRS medium or milk acidified with Lc. cremoris WG2. In MRS culture, the addition of $CuSO_4 \cdot H_2O$ and sodium ascorbate stimulated diacetyl production. Since the addition of sodium ascorbate to milk cultures with $CuSO_4 \cdot H_2O$ did not further increase diacetyl, $CuSO_4 \cdot H_2O$ was concluded to be the main cause for the stimulation. Mn^{2+} also has been reported to stimulate citrate metabolism in Leuconostoc by Reiter and Moller-Madsen (1963), but Cogan (1975) found that Mn^{2+} did not stimulate citrate utilization nor diacetyl and acetoin production by Lc. diacetylactis strains. In the experiments by Kaneko et al. (1987), the addition of Cu^{2+} to medium stimulated diacetyl production but suppressed growth and citrate utilization as compared to the medium without Cu^{2+}. The higher pH of MRS culture with added $CuSO_4 \cdot H_2O$ and sodium ascorbate than that without them supported the observed growth suppression in these experiments. Whether or not citrate utilization was suppressed was not examined in this study. Milk culture with Lc. diacetylactis 18-16 and Lc. cremoris WG2 contained less diacetyl but more acetoin than the MRS culture with the former strain. A reason for this was not found.

Thornhill and Cogan (1984) suggested the possibility of distinguishing between heterofermentative and homofermentative lactic acid bacteria by the amounts of ethanol produced. Amounts of this compound produced in the MRS cultures along with the production of gas by strains in the present study confirmed this.

However, Cogan (1987) found that some citrate-utilizing heterofermenters produced little or no ethanol when citrate was metabolized.

In milk cultures acidified with Lc. cremoris WG2, all strains of Leuconostoc produced ethanol, but less than they produced in the MRS cultures. It is likely that even with 0.1% added yeast extract, strains of Leuconostoc did not metabolize lactose as actively as glucose in the MRS broth. In a report by Thornhill and Cogan (1984), the author also proposed to distinguish between citrate-utilizing and non-citrate-utilizing lactic acid bacteria from the amounts of acetate produced from citrate. In MRS cultures, Leuconostoc strains clearly fell into two groups based on the amounts of acetic acid produced; one group held strains producing around 2,100 ppm, the other strains producing around 950 ppm. In milk cultures acidified with Lc. cremoris WG2, samples also were divided into two groups, one producing around 1,000 ppm and the other producing around 200 ppm of acetic acid. In this regard, strains 180, 181 and cj-1 of Leuconostoc were considered non-citrate-utilizing bacteria.

Milk culture inoculated with Lc. cremoris WG2 alone contained acetoin, but addition of Leuconostoc did not support acetoin production in the case of most strains. Only samples with strain CAF7 or 16-9 contained acetoin. Since the concentrations of acetoin in these two samples were higher than that with Lc. cremoris WG2 alone, most of the acetoin produced in the two samples apparently was produced by strains of the Leuconostoc. The reason why acetoin was not produced by Lc. cremoris WG2 in milk cultures

inoculated with this bacterium and Leuconostoc was not clear, but it might be due to competition between Lc. cremoris WG2 and Leuconostoc for citrate.

Milk medium inoculated with Leuconostoc strains were acidified with H_3PO_4 and analyzed for volatile compounds produced. All strains except CAF7 failed to produce diacetyl or acetoin in the milk cultures. Strain CAF7 produced diacetyl and acetoin, and much less ethanol than other strains. In solutions containing citric acid, acetic acid and glucose, all strains thought to be citrate-utilizing Leuconostoc produced diacetyl and acetoin, and less ethanol compared to that produced in other types of cultures. When strain CAF7 in milk culture acidified with H_3PO_4 and citrate-utilizing strains of Leuconostoc in the citrate solutions produced diacetyl, they always produced very small amounts of ethanol, suggesting diminished glucose metabolism. These findings supported the proposed theory of Cogan et al. (1981) that glucose metabolism inhibits diacetyl and acetoin production of Leuconostoc. Montville et al. (1987b) observed the same phenomenon in Lb. plantarum strains. Strains thought to be non-citrate-utilizing Leuconostoc did not produce diacetyl and acetoin in the citrate solutions.

Lactococcus diacetylactis strains18-16 and 14AM (the latter, re-classified as Lc. diacetylactis from results of the present study) produced much more diacetyl in citrate solutions than citrate-utilizing Leuconostoc strains, and also produced no ethanol. Since the number of cells in the citrate solutions was not determined, it was not appropriate to rank them by the amounts of diacetyl produced. Nonetheless, Lc. diacetylactis strains seemed to have

much greater ability to produce diacetyl than Leuconostoc strains. Collins (1972) reported that Lc. diacetylactis metabolized citrate in the presence of an additional energy source such as glucose or lactose. Since the citrate solutions contained only 1 mM glucose (0.018%), the mechanisms by which Lc. diacetylactis produced diacetyl in the citrate solutions was not clear.

Although the proposed inhibitory effect of glucose metabolism on diacetyl and acetoin production (Cogan et al., 1981) was supported in this study, Leuconostoc strains, especially Lu. cremoris, are known to produce diacetyl in milk acidified with Lc. cremoris or Lc. lactis (Pack et al., 1968; Cogan, 1985). Also, Walker and Gilliland (1987) reported diacetyl production by Lu. cremoris in acidified whey and buttermilk. They observed that Lu. cremoris grew in the whey acidified with lactic or acetic acids. These findings contradict results in the present study that almost all Leuconostoc strains did not produce diacetyl in milk cultures acidified with Lc. cremoris WG2 or H_3PO_4. Cogan et al. (1981) grew Lu. lactis in modified MRS broth at 30°C for 16 hr, then added citric acid to the culture, and reincubated. They found that after the addition of citric acid, the strain started to produce acetoin. Cultures grown before adding citric acid contained 34 mM of glucose (0.612%) which was supposed to inhibit acetoin production. They suggested that the acetoin production was caused by the balance between the inhibitory effect of glucose metabolism and the stimulatory effect of the medium components plus the lower pH. There was another factor to consider in diacetyl and acetoin production by Leuconostoc; this was the period of incubation. It was reported that even though Leuconostoc

strains produced diacetyl and acetoin, once citrate was depleted, those compounds were reduced by reductase enzymes and disappeared (Cogan, 1975; Cogan et al., 1981). The reason why milk cultures acidified with Lc. cremoris WG2 containing strain CAF7 or 16-9 contained acetoin but those with other citrate-utilizing strain did not might relate to these factors. Cogan (1987) proposed that more important reasons exist to explain why Leuconostoc do not produce diacetyl and acetoin in the presence of glucose or lactose. As far as the theory of the inhibitory effect of carbohydrate metabolism is concerned, among Leuconostoc strains able to produce diacetyl and acetoin, slow-growing or non-lactose-metabolizing strains should be good aroma producers in milk cultures. Actually, Leuconostoc strain CAF7 which was a slow grower, produced acetoin in milk acidified with Lc. cremoris WG2 and both diacetyl and acetoin when acidified with H_3PO_4. Although the mechanisms of diacetyl and acetoin production by Leuconostoc need to be studied further, GLC detection of diacetyl and acetoin produced in the citrate solutions was found to be very simple and exact. It should provide a useful method to study further diacetyl production by these bacteria.

Table 3.1. Strains used in this study.

Strain	Source	Gas from glucose	Dextran from sucrose
Lactococcus diacetylactis 18-16	This laboratory	-	-
Leuconostoc sp. strain 16-9	This laboratory	+	+
Leuconostoc sp. strain 14AM	This laboratory	-	-
Leuconostoc sp. strain p325	This laboratory	+	-
Leuconostoc cremoris 104	This laboratory	+	+
Leuconostoc cremoris J	S. E. Gilliland[a]	+	-
Leuconostoc cremoris CAF7	This laboratory	+	-
Leuconostoc dextranicum 180	This laboratory	+	+
Leuconostoc dextranicum 181	This laboratory	+	+
Leuconostoc mesenteroides cj-1	This laboratory	+	+

[a] Department of Animal Science, Oklahoma State University.

Table 3.2. Reproducibility[a] of the known amounts of volatile compounds in different media as determined by the Internal Standard calculation procedure.

	Std. solut.		MRS broth		Milk with lactate		Milk with H_3PO_4	
Compound	Av.	SD	Av.	SD	Av.	SD	Av.	SD
Acetaldehyde	9.90	0.50	8.79	1.13	11.01	1.65	NC	
Ethanol	950.0	8.2	955.2	8.7	951.5	13.4	1004.4	4.95
Diacetyl	5.00	0.13	4.97	0.15	5.38	0.23	5.58	0.13
Propanol	5.00	0.19	4.58	0.16	NC		NC	
Acetoin	92.5	3.6	76.2	0.7	81.9	3.4	100.4	3.4
Acetic acid	999.0	17.5	NC		1016.0	88.0	1021.7	75.0

[a] Data are shown in ppm (v/v). Data for standard solution were from 3 injections and those for other media were from 5 injections. Abbreviation: Std. solut. = standard solution; Av. = average; SD = standard deviation; NC = not checked.

Table 3.3. Concentration[a](v/v) of volatile compounds produced by various strains in MRS medium incubated at 30°C for 20 hr.

Strain	pH	Acet-aldeh. (ppm)	Ethyl alcoh. (ppm)	Diac-etyl (ppm)	Pro. alcoh. (ppm)	Acet-oin (ppm)	Acetic acid (ppm)
Lc. diacet. 18-16	4.39	10.9	ND	1.6	ND	634.2	1,950
Lc. diacet. 18-16 (with $CuSO_4.H_2O$ and sodium ascorbate)	4.73	8.9	ND	4.7	ND	514.9	2,080
Lu. sp. strain 16-9	4.13	5.7	3,410	ND	0.3	ND	2,120
Lu. sp. strain 14AM	4.29	19.8	10	1.2	ND	338.3	1,980
Lu. sp. strain p325	4.32	6.5	2,520	ND	0.4	ND	2,120
Lu. cremoris 104	4.02	2.8	4,220	ND	0.6	ND	2,120
Lu. cremoris J	4.15	ND	4,590	ND	0.6	ND	2,200
Lu. cremoris CAF7[b]	5.03	ND	910	ND	ND	ND	1,540
Lu. dextran. 180	4.45	ND	2,330	ND	ND	ND	950
Lu. dextran. 181	4.55	5.6	1800	ND	0.2	ND	920
Lu. mesent. cj-1	4.04	7.7	3,940	ND	0.6	ND	970

[a] Average of two injections. [b] Incubated for 43 hr.
Abbreviation: Acetaldeh. = acetaldehyde; Ethyl alcoh. = ethanol; Pro. alcoh. = propanol; Lc. diacet. = Lactococcus diacetylactis; Lu. = Leuconostoc; Lu. dextran. = Leuconostoc dextranicum; Lu. mesent. = Leuconostoc mesenteroides; ND = not detected.

Table 3.4. Concentration[a](v/v) of volatile comounds produced by various strains in milk cultures acidified with Lactococcus cremoris WG2 and incubated at 30°C for 24hr.

Strain	pH	Acet-aldeh. (ppm)	Ethyl alcoh. (ppm)	Diac-etyl (ppm)	Acet-oin (ppm)	Acetic acid (ppm)
Lc. cremoris WG2 alone	4.42	ND	6.0	ND	8.0	65.5
+Lc. diacetylactis 18-16	4.66	8.9	ND	0.6	917.5	801.3
+Lc. diacetylactis 18-16 (with $CuSO_4 \cdot H_2O$)	4.50	8.5	ND	1.5	880.9	827.1
+Lc. diacetylactis 18-16 (with $CuSO_4 \cdot H_2O$ and sodium ascorbate)	4.47	7.6	0.7	1.6	1,183.8	1,162.8
+Lu. sp. strain 16-9	4.43	ND	1,497.9	ND	26.5	1,063.4
+Lu. sp. strain 14AM	4.48	8.3	1.8	1.0	927.7	1,054.3
+Lu. sp. strain p325	4.54	ND	971.6	ND	ND	1,066.8
+Lu. cremoris 104	4.48	ND	919.7	ND	ND	1,039.5
+Lu. cremoris J	4.34	ND	1,626.3	ND	ND	1,516.8
+Lu. cremoris CAF7	4.53	ND	220.2	ND	73.1	1,157.9
+Lu. dextranicum 180	4.30	ND	580.8	ND	ND	150.0
+Lu. dextranicum 181	4.30	ND	585.5	ND	ND	166.6
+Lu. mesenteroides cj-1	4.29	ND	1,069.9	ND	ND	240.8

[a] Average of two injections.

Abbreviation: Acetaldeh. = acetaldehyde; Ethyl alcoh. = ethanol; Lc. = Lactococcus ; Lu. = Leuconostoc; ND = not detected.

Table 3.5. Concentration[a](v/v) of volatile comounds produced by various strains in milk cultures[b] acidified with H_3PO_4 (pH=4.5) and incubated at 30°C for 9 hr.

Strain	Ethyl alcoh. (ppm)	Diac-etyl (ppm)	Acet-oin (ppm)	Acetic acid (ppm)
Lu. sp. strain 16-9	492.0	ND	ND	1,712.0
Lu. sp. strain p325	1,582.1	ND	ND	1,194.8
Lu. cremoris 104	970.6	ND	ND	1,264.1
Lu. cremoris J	254.7	ND	ND	1,716.0
Lu. cremoris CAF7	31.3	2.4	52.8	1,489.6
Lu. dextranicum 180	560.4	ND	ND	175.4
Lu. dextranicum 181	743.8	ND	ND	161.4

[a] Average of two injections. [b] Preincubated at 30°C for 24 hr before adding H_3PO_4.
Abbreviation: Ethyl alcoh. = ethanol; Lu. = Leuconostoc; ND = not detected.

Table 3.6. Concentration[a](v/v) of volatile compounds produced by various strains in citrate solutions incubated at 30°C for 4 hr.

Strain	Ethyl alcoh. (ppm)	Diac-etyl (ppm)	Acet-oin (ppm)	Acetic acid (ppm)
Control	ND	ND	ND	2,787.5
Lc. diacetylactis 18-16	ND	26.9	423.0	1,779.2
Lu. sp. strain 16-9	21.6	3.0	253.0	1,696.5
Lu. sp. strain 14AM	ND	25.3	494.5	939.9
Lu. sp. strain p325	9.9	7.0	415.6	1,717.9
Lu. cremoris 104	21.6	12.8	481.9	1,408.6
Lu. cremoris J	4.8	7.9	192.2	2,560.9
Lu. cremoris CAF7	4.1	4.6	43.8	2,103.4
Lu. dextranicum 180	71.6	ND	ND	1,716.4
Lu. dextranicum 181	51.6	ND	ND	2,617.9
Lu. mesenteroides cj-1	62.3	ND	ND	1,898.8

[a] Average of two injections.
Abbreviation: Ethyl alcoh. = ethanol; Lc. = Lactococcus; Lu. = Leuconostoc; ND = not detected.

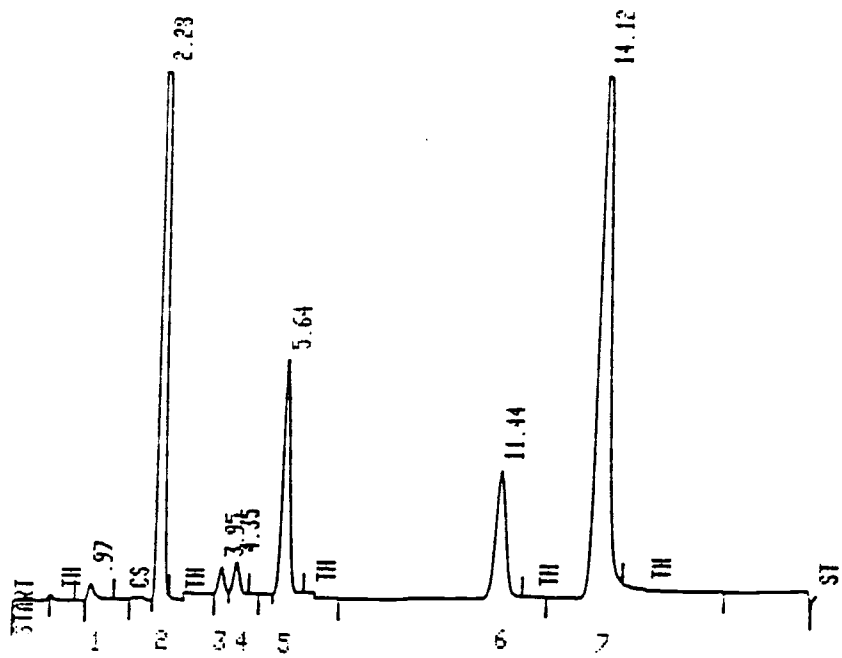

Fig. 3.1. Chromatogram of standard solution.
Peaks: 1 = acetaldehyde (9.9 ppm); 2 = ethanol (950 ppm); 3 = diacetyl (5 ppm); 4 = propanol (5 ppm); 5 = sec-butanol as Internal Standard (49.5 ppm); 6 = acetoin (92.5 ppm); 7 = acetic acid (999 ppm).

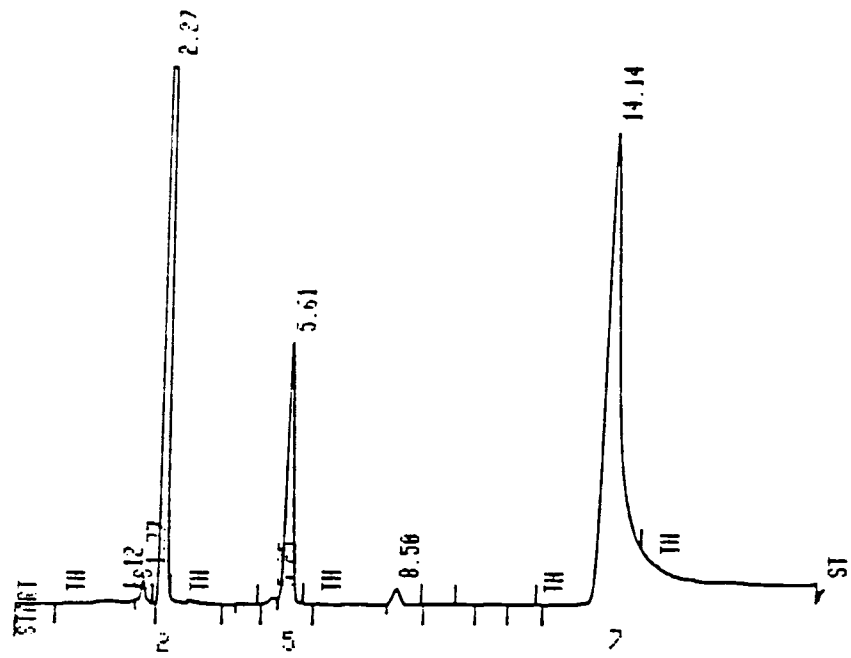

Fig. 3.2. Chromatogram of MRS culture of Leuconostoc cremoris CAF7.
Peaks: 2 = ethanol; 5 = sec-butanol as Internal Standard; 7 = acetic acid.

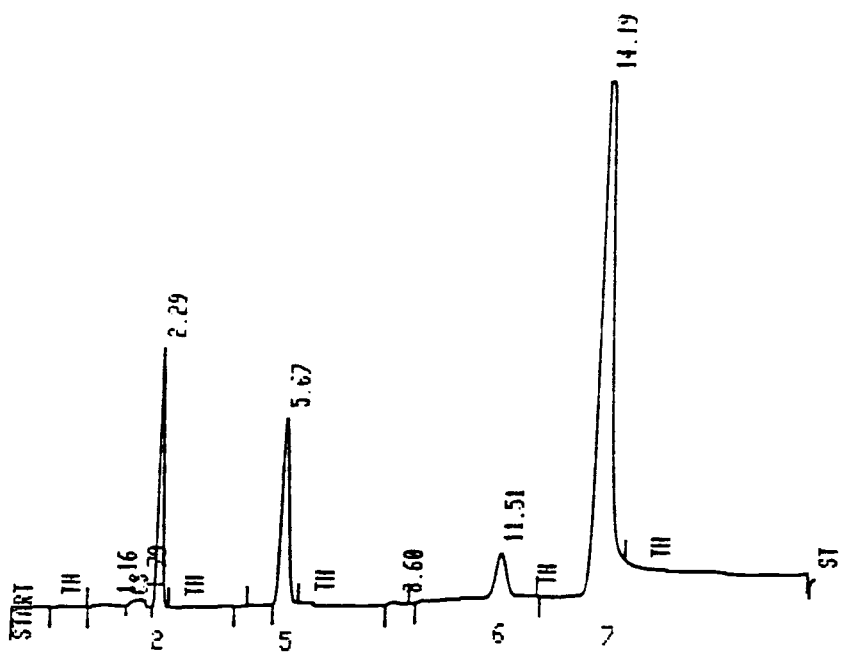

Fig. 3.3. Chromatogram of milk culture of Leuconostoc cremoris CAF7 acidified with Lactococcus cremoris WG2.
Peaks: 2 = ethanol; 5 = sec-butanol as Internal Standard; 6 = acetoin; 7 = acetic acid.

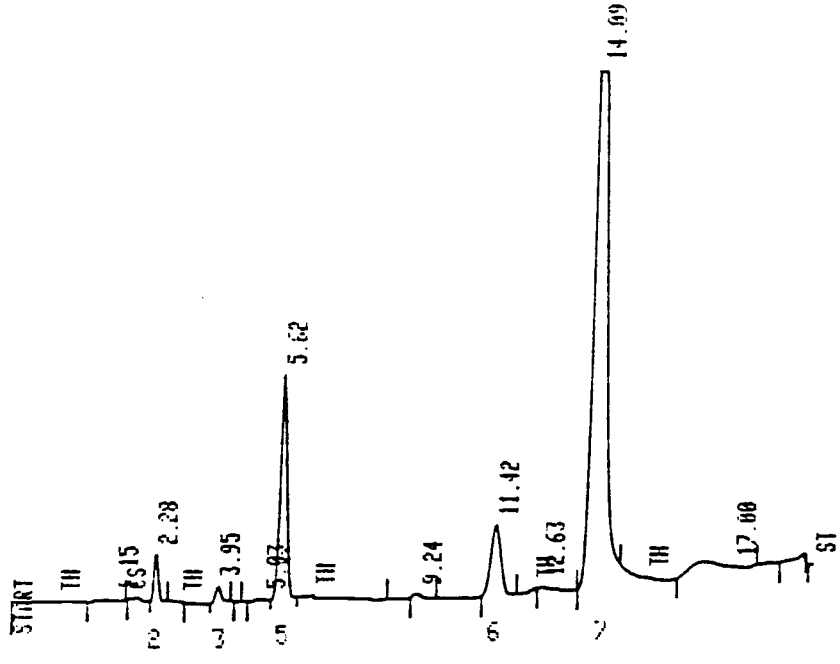

Fig. 3.4. Chromatogram of milk culture of Leuconostoc cremoris CAF7 acidified with H_3PO_4 .
Peaks: 2 = ethanol; 3 = diacetyl; 5 = sec-butanol as Internal Standard; 6 = acetoin; 7 = acetic acid.

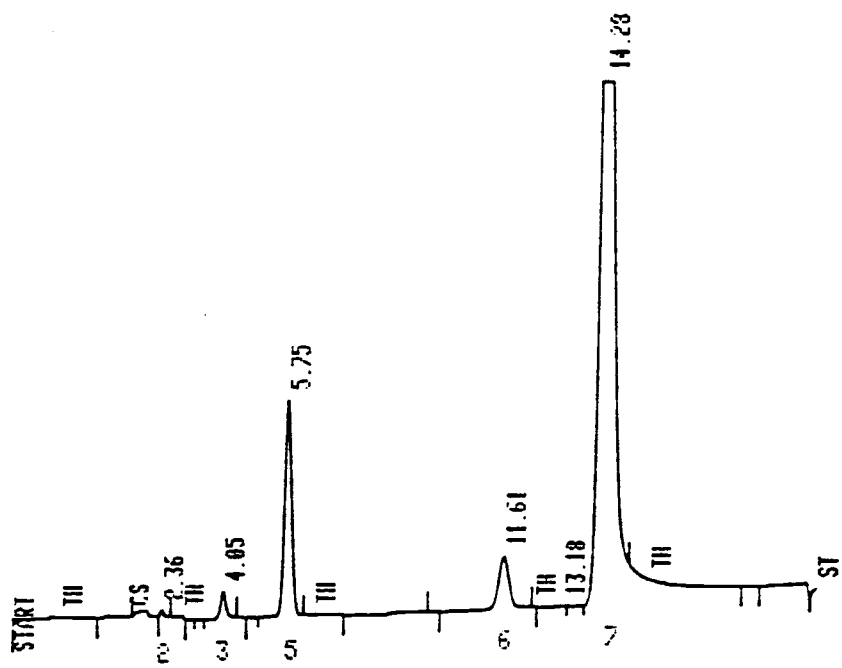

Fig. 3.5. Chromatogram of citrate solution containing Leuconostoc cremoris CAF7.
Peaks: 2 = ethanol; 3 = diacetyl; 5 = sec-butanol as Internal Standard; 6 = acetoin; 7 = acetic acid.

REFERENCES

Cogan, T. M. 1975. Citrate utilization in milk by Leuconostoc cremoris and Streptococcus diacetylactis. J. Dairy Res. 42:139-146.

Cogan, T. M., M. O'Dowd, and D. Mellerick. 1981. Effects of pH and sugar on acetoin production from citrate by Leuconostoc lactis. Appl. Environ. Microbiol. 41:1-8.

Cogan, T. M. 1985. The leuconostocs: milk products, P.25-40. In S. E. Gilliland (ed.), Bacterial Starter Cultures for Foods. CRC Press, Inc., Boca Raton, Fla.

Cogan, T. M. 1987. Co-metabolism of citrate and glucose by Leuconostoc spp.: effects on growth, substrates and products. J. Appl. Bacteriol. 63:551-558.

Collins, E. B. 1972. Biosynthesis of flavor compounds by microorganisms. J. Dairy Sci. 55:1022-1028.

Di Corcia, A., R. Samperi, and C. Severini. 1980. Gas chromatographic column for the rapid determination of congeners in potable spirits. J. Chromatogr. 198:347-353.

Doelle, H. W. 1969. Improved gas chromatographic separation and determination of acetone, diacetyl and C_1-C_7 alcohols. J. Chromatogr. 42:541-543.

Hill, E. C., C. W. Wenzel, and A. Barreto. 1954. Colorimetric method for detection of microbiological spoilage in citrus juices. Food Technol. 8:168-171.

Jordan, K. M. and T. M. Cogan. 1988. Production of acetolactate by Streptococcus diacetylactis and Leuconostoc spp. J. Dairy Res. 55:227-238.

Kaneko, T., H. Suzuki, and T. Takahashi. 1986. Diacetyl formation and degradation by Streptococcus lactis subsp. diacetylactis 3022. Agric. Biol. Chem. 50:2639-2641.

Kaneko, T., H. Suzuki, and T. Takahashi. 1987. The effects of metal ions on diacetyl production by Streptococcus lactis subsp. diacetylactis 3022. Agric. Biol. Chem. 51:2315-2320.

Keen, A. R. and N. J. Walker. 1973. Separation of diacetyl, acetoin and 2,3-butylene glycol by ion-exchange chromatography. Anal. Biochem. 52:475-481.

Kempler, G. M. and L. L. McKay. 1981. Biochemistry and genetics of citrate utilization in Streptococcus lactis subsp. diacetylactis. J. Dairy Sci. 64:1527-1539.

Lee, S. M. and D. B. Drucker. 1975. Analysis of acetoin and diacetyl in bacterial culture supernatants by gas-liquid chromatography. J. Clin. Microbiol. 2:162-164.

de Man, J. C., M. Rogosa, and M. E. Sharpe, 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. 23:130-135.

McKay, L. L. and K. A. Baldwin. 1974. Altered metabolism in Streptococcus lactis C2 mutant deficient in lactate dehydrogenase. J. Dairy Sci. 57:181-186.

Montville, T. J., M. E. Meyer, A. H.-M. Hsu, and G. T.-C. Huang. 1987a. High pressure liquid chromatography methods for quantification of acetoin and diacetyl from bacterial cultures. J. Microbiol. Methods 7:1-8.

Montville, T. J., M. E. Meyer, and A. H.-M. Hsu. 1987b. Influence of carbon substrates on lactic acid, cell mass and diacetyl-acetoin production in Lactobacillus plantarum. J. Food Protect. 50:42-46.

Orberg, P. K. and W. E. Sandine. 1984. Common occurrence of plasmid DNA and vancomycin resistance in Leuconostoc spp. Appl. Environ. Microbiol. 48:1129-1133.

Owades, J. L. and J. A. Jakovac. 1963. Microdetermination of diacetyl in beer. Proc. Am. Soc. Brewing.

Pack, M. Y., W. E. Sandine, P. R. Elliker, E. A. Day, and R. C. Lindsay. 1964. Owades and Jakovac method for diacetyl determination in mixed strain starters. J. Dairy Sci. 47:981-986.

Pack, M. Y., E. R. Vedamuthu, W. E. Sandine, P. R. Elliker, and H. Leesment. 1968. Effect of temperature on growth and diacetyl production by aroma bacteria in single and mixed strain lactic cultures. J. Dairy Sci. 51:339-344.

Palo, V. and H. Ilkova. 1970. Direct gas chromatographic estimation of lower alcohols, acetaldehyde, acetone and diacetyl in milk products. J. Chromatogr. 53:363-367.

Reiter, B. and A. Moller-Madsen. 1963. Reviews on the progress of dairy science. Section B. Cheese and butter starters. J. Dairy Res. 30:419-456.

Speckman, R. A. and E. B. Collins. 1968. Separation of diacetyl, acetoin, and 2,3-butylene glycol by salting out chromatography. Anal. Biochem. 22:154-160.

Terzaghi, B. E. and W. E. Sandine. 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. 29:807-813.

Thornhill, P. J. and T. M. Cogan. 1984. Use of gas-liquid chromatography to determine the end products of growth of lactic acid bacteria. Appl. Environ. Microbiol. 47:1250-1254.

Walker, D. K. and S. E. Gilliland. 1987. Buttermilk manufacture using a combination of direct acidification and citrate fermentation by Leuconostoc cremoris. J. Dairy Sci. 70:2055-2062.

Walsh, B. and T. M. Cogan. 1974a. Separation and estimation of diacetyl and acetoin in milk. J. Dairy Res. 41:25-30.

Walsh, B. and T. M. Cogan. 1974b. Further studies on the estimation of diacetyl by the methods of Prill and Hammer and Owades and Jakovac. J. Dairy Res. 41:31-35.

Westerfeld, W. W. 1945. A colorimetric determination of blood acetoin. J. Biol. Chem. 161:495-502.

BIBLIOGRAPHY

Baker, J. C. 1957. The effects of yeast on bread flavor. Bakers Digest 31(5):64-68, 79.

Bundus, R. H. and A. J. Luksas. 1969. U.S. Patent 3,466,174.

Carlin, G. T. 1958. The fundamental chemistry of bread making I. Proc. Am. Soc. Bakery Engr.:56-63.

Carlin, G. T. 1959. The fundamental chemistry of bread making II. Proc. Am. Soc. Bakery Engr.:136-147.

Cogan, T. M. 1975. Citrate utilization in milk by Leuconostoc cremoris and Streptococcus diacetylactis. J. Dairy Res. 42:139-146.

Cogan, T. M., M. O'Dowd, and D. Mellerick. 1981. Effects of pH and sugar on acetoin production from citrate by Leuconostoc lactis. Appl. Environ. Microbiol. 41:1-8.

Cogan, T. M. 1985. The leuconostocs: milk products, P.25-40. In S. E. Gilliland (ed.), Bacterial Starter Cultures for Foods. CRC Press, Inc., Boca Raton, Fla.

Cogan, T. M. 1987. Co-metabolism of citrate and glucose by Leuconostoc spp.: effects on growth, substrates and products. J. Appl. Bacteriol. 63:551-558.

Collins, E. B. 1972. Biosynthesis of flavor compounds by microorganisms. J. Dairy Sci. 55:1022-1028.

Di Corcia, A., R. Samperi, and C. Severini. 1979. Modified graphitized carbon black for the gas chromatographic analysis of alcoholic beverages. J. Chromatogr. 170:245-248.

Di Corcia, A., R. Samperi, and C. Severini. 1980. Gas chromatographic column for the rapid determination of congeners in potable spirits. J. Chromatogr. 198:347-353.

Doelle, H. W. 1969. Improved gas chromatographic separation and determination of acetone, diacetyl and C_1-C_7 alcohols. J. Chromatogr. 42:541-543.

Dziezak, J. D. 1986. Biotechnology and flavor development; an industrial research perspective. Food Technol. 40(4):108-113, 120.

Fowell, M. S. 1967. Infection control in yeast factories and breweries. Process Biochem. 2:11-15.

Gilliland, S. E. and M. L. Speck. 1977. Use of Minitek system for characterizing lactobacilli. Appl. Environ. Microbiol. 33:1289-1292.

Gilliland, S. E. 1985. Role of starter culture bacteria in food preservation, p.175-185. In S. E. Gilliland (ed.), Bacterial Starter Cultures for Foods. CRC Press, Inc., Boca Raton, Fla.

Harvey, R. J. and E. B. Collins. 1961. Role of citritase in acetoin formation by Streptococcus diacetylactis and Leuconostoc citrovorum. J. Bacteriol. 82:954-959.

Harvey, R. J. and E. B. Collins. 1962. Citrate transport system of Streptococcus diacetylactis. J. Bacteriol. 83:1005-1009.

Hill, E. C., C. W. Wenzel, and A. Barreto. 1954. Colorimetric method for detection of microbiological spoilage in citrus juices. Food Technol. 8:168-171.

Hino, A., H. Takano., and Y. Tanaka. 1987. New freeze-tolerant yeast for frozen dough preparations. Cereal Chem. 64:269-275.

Hironaka, Y. 1985a. Effect of fermentation conditions on flavor substances in French bread produced by the straight dough method. Nippon Shokuhin Kogyo Gakkaishi 32:486-492.

Hironaka, Y. 1985b. Effect of fermentation conditions on flavor substances in sweet baked goods produced by the sugar containing sponge dough method. Nippon Shokuhin Kogyo Gakkaishi 32:592-596.

Imrie, F. K. E. and R. H. Tilbury. 1972. Polysaccharides in sugar cane and its products. Sugar Technol. Rev. 1:291-361.

Jackel, S. S. 1963. U.S. Patent 3,102,033.

Jackel, S. S. 1969. Fermentation flavors of white breads. Bakers digest 43(5):24-28, 64.

Johnson, J. A. and B. S. Miller. 1957. Pre-ferments. Bakers Digest 31(3):30-35, 76-77.

Jordan, K. M. and T. M. Cogan. 1988. Production of acetolactate by Streptococcus diacetylactis and Leuconostoc spp. J. Dairy Res. 55:227-238.

Kaneko, T., H. Suzuki, and T. Takahashi. 1986. Diacetyl formation and degradation by Streptococcus lactis subsp. diacetylactis 3022. Agric. Biol. Chem. 50:2639-2641.

Kaneko, T., H. Suzuki, and T. Takahashi. 1987. The effects of metal ions on diacetyl production by Streptococcus lactis subsp. diacetylactis 3022. Agric. Biol. Chem. 51:2315-2320.

Keen, A. R. and N. J. Walker. 1973. Separation of diacetyl, acetoin and 2,3-butylene glycol by ion-exchange chromatography. Anal. Biochem. 52:475-481.

Keenan, T. W. 1968. Production of acetic acid and other volatile compounds by Leuconostoc citrovorum and Leuconostoc dextranicum. Appl. Microbiol. 16:1881-1885.

Kempler, G. M. and L. L. McKay. 1980. Improved medium for detection of citrate-fermenting Streptococcus lactis subsp. diacetylactis. Appl. Environ. Microbiol. 39:926-927.

Kempler, G. M. and L. L. McKay. 1981. Biochemistry and genetics of citrate utilization in Streptococcus lactis subsp. diacetylactis. J. Dairy Sci. 64:1527-1539.

Kohn, F. E., L. Wiseblatt., and L. S. Fosdick. 1961. Some volatile carbonyl compounds arising during panary fermentation. Cereal Chem. 38:165-169.

Lee, S. M. and D. B. Drucker. 1975. Analysis of acetoin and diacetyl in bacterial culture supernatants by gas-liquid chromatography. J. Clin. Microbiol. 2:162-164.

Liebs, P., M. Krueger, and H. C. Wolter. 1969. Removal of diacetyl from beer. German (East) Patent 1,801,509. Nov. 6.

Linko, Y., B. S. Miller, and J. A. Johnson. 1962. Quantitative determination of certain carbonyl compounds in pre-ferments. Cereal Chem. 39:263-272.

Maga, J. A. 1974. Bread flavor. CRC Crit. Rev. Food Technol. 5:55-142.

de Man, J. C., M. Rogosa, and M. E. Sharpe, 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. 23:130-135.

McKay, L. L. and K. A. Baldwin. 1974. Altered metabolism in Streptococcus lactis C2 mutant deficient in lactate dehydrogenase. J. Dairy Sci. 57:181-186.

Mitsuoka, T. 1969. Recent trends in the taxonomy of lactobacilli. J. of the Food Hygienic Society of Japan 10:147-156.

Montville, T. J., M. E. Meyer, A. H.-M. Hsu, and G. T.-C. Huang. 1987a. High pressure liquid chromatography methods for quantification of acetoin and diacetyl from bacterial cultures. J. Microbiol. Methods 7:1-8.

Montville, T. J., M. E. Meyer, and A. H.-M. Hsu. 1987b. Influence of carbon substrates on lactic acid, cell mass and diacetyl-acetoin production in Lactobacillus plantarum. J. Food Protect. 50:42-46.

Orberg, P. K. and W. E. Sandine. 1984. Common occurrence of plasmid DNA and vancomycin resistance in Leuconostoc spp. Appl. Environ. Microbiol. 48:1129-1133.

Owades, J. L. and J. A. Jakovac. 1963. Microdetermination of diacetyl in beer. Proc. Am. Soc. Brewing.

Pack, M. Y., W. E. Sandine, P. R. Elliker, E. A. Day, and R. C. Lindsay. 1964. Owades and Jakovac method for diacetyl determination in mixed strain starters. J. Dairy Sci. 47:981-986.

Pack, M. Y., E. R. Vedamuthu, W. E. Sandine, P. R. Elliker, and H. Leesment. 1968. Effect of temperature on growth and diacetyl

production by aroma bacteria in single and mixed strain lactic cultures. J. Dairy Sci. 51:339-344.

Palo, V. and H. Ilkova. 1970. Direct gas chromatographic estimation of lower alcohols, acetaldehyde, acetone and diacetyl in milk products. J. Chromatogr. 53:363-367.

Reed, G. and H. J. Peppler. 1973. Yeast Technology. AVI Publishing Company, Inc., Westport, Connecticut.

Reiter, B. and A. Moller-Madsen. 1963. Reviews on the progress of dairy science. Section B. Cheese and butter starters. J. Dairy Res. 30:419-456.

Robinson, R. J., T. H. Lord, J. A. Johnson, and B. S. Miller. 1958. The aerobic microbiological population of pre-ferments and the use of selected bacteria for flavor production. Cereal Chem. 35:295-305.

Rogosa, M. 1974. Lactobacillus, p.576-593. In R. E. Buchanan and N. E. Gibbons (ed.), Bergey's Manual of Determinative Bacteriology, 8th ed. The Williams and Wilkins Co., Baltimore.

Samson, F. E., A. M. Katz, and D. L. Harris. 1955. Effects of acetate and other short-chain fatty acids on yeast metabolism. Archives of Biochemistry and Biophysics 54:406-423.

Sandine, W. E. and P. R. Elliker. 1970. Microbially induced flavors in fermented foods. Agri. and Food Chem. 18:557-562.

Schulz, A. 1966. Fundamentals of rye bread production. Baker's Digest 40(4):77-80.

Sharpell, F. H. 1985. Microbial flavors and fragrances, p.965-981. In M. Moo-Young et al. (ed.), Comprehensive Biotechnology, Vol. 3. Pergamon Press.

Smith, D. E. and J. R. Coffman. 1960. Analysis of food flavors by gas-liquid chromatography. Analytical Chemistry 32:1733-1737.

Speckman, R. A. and E. B. Collins. 1968. Separation of diacetyl, acetoin, and 2,3-butylene glycol by salting out chromatography. Anal. Biochem. 22:154-160.

Sugihara, T. F., L. Kline, and L. B. Mcgready. 1970. Nature of the San Francisco sour dough French Bread process. II. Microbiological aspects. Bakers Digest 44(2):51-57.

Sugihara, T. F. 1978. Microbiology of the soda cracker process. J. Food Protect. 41:977-979.

Sugihara, T. F. 1985. The lactobacilli and streptococci: bakery products, p.119-125. In S. E. Gillilanbd (ed.), Bacterial Starter Cultures for Foods. CRC Press, Inc., Boca Raton, Fla.

Takeda, T., S. Okada, and M. Kozaki. 1984. Characteristics of lactic acid bacteria isolated from bread-dough, compressed-yeast and active dry-yeast. Nippon Shokuhin Kogyo Gakkaishi 31:642-648.

Terzaghi, B. E. and W. E. Sandine. 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. 29:807-813.

Thomas, B. and M. Rothe. 1957. Zur frage der beteiligung von acetoin and diacetyl am aroma des weiβbrotes. Ernahrungsforschung 2:751-757.

Thornhill, P. J. and T. M. Cogan. 1984. Use of gas-liquid chromatography to determine the end products of growth of lactic acid bacteria. Appl. Environ. Microbiol. 47:1250-1254.

Vedamuthu, E. R. 1982. Fermented milks, p.199-226. In A. H. Rose (ed.), Fermented Foods. Academic Press.

Visser't Hooft, F. and F. J. G. deLeeuw. 1935. The occurrence of acetylmethylcarbinol in bread and its relation to bread flavor. Cereal Chem. 12:213-229.

Walker, D. K. and S. E. Gilliland. 1987. Buttermilk manufacture using a combination of direct acidification and citrate fermentation by Leuconostoc cremoris. J. Dairy Sci. 70:2055-2062.

Walsh, B. and T. M. Cogan. 1974a. Separation and estimation of diacetyl and acetoin in milk. J. Dairy Res. 41:25-30.

Walsh, B. and T. M. Cogan. 1974b. Further studies on the estimation of diacetyl by the methods of Prill and Hammer and Owades and Jakovac. J. Dairy Res. 41:31-35.

Westerfeld, W. W. 1945. A colorimetric determination of blood acetoin. J. Biol. Chem. 161:495-502.

White, J. 1954. Yeast Technology. Champan and Hall, London.

Wiseblatt, L. and F. E. Kohn. 1960. Some volatile aromatic compounds in fresh bread. Cereal Chem. 37:55-66.