AN ABSTRACT OF THE THESIS OF

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Title: EFFECT OF SUCROSE ON THE PRODUCTION OF FLAVOR COMPOUNDS BY YOGURT CULTURE BACTERIA

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Organoleptically, flavored yogurts appear to contain lower levels of acetaldehyde than plain yogurt. This study was undertaken to seek the reasons for this phenomenon by focusing on the analyses of acetaldehyde production by yogurt culture bacteria in yogurt base containing added sucrose and by performing other analyses such as the determination of cell numbers, pH, titratable acidity, volatile acidity and average flavor threshold (AFT) values of acetaldehyde in various media.

The volatile compounds produced by yogurt culture bacteria were trapped and chromatographed by a gas entrainment on-column trapping gas-liquid chromatographic technique. Acetaldehyde was identified by coincidence of retention time with that of the authentic compound and quantitated using methyl acetate as an internal standard.

Acetaldehyde production by mixed cultures was first

detectably inhibited by 8% sucrose. However, acid production and cell counts of both species, grown together in the mixed culture, were inhibited by concentrations of sucrose of 4% and higher. A rapid production of acetaldehyde at concentrations between 0% and 8% sucrose occurred between 2 and 6 hr incubation times. The level of this compound then decreased up to 15 hr incubation and leveled off with continued incubation up to 24 hr.

By itself, Lactobacillus bulgaricus was stimulated by 4%, 8% and 12% sucrose resulting in the high production of acetaldehyde and lactic acid and increased cell numbers in comparison with cultures grown in media containing no added sucrose. A level of 8% sucrose was most effective. The rod continuously produced acetaldehyde up to 24 hr, suggesting it is responsible for the production of high concentrations of acetaldehyde in yogurt.

Acetaldehyde production by Streptococcus thermophilus varied from strain to strain. In general, however, less acid was produced and microbial numbers were lower, as sucrose content increased. The coccus produced much lower amounts of acetaldehyde at equivalent incubation times as compared with the rod.

During refrigerated storage of from 1 to 14 days, acetaldehyde concentration greatly decreased in media containing both 0% and 8% sucrose.

Values for volatile acidity of yogurts containing 0% to 8%

sucrose were low. There was little or no difference in volatile acidities between yogurts containing 0% and 8% sucrose.

Average flavor threshold values for acetaldehyde were slightly higher in 2%-fat milk with 8% added sucrose than in plain 2%-fat milk. With the addition of both strawberry flavor and 8% sucrose, the AFT of acetaldehyde was much higher.

Since there were no great differences in the levels of acetaldehyde found in yogurts containing 0%, 4% or 8% sucrose (8% being the amount ordinarily added to flavored yogurt), it is suggested that the strong masking effect exerted by fruit, fruit flavor and sucrose, as demonstrated by AFT values, is largely responsible for the organoleptic sensation of lower levels of acetaldehyde in flavored yogurt.

Effect of Sucrose on the Production of Flavor Compounds by Yogurt Culture Bacteria

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INTRODUCTION

Yogurt is commonly manufactured from milk adjusted to contain about 2%-fat and 12% solid-not-fat. Flavored yogurt contains, in addition, flavoring agents which are most frequently fruit flavor and bits of fruit plus added sucrose to enhance the sweetness. A few non-fruit flavors such as vanilla-flavored yogurt are produced. The latter also contain added sucrose. In the manufacture of fruit-flavored yogurt, fruit may be added to the yogurt carton before fermentation or mixed with plain yogurt after incubation.

Experienced dairy products judges have noted that flavored yogurt seems, organoleptically, to contain less acetaldehyde than plain yogurt. Since the flavor of plain yogurt depends so heavily on the level of acetaldehyde, it is of interest to know the reason for the observed lower level of acetaldehyde in flavored yogurt.

Since sucrose is generally known to inhibit microorganisms at moderate or high concentrations, the added sucrose might be expected to influence cell growth and acetaldehyde production during the fermentation process. Up to the present time, the effect of sucrose on acetaldehyde production by yogurt culture bacteria has not been reported in the literature.

The purpose of this study was to investigate the effect of sucrose on acetaldehyde production by single strains and mixed cultures of yogurt culture bacteria.

REVIEW OF LITERATURE

Flavor of Yogurt

Yogurt is a cultured dairy product fermented by the symbiotic action of Strepococcus thermophilus and Lactobacillus bulgaricus. The important characteristics which determine the quality of yogurt are aroma, taste, consistency and appearance. According to Davis (1967), desirable characteristics are achieved through the use of: (a) good quality milk supply, free from antibiotics and other bactericidal substances, (b) correct heat treatment of the milk, (c) vigorous pure cultures or a controlled mixture, (d) clean, sterile equipment, (e) proper inoculation and incubation (time and temperature).

Lactic acid is the major metabolic product of the fermentation of lactose by lactic acid bacteria. Pure lactic acid is odorless and non-volatile, thus it does not contribute to the odor, but is responsible for the acid taste of cultures and cultured dairy products (Hammer and Babel, 1943).

Pette and Lolkema (1950c), during a study of the carbonyl compounds of yogurt, found acetaldehyde in the steam distillate. These scientists correlated flavor and odor with acidity and with the amount of 2,4 dinitrophenlyhydrazone derivatives of carbonyl compounds in the distillate.

A few years later, Schulz and Hingst (1954) confirmed that acetaldehyde is a major, important flavor component of yogurt and obtained the characteristic flavor of yogurt when they added 50 parts per million (ppm) of acetaldehyde to milk soured with S. thermophilus. They concluded that although acetaldehyde is a flavor component, it alone is not entirely responsible for typical yogurt flavor.

Further studies have been pursued and have shown that acetaldehyde is a very important flavor component required to give the full flavor of yogurt and that it is produced mainly by L. bulgaricus rather than S. thermophilus (Schulz and Hingst, 1954; Davis, 1956b; Bottazzi and Vescovo, 1969; Görner et al., 1968). Görner et al. (1968) reported that acetaldehyde increases continuously during the first 6-8 hours of incubation and then decreases upon continued incubation. The best flavor was obtained when the concentration of acetaldehyde was between 23.1 ppm and 41.0 ppm.

Acetaldehyde can be produced by homofermentative lactic acid bacteria by decarboxylation of pyruvic acid which is formed by transamination of alanine or mainly by the metabolism of glucose or lactose (Harvey, 1960; Keenan and Bills, 1968b; Görner et al., 1968). Also it can be produced by streptococci from thymidine by thymidine phosphorylase, deoxyriboadolase and deoxyribomutase (Sandine and Elliker, 1970).

It is well known that acetaldehyde can be reduced to ethanol by

alcohol dehydrogenase. Lundstedt (1969) reported that S. thermophilus will reduce the level of acetaldehyde that is produced by L. bulgaricus. The decrease in the quantity of acetaldehyde from 56.1 to 19.8 ppm for 23 hours incubation, reported by Görner et al. (1968) supports the presence of the dehydrogenase activity of yogurt bacteria.

The production of acetaldehyde by S. thermophilus, S. lactis, S. cremoris, S. diacetilactis, S. faecalis, S. lactis var. maltigenes, L. lactis, L. brevis, and L. plantarum have been reported (Bottazzi and Dellaglio, 1967; Bottazzi and Vescovo, 1969; Harvey, 1960; Bills and Day, 1966; Keenan et al., 1966a,b, 1967a, 1968b; Badings and Galesloot, 1962; Zuraw and Morgan, 1952; Jackson and Morgan, 1954).

It is noteworthy that S. lactis var. maltigenes produces 2- and 3-methyl butanal, 2-methyl propanal and phenylacetaldehyde which are responsible for a malty flavor defect in dairy products (Jackson and Morgan, 1954; Morgan et al., 1966; Sheldon, 1968). These aldehydes are produced from the corresponding amino acids via transamination and decarboxylation.

Bassette and Harper (1958) suggested that acetone, diacetyl and butanone-2 are metabolic products of the decarboxylation of the corresponding beta-ketoacids. Acetoin is produced by irreversible reduction of diacetyl or by decarboxylation of alpha-acetolactic acid from pyruvic acid (Seitz, 1962; Speckman and Collins, 1968).

The production of acetone by lactic acid bacteria is a controversial topic. Several reports have shown the production of acetone by streptococci (Harvey, 1960; Keenan et al., 1966a; Bottazzi and Dellaglio, 1967; Bottazzi and Vescovo, 1969; Vedamuthu et al., 1966). The production of the compound by L. bulgaricus has been reported by Bottazzi and Vescovo (1969). However, Bassette and Clayton (1965) collected volatile compounds from heat-treated milk before inoculation and found that S. lactis and S. diacetilactis did not produce a detectable amount of acetone. Also, Keenan et al. (1967b) later reported little or no evidence for acetone production by any of S. lactis, S. cremoris and S. diacetilactis. These microorganisms did not decarboxylate acetoacetate which is the most possible precursor of acetone. More recently Görner et al. (1968) stated that acetone was not produced as a metabolic product during the fermentation by S. thermophilus, L. bulgaricus or mixed cultures of the two organisms. The level of acetone during fermentation did not increase and was almost the same as the original milk itself.

Diacetyl is produced by S. thermophilus (Görner et al., 1968; Bottazzi and Vescovo, 1969; Bottazzi and Dellaglio, 1967; Davis, 1956b, 1967), but not by L. bulgaricus (Görner et al., 1968; Bottazzi and Vescovo, 1969). Davis (1956b, 1967) reported that S. thermophilus produces a slight buttery aroma due to diacetyl, thus giving yogurt an over-all flavor with acetaldehyde and acetic acid. However,

Görner et al. (1968) and Bottazzi and Vescovo (1969) found only an insignificant trace of diacetyl in cultures of S. thermophilus and in yogurt.

Diacetyl is known to be a very important flavor component in butter, sour cream and cottage cheese. Since Badings and Galesloot (1962) reported a "yogurt" or "green apple" flavor defect in butter, acetaldehyde production by lactic starter cultures has been extensively studied. Lindsay et al. (1965) focused on the ratio of diacetyl to acetaldehyde. A harsh flavor occurs when the ratio is 13:1 to 5.5:1, while green flavor is apparent when it is below 3.2:1. A desirable butter flavor is obtained when the ratio is between 4.5:1 and 3.2:1.

Acetoin was found to be produced by S. thermophilus, but not by L. bulgaricus (Bottazzi and Vescovo, 1969). A considerable quantity of acetoin produced in the culture of S. thermophilus prompted them to designate an "acetoin-type" flavor for cultures of S. thermophilus.

Butanone-2 was found to be present in cultures of S. thermophilus and L. bulgaricus and in mixed cultures of the two organisms (Görner et al. 1968). The authors explained that butanone-2 is from the milk used and not a metabolic product.

Möhler and Denbsky (1970) reported that yogurt examined contained 0.2 to 3.0 mg of formaldehyde per kg.

Ethanol appears to be a metabolic product in yogurt. Görner

et al. (1968) found it in cultures of S. thermophilus and L. bulgaricus and in yogurt, but the quantity produced was low. Since ethanol has a high flavor threshold (Bills and Day, 1966), it would not be expected to contribute to the flavor of yogurt. As previously mentioned, ethanol is produced from acetaldehyde by alcohol dehydrogenase. Bills and Day (1966) added acetaldehyde and propanal to cultures of S. cremoris, S. lactis and S. diacetilactis. Added aldehydes decreased and the corresponding alcohols increased after incubation for 14 or 36 hours. All strains examined had dehydrogenase activity with S. diacetilactis having the lowest activity. Morgan et al. (1966) observed high levels of alcohols, such as ethanol, 2-methyl propanol and 3-methyl butanol in the culture of S. lactis var. maltigenes, and stated that this organism probably has a yeast-like alcohol dehydrogenase.

In cheese and in cultures of Pseudomonas fragi, a high level of ethanol causes fruity flavor due to the formation of esters, such as ethyl butyrate and ethyl hexanoate, with fatty acids (Bills et al., 1965; Reddy, 1970).

Along with volatile flavor components, pH or acidity should be dealt with, since desirable flavor is also dependent upon an appropriate acidity level. Mocquot and Hurel (1970) and Crawford (1962) reported that the desirable pH for a full flavor ranges from pH 4.5 to 4.7. However, Ross (1971), Lundstedt (1969), Stocklin (1969) and Davis (1967) consider pH 4.0 to 4.4 to be a better range.

Fatty acids in dairy products play an important role in contributing to flavor. All lactic acid bacteria studied by Fryer et al. (1967) had lipolytic activity. Acetic acid is known to be the most abundant aliphatic acid in fermented dairy products. Propionic acid produced by Propionibacterium shermanii is important to Swiss cheese. Little work has been performed on fatty acids in yogurt and their role in flavor.

Turčić et al. (1969) investigated volatile fatty acids in yogurt and found that acetic, butyric, caproic and capric acids increased, while propionic and caprylic acids decreased, as compared with those present in milk. It was reported that L. bulgaricus, rather than S. thermophilus, is mainly responsible for the hydrolysis of milk fat which contributes to the typical flavor of yogurt.

Amino acids are known to contribute to the taste of some dairy products. Although they do not contribute to the odor of foods because of lack of volatility, amino acids may be the precursors of volatile flavor components. Amino acids in yogurt have been studied chiefly by Miller et al. (1964, 1966) who analyzed qualitatively and quantitatively amino acids in milk, L. bulgaricus and S. thermophilus cultures and yogurt. S. thermophilus utilizes free amino acids present in milk for growth during the early period of incubation. At the end of incubation, however, the coccus produces an increased amount of free proline and alanine. On the contrary, L. bulgaricus

produced a high amount of all free amino acids, particulary proline and glutamic acid. Also valine increased to some extent. The total quantity of free amino acids at pH 4. 1 in the culture of S. thermophilus was 115 mg/l, while that of L. bulgaricus was 963 mg/l. They concluded that amino acids are produced mainly by L. bulgaricus rather than S. thermophilus in yogurt.

Flavor Defects in Yogurt

Often an absence of flavor and odor in conjunction with inadequate acid production is observed in yogurt. Davis (1967) described possible reasons for these defects: (a) yogurt prepared at the wrong acidity, either too early or too late; (b) a failure to obtain the proper balance of yogurt bacteria, (c) a failure of one or both of the bacteria to grow.

A harsh acid flavor occurs when L. bulgaricus predominates, incubation time is too long, temperature is too high, storage temperature is too high, or excessive amounts of starter are used (Pette and Lolkema, 1951; Schulz and Hingst, 1954; Crawford, 1962; Davis, 1956b, 1967; Stocklin, 1969). Absence of flavor and odor and flat taste are caused by the use of strains of L. bulgaricus which produce little of the flavor and aroma substances, too short an incubation time, too low a temperature, or the failure of yogurt bacteria due mostly to the presence of bacteriophage or antibiotics.

Little work has been reported regarding bacteriophage activity in yogurt. S. thermophilus is known to be sensitive to phage. Pette and Kooy (1952) found two cases of slow acid production because of S. thermophilus attack by phage. Stolk (1955) isolated phages of S. thermophilus in yogurt, but not of L. bulgaricus which is known to be resistent to phage.

Concerning antibiotics, Mocquot (1970) stated that both bacteria are about equally sensitive and are inhibited by the following amounts; penicillin 0.005 i.u./ml, aureomycin 0.066 ug/ml and streptomycin 0.38 i.u./ml. Emmons and Turkey (1967) reported that S. thermophilus is more sensitive to penicillin than L. bulgaricus. The range is 0.01 to 0.05 i.u./ml for S. thermophilus and 0.30 to 0.60 for L. bulgaricus. Ross (1971) reported that L. bulgaricus is susceptible to 0.0025 ppm of penicillin and theromycin.

Schulz (1949) noted that a culture of L. bulgaricus which was responsible for slow acid development formed a long filiform, as seen under microscope. The reasons given for degeneration of the culture were excessively long storage of the culture, incubation at too low a temperature or defects in the milk.

Bitter flavor is caused by the use of milk of poor quality, by culture strains which produce bitterness or by contaminants which are mainly coliforms, sporeforming organisms, yeasts, and molds (Pette and Lolkema, 1950c; Davis, 1956c). Also, the defect of

bitterness is assigned to a high proportion of L. bulgaricus as compared with S. thermophilus, to too high a temperature or prolonged cooling time after incubation (Stocklin, 1969; Ross, 1971).

Unclean flavors can arise from milk of poor quality (Pette and Lolkema, 1950c) or poor sanitation during manufacture (Foster, 1964).

Bacteriology of Yogurt Culture

Taxonomy

Generally accepted yogurt bacteria are S. thermophilus and L. bulgaricus. Humphreys and Plunkett (1969) listed other bacteria which have been cited for yogurt manufacture. They are Bacterium caucasium, Bacterium bulgaricum, Procamobacterium jogourii, Thermobacterium jugurt, Thermobacterium bulgaricum, Thermobacterium lactis, L. yoghourti, L. jugurti, L. acidophilus, S. lactis, S. lactis thermophilus, and Diplococcus yogurt. Recently S. diacetilactis also has been used (Rašić et al., 1966).

Criteria, such as growth in different sugar media, tolerance to high levels of sodium chloride, response to specific chemicals, ability to grow at various temperatures and pH values and reactions in litmus milk have been useful in classifying species within a genus or in classifying similar bacteria into a genus. However, classification and nomenclature of yogurt bacteria, especially L. bulgaricus, has

been a controversial problem.

Taxonomy of streptococci has been discussed in detail by Orla-Jenson (1942), Breed et al (1957), Sherman (1937) and Davis (1955). Taxonomy of lactobacilli has been reported by Orla-Jenson (1942), Sharpe (1962a, b), Davis (1955, 1960, 1962), Briggs(1953), and Rogosa and Sharpe (1959).

It is easy to visually distinguish S. thermophilus from L. bulgaricus since the former is a coccus which forms pairs to long chains and the latter is a rod. The coccus, which is a homofermentative lactic acid bacteria, is found in dairy products, calf stomach or the alimentary tract of the horse (Mocquot, 1970; Breed et al., 1957). The characteristics of S. thermophilus, listed by Breed et al. (1957), are growth at high temperature, strong thermal tolerance, extreme sensitivity to sodium chloride and inability to ferment maltose. It produces acid from glucose, fructose, lactose and sucrose. Litmus milk is curdled, acidified and reduced slightly (Davis, 1967). It is known that the coccus grows at 45°C, but not at 15°C. The optimal temperature varies. According to Ashton (1963), Mocquot (1970), and Stocklin (1969), it is 37°C.

L. bulgaricus, which is a homofermentative lactic acid bacteria, is found in dairy products or calf stomach. Breed et al. (1957) stated that the rod ferments glucose and lactose, but usually not sucrose, maltose and unheated fructose. Earlier workers reported

it ferments sucrose, but later workers noted variables or negative results. Wheater (1955) examined 34 strains of L. bulgaricus, none of which fermented sucrose. Differentiation between L. bulgaricus and L. acidophilus has been controversial. Wheater (1955) found that L. bulgaricus does not grow in 2% sodium chloride or in 2% sodium tauroglycholate, while L. acidophilus grows in both the media. Also they reported that L. bulgaricus does not grow below 20°C or over 50°C. Recently, a nucleic acid homology method has been used to distinguish species in the genus lactobacillus (Miller III et al., 1971). The optimum temperature for growth is 43°C (Ashton, 1963; Mocquot, 1970; Stocklin, 1969).

Bacteria Composition of Yogurt Culture

Pette and Lolkema (1951) first observed that a ratio of cell numbers of one-to-one between L. bulgaricus and S. thermophilus is desirable to produce a good flavored yogurt. Since then, many food scientists have noticed and recommended the one-to-one ratio (Schulz and Hingst, 1954; Görner et al., 1968; Stocklin, 1969; Davis, 1956b; Ross, 1971). Others have recommended ratios of 1:2, 1:3, 2:3 or 1:5 (Emmons and Tuckey, 1967; Davis, 1967; Jankov and Stoyanov, 1966).

Davis (1967) explained that the ratio is influenced by temperature, incubation time, inoculum, nutritional quality of milk, relative

volume of two bacteria (if these are cultured separately), acidity of milk and intensity of heat treatment. It has been recognized by several authors (Pette and Lolkema, 1951; Davis, 1956b, 1967; Stocklin, 1969; Mocquot, 1970; Schulz and Hingst, 1954) that short incubation time, low temperature and small inoculum favor S. thermophilus and vice versa. If short incubation and low temperature are maintained, the rod will decrease and finally disappear from the culture. On the contrary, if long incubation and high temperature are applied, the coccus will decrease and disappear. Therefore, it is very important to control effectively the ratio between the rod and the coccus by adjusting temperature and incubation time. Pette and Lolkema (1951) suggested that if S. thermophilus predominates, an increase in temperature is necessary and if L. bulgaricus predominates, a decrease is necessary. They found that the coccus outnumbered the rod 3 to 4: 1 at the end of the first hour of incubation at 46°C and then the latter multiplied rapidly, resulting in the ratio of approximately 1 to 1 at the end of incubation. Pette and Lolkema (1951) also found that if milk is incubated for a long period, the high acidity produced by L. bulgaricus will kill the coccus. Davis (1967) and Mocquot (1970) reported a more detailed feature of the culture; S. thermophilus, which grows fastest at pH 6.5 and is stimulated by L. bulgaricus, grows rapidly up to pH 5.5 and then ceases to grow at pH 4.2. L. bulgaricus, which is more resistent to acid and grows

best at pH 5.5, takes over the acid production in the late fermentation process and produces high acid.

Optimum temperature, incubation time and inoculum vary, depending upon the nature of strains or upon the type of product.

It appears that the range of incubation temperature for yogurt manufacture is anywhere from 41°C to 50°C. Pette and Lolkema (1951) and Kosikowski (1966) recommended 45°C, Crawford (1962) and Davis (1956b, 1967) 43°C, Mocquot (1970) 42.5°C.

Incubation time ranges from 2 to 4 hours. Pette and Lolkema (1951) incubated 3 to 4 hours, Davis (1956a) 2-1/2 to 3 hours and Ross (1971) 2 hours.

Percentage inoculum ranges from 1 to 5%. 1-2, 2, 2.5 and 3% have been used by Ross (1971), Davis (1967), Pette and Lolkema (1951) and Crawford (1962), respectively.

Intensity of heat treatment of the milk must also be considered. Severely heated milk favors L. bulgaricus due to exclusion of oxygen and the production of sulfhydryl compounds and vice versa (Davis, 1956a).

Addition of Sucrose

Sucrose may be added to milk in amounts ranging from 4 to 10.5% for the manufacture of yogurt (Crawford, 1962; Ross, 1971; Ueno et al., 1966). Traditionally, yogurt does not contain added

sucrose, but the introduction of flavored yogurt has encouraged the addition of sucrose in recent years. Sucrose is used as a preservative since it ties up moisture, making it unavailable to microorganisms, and exerts a high osmotic pressure on bacterial cells. Ueno et al. (1966) reported that 10.5% sucrose is added in Japan, resulting in a required incubation time of 3-1/2 to 4 hours instead of 2-1/2 to 3-1/2 hours. Added sucrose not only retards yogurt microorganisms, but also makes yogurt more palatable and prevents too high acid production (Stocklin, 1969).

Symbiosis of Yogurt Bacteria

Several workers have found that symbiotic action of both yogurt bacteria is necessary to obtain a full, desirable flavor and acidity of yogurt (Pette and Lolkema, 1950a,b,c; Schulz and Hingst, 1954; Görner et al., 1968; Davis, 1956b).

Pette and Lolkema (1950b) noticed that L. bulgaricus produces from protein a water soluble, heat stable stimulant for S. thermophilus resulting in the faster and higher production of flavor compounds and acidity in yogurt. The number of cocci increased in mixed culture, compared with single strain culture of S. thermophilus. By adding amino acids one-by-one to milk containing all other amino acids, they observed that valine is most stimulative for streptococcus. Bautista et al. (1966) obtained a different result. The latter scientists

found either histidine and glycine, but not valine, obtained from the filtrate of L. bulgaricus culture or the filtrate itself stimulated S. thermophilus, resulting in faster and higher acid production.

S. thermophilus may produce a stimulant for L. bulgaricus, even though Pette and Lolkema (1950b) and Bautista et al., (1966) report a negative stimulative effect on L. bulgaricus by S. thermophilus In skimmilk heated at 90°C for 10 minutes, Galesloot et al., (1968) observed that S. thermophilus produced a stimulant for L. bulgaricus under nitrogen instead of air or under air where oxygen in skimmilk had been used up by S. thermophilus during a long incubation. L. bulgaricus grown in skimmilk to which was added a filtrate of S. thermophilus produced a higher level of acid compared to a control that did not contain the filtrate. This stimulant was identified as formic acid by Verigna et al., (1968).

Auclair and Portmann (1957) noted that L. lactis was stimulated in an autoclaved milk and the stimulant was found to be formic acid which represents about 80% of all free fatty acids generated by autoclaving.

Another symbiotic action exerted by S. thermophilus, as reported by Davis (1956a), is that the coccus removes oxygen thus favoring L. bulgaricus, and also hydrolyzes protein, thus providing the rod with more easily assimilable nitrogen sources.

Biochemistry of Yogurt

Associative growth of S. thermophilus and L. bulgaricus produces lactic acid from the metabolism of lactose. The breakdown of lactose into glucose and galactose is the first step. Glucose is immediately metabolized. However, galactose must be converted to glucose by the following steps, outlined by Kandler (1961).

1. $\text{Galactose} + \text{ATP} \xrightarrow{\text{Galactokinase}} \text{Galactose-1-PO}_4 + \text{ADP}$

2. $\text{Galactose-1-PO}_4 + \text{UDP-glucose} \xrightarrow[\text{transuridylase}]{\text{Galactose-1-PO}_4} \text{UDP-galactose} + \text{Glucose-1-PO}_4$

3. $\text{UDP-galactose} \xrightarrow[\text{epimerase}]{\text{UDP-galactose-4-}} \text{UDP-glucose}$

Carbohydrate Metabolism by Homofermentative Lactic Acid Bacteria

It is well known that homofermentative lactic acid bacteria produce exclusively L(+) lactic acid as a chief metabolic product from glucose. Homofermentative lactic acid bacteria are distinguished from heterofermentative lactic acid bacteria which produce D(-) lactic acid. Besides lactic acid, homofermentative lactic acid bacteria produce minor metabolites which are important to flavor in dairy products.

70-90% of lactose is converted to lactic acid through the Embden Meyerhof-Parnes (EMP) scheme. In the EMP scheme, a tight coupling reaction concerning nicotinamide adenine dinucleotide (NAD)

forces pyruvic acid to be converted to lactic acid. Reduced NADH generated by the conversion of glyceraldehyde-3-phosphate to 1, 3 diphosphoglyceraldehyde has to be oxidized to convert pyruvic acid to lactic acid. Kandler (1961) reported that homofermentative lactic acid bacteria definitely possess enzymes that oxidize and decarboxylate glucose-6-phosphate to ribulose-5-phosphate. This fact could explain the formation of compounds other than lactic acid. Also utilization of glucose via the Entner-Doudoroff pathway, shown in Figure 1, by homofermentative lactic acid bacteria was reported by Reiter and Møller-Madsen (1963). The main products other than lactic acid in the latter pathway are ethanol and carbon dioxide.

Carbohydrate Metabolism by Heterofermentative Lactic Acid Bacteria

It may be necessary to cite the metabolism of heterofermentative lactic acid bacteria since products other than lactic acid have been found in yogurt.

Glucose is metabolized via the hexose monophosphate shunt (HMP), summarized by Kandler (1961) as shown in Figure 2.

Heterofermentative lactic acid bacteria lack aldolase which catalyzes the conversion of fructose-1, 6-diphosphate to 3-phosphoglyceraldehyde and dihydroxyacetone phosphate. In addition, they lack triosephosphate isomerase which catalyzes the reversible

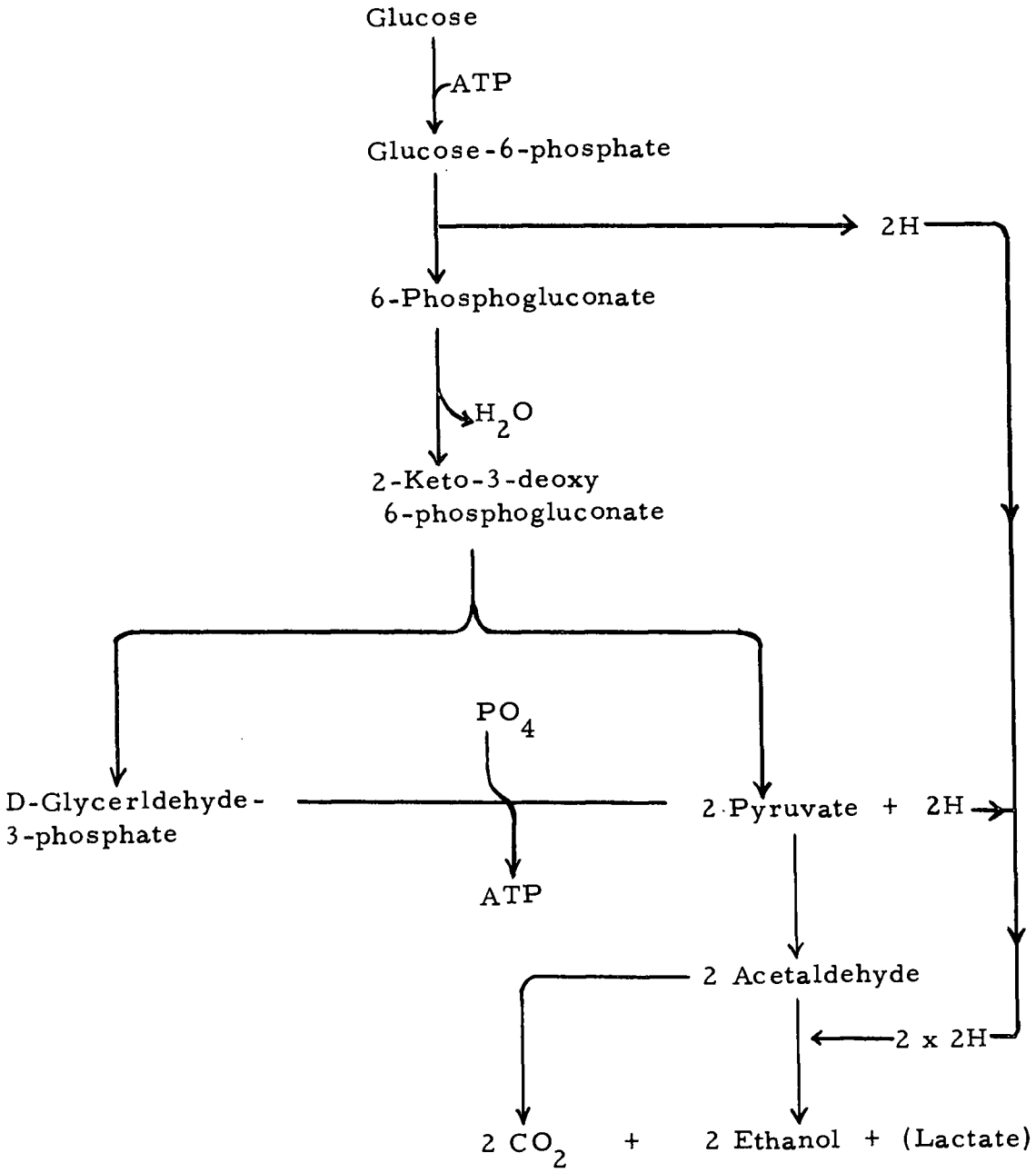

Figure 1. The Entner-Doudoroff pathway. (from Wood, 1961)

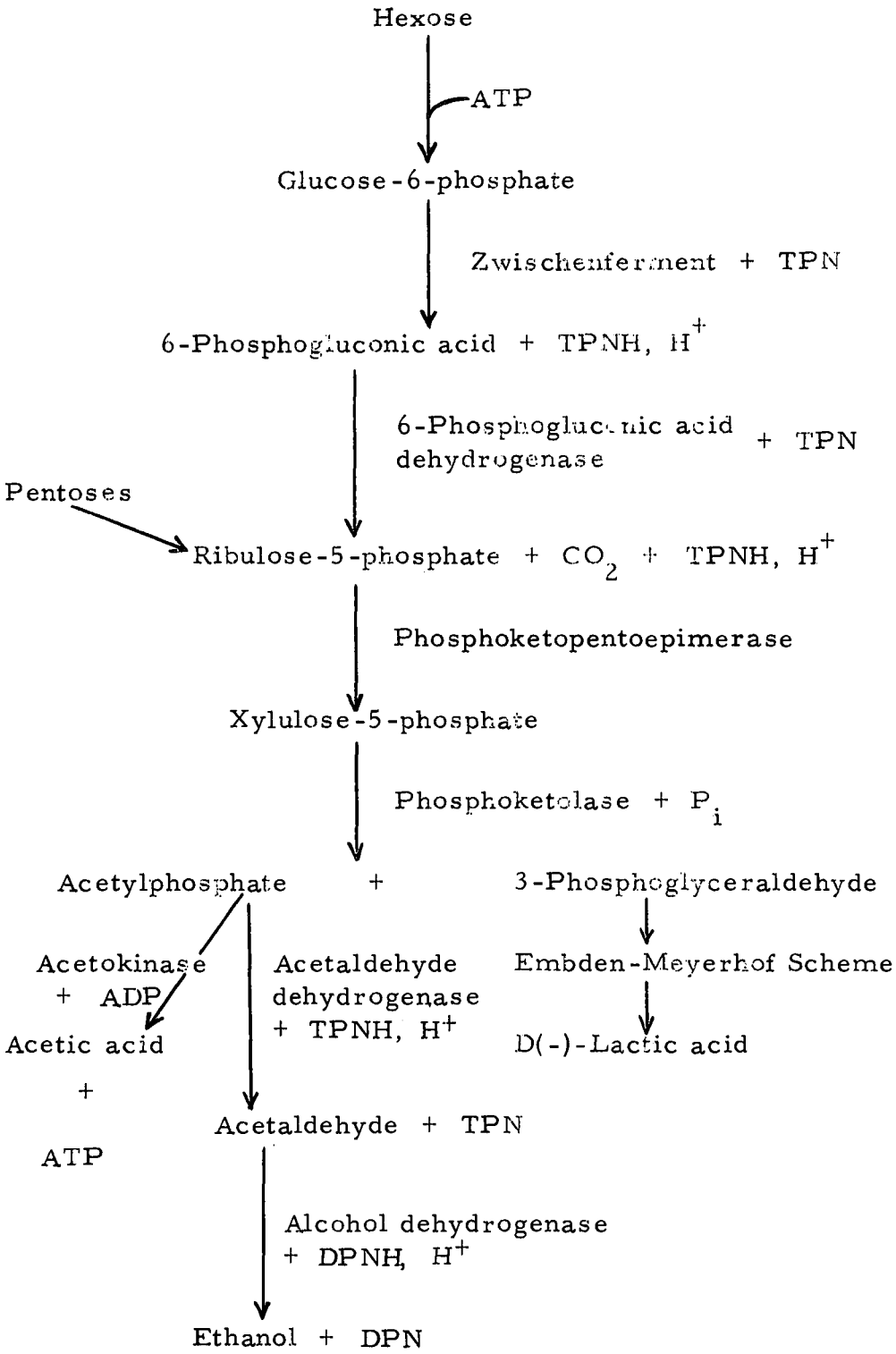

Figure 2. Carbohydrate metabolism of heterofermentative lactic acid bacteria. (from Kandler, 1961)

reaction between the above trioses in the EMP pathway. Glucose-6-phosphate is converted to 6-phosphogluconic acid rather than fructose-1, 6-diphosphate. By decarboxylation, the latter is converted to pentose phosphates which go to acetylphosphate and 3-phosphoglyceraldehyde by 3-2 carbon cleavage. Acetylphosphate is converted to acetaldehyde in an anaerobic condition which results in the production of ethanol or to acetic acid in an aerobic condition.

Citric Acid Fermentation

S. diacetilactis and Leuconostoc sp. can utilize citric acid present in milk. Since the mechanism for the production of diacetyl and acetoin by yogurt microorganisms has not been investigated, this pathway could be a route for their production. The pathways for conversion of citric acid to diacetyl by S. diacatilactis have been extensively studied by Seitz (1962). Figure 3 shows the utilization of citric acid and pyruvic acid, as summarized by Seitz (1962).

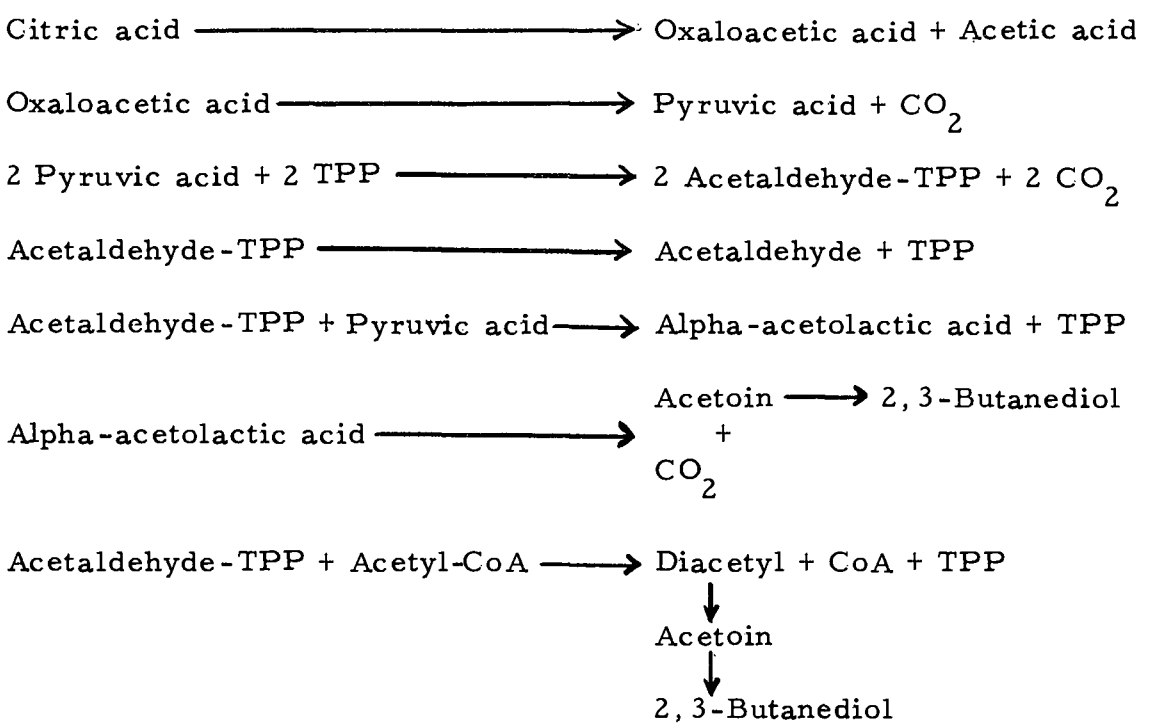

Figure 3. Pathways for enzymatic conversion of citric acid by S. diacetilactis and L. citrovorum. (from Seitz, 1962 and Speckman and Collins, 1968)
TPP=thiamine pyrophosphate. CoA=coenzyme A

According to Galesloot (1962), the mechanism for citric acid utilization by Leuconostoc sp. in the butter culture is identical to that shown in Figure 3. Recently, Speckman and Collins (1968) showed that neither alpha-acetolactic acid nor acetoin is the precursor of diacetyl with S. diacetilactis and L. citrovorum. They found that the cell free extract of these bacteria produced diacetyl and acetoin when acetyl-CoA was added to the system consisting of thiamine pyrophosphate (TPP) and manganese. They confirmed that diacetyl is produced by the direct reaction of acetaldehyde-TPP with acetyl-

CoA and acetoin is produced either from the reduction of diacetyl or from the decarboxylation of alpha-acetolactic acid.

EXPERIMENTAL

Cultures

Cultures Employed

Four single strains of S. thermophilus, four single strains of L. bulgaricus and four mixed yogurt cultures were utilized. Table 1 shows cultures and their origin.

Cultivation of Single Strains of S. thermophilus and L. bulgaricus

The medium for the cultivation of single strains of S. thermophilus and L. bulgaricus was reconstituted 11% nonfat milk, which was prepared by dissolving nonfat dry milk in distilled water and autoclaving at 121°C for 10 min. Cocci and rods were incubated in the media for 15 hr at 37-38°C and 43-44°C, respectively. Cultures were immediately cooled and held at 5°C. Successive transfers were made every three days using 1.5% inoculum.

Cultivation of Commercial Mixed Cultures

The medium for the cultivation of commercial mixed cultures was 2%-fat homogenized milk, which was prepared by autoclaving at 121°C for 10 min.

An adequate amount of lyophylized mixed cultures "A", "C-a"

Table 1. Cultures employed

Cultures	Origin
Commercial mixed culture "A"	Chr. Hansen's Lab., Milwaukee, Wis.
Commercial mixed culture "B"	Sunshine Dairy, Portland, Ore.
Commercial mixed culture "C-a"	Mosely Lab., Indianapolis, Ind.
Commercial mixed culture "C-b"	Mosely Lab., Indianapolis, Ind.
S. thermophilus "A"[a]	Chr. Hansen's Lab., Milwaukee, Wis.
S. thermophilus "B"[b]	Sunshine Dairy, Portland, Ore.
S. thermophilus "N"	Nordica Food Co., Sioux Fall, S. D.
S. thermophilus C3	Department of Microbiology, Oregon State University
L. bulgaricus "A"[a]	Chr. Hansen's Lab., Milwaukee, Wis.
L. bulgaricus "B"[b]	Sunshine Dairy, Portland, Ore.
L. bulgaricus "N"	Nordica Foods Co., Sioux Fall, S. D.
L. bulgaricus ATCC 7993	Department of Microbiology, Oregon State University

[a] Isolated from the commercial mixed culture "A"

[b] Isolated from the commercial mixed culture "B"

and "C-b" were dissolved in the milk medium and incubated at 43-44°C until the ratio of S. thermophilus to L. bulgaricus reached 1:1. A frozen commercial mixed culture "B" was inoculated (using 2% inoculum) into the milk medium and incubated at 47-48°C until the ratio of the two bacteria became 1:1. Thereafter, all mixed cultures were transfered three or four times using 2% inoculum. They were held at 5°C.

Isolation of Single Strains from Commercial Mixed Cultures

Dilutions of commercial mixed culture "A" and "B" were made and plated on Elliker agar (Elliker et al., 1956). After incubation at 43-44°C for 48 hr, rough-edged colonies typical of L. bulgaricus and smooth-edged colonies typical of S. thermophilus were picked into sterile milk medium and incubated in the manner described in the previous section. Buffered distilled water blanks prepared according to American Public Health Association (1967) were used to make all dilutions.

pH and Acidity Measurement

Titratable acidity was determined in accordance with Milk Industry Foundation (1964). Nine gram samples were weighed and titrated with 0.1 N NaOH using 5 drops of phenolphthalein as indicator. The titratable acidity was expressed as percent lactic acid. pH was

measured with a Beckman Expandomatic pH meter.

Determination of Microbial Population

The microbial population of single strain cultures were determined by plating dilutions of cultures on Elliker agar (Elliker et al., 1956), otherwise specified. Plates were incubated for 48 hr at 37-38°C for S. thermophilus and at 43-44°C for L. bulgaricus, respectively.

Microbial numbers of S. thermophilus and L. bulgaricus in mixed cultures were determined by means of the direct microscopic clump count as described in American Public Health Association (1967) following several dilutions, otherwise specified. The ratio of S. thermophilus to L. bulgaricus was calculated by dividing the number of cocci by the number of rods.

Analysis of Acetaldehyde

Preparation of Medium (Yogurt Base)

A medium (yogurt base) consisted of 2%-fat homogenized milk which was fortified with 2% nonfat dry milk. The following concentrations of sucrose were added so that the final weight of the medium became 100 gram; 0, 4, 8, 12 and 16%. All the media were heat-treated at 85°C for 30 min and cooled to a temperature corresponding

to the incubation of a culture.

Gas Liquid Chromatography

The volatile components were analyzed by gas liquid chromatography (GLC). The gas entrainment on-colum trapping technique developed by Morgan and Day (1965) was employed. Nitrogen was bubbled at a rate of 10 ml per min for 5 min through 4 ml of a sample diluted to 8 ml with distilled water containing 15 ppm methyl acetate[1/] as internal standard. Approximately one gram of 1-tetradecanol was added to the sample to serve as an antifoam agent and the sample was equilibrated for 10 min at 60°C prior to nitrogen ebullition and held at this temperature during the trapping period. A 1/8-inch by 12 ft (0.318 cm x 366 cm) column packed with 20% 1,2,3,-tris (2-cyanoethoxy) propane on 60-80 mesh Celite 545 was employed. The volatiles were entrained on this column and chromatographed at an oven temperature of 50°C with a Varian Aerograph model 1200 gas chromatograph. Acetaldehyde was identified by coincidence of retention time with that of the authentic compound. Quantities of acetaldehyde eluted were determined by means of a standard curve prepared from known concentrations of the authentic compound. Normalization of fluctuation between analyses was accomplished by comparing the

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[1/] Matheson, Coleman and Bell, Vorwood, Ohio.

peak height of acetaldehyde with that of methyl acetate, the internal standard.

Acetaldehyde Production by Commercial Mixed Cultures

Four commercial mixed cultures were studied for acetaldehyde production. Each of the mixed cultures "A", "B", "C-a", and "C-b", which maintained approximately 1:1 ratios of S. thermophilus to L. bulgaricus was inoculated at the rate of 2% inoculum into yogurt bases containing 0, 4, 8, 12 and 16% sucrose. Incubation temperature was 43-44°C for cultures "A", "C-a" and "C-b" and 47-48°C for the culture "B". The former three cultures and the latter culture were incubated for 3 hr 30 min and for 2 hr 45 min, respectively. All were held at room temperature for 1 hr and at 5°C for 1 hr. The acetaldehyde content, pH, titratable acidity, microbial population and the ratio of two species were determined.

Acetaldehyde Production by Single Strains of S. thermophilus and L. bulgaricus

The strains of S. thermophilus studied were "B", "N" and C3 and the strains of L. bulgaricus studied were "B", "N" and ATCC 7993. All single strains were inoculated (using 1.5% inoculum) into yogurt bases containing 0, 4, 8, 12 and 16% sucrose. Strains of S. thermophilus and L. bulgaricus were incubated at 37-38°C and at

43-44°C, respectively, until coagulation took place. Immediately after incubation, all were cooled and held at 5°C for 12 hr. The concentration of acetaldehyde, standard plate count, pH and titratable acidity were determined.

Aectaldehyde Production by a Single Strain of *S. thermophilus* and *L. bulgaricus* and by the Mixed Culture as a Function of Incubation Time

A single strain of *S. thermophilus* and a single strain of *L. bulgaricus* isolated from the mixed culture designated as "A" and a mixed culture of the two organisms were employed to examine and compare the acetaldehyde production as a function of time. A single strain of *S. thermophilus* "A" and *L. bulgaricus* "A" was inoculated (using 1.5% inoculum) into yogurt bases containing 0% and 8% sucrose and incubated at 37-38°C and at 43-44°C, respectively, for 1-1/2, 3, 4-1/2, 6, 7-1/2, 9, 10-1/2, 12, and 24 hr. All cultures were held at 5°C for 12 hr. The mixed culture was inoculated (using 2% inoculum) into yogurt bases containing 0% and 8% sucrose and incubated at 43-44°C for 1, 2, 3, 4, 6, 9, 15 and 24 hr. All were held at room temperature for 1 hr and then at 5°C for 1 hr.

The acetaldehyde production and microbial population for all cultures and the ratio of the two species in the mixed culture were determined.

Production of Acetaldehyde During Refrigerated Storage

Mixed culture "A" was inoculated at the rate of 2% inoculum into yogurt bases containing 0% and 8% sucrose and incubated at 43-44°C for 3 hr 30 min. After being held at room temperature for 1 hr, they were refrigerated at 5°C and the acetaldehyde content was analyzed after 1, 2, 4, 7, 10 and 14 days.

Determination of Volatile Acidity

Yogurt containing 0% and 8% sucrose was made with mixed cultures "A", "C-a" and "C-b" in the manner described in the previous section. One hundred grams of yogurt was acidified to pH 2 with concentrated phosphoric acid and made up to 150 ml with the addition of distilled water. An amount of 100 ml distillate was collected by means of steam distillation and titrated with 0.1 N NaOH using 5 drops of phenolphthalein as indicator. Titratable acidity was expressed as ml of 0.1 N NaOH required to neutralize 100 ml of distillate, as described by Keenan et al. (1968a).

Determination of Average Flavor Threshold of Acetaldehyde

Average flavor threshold (AFT) values of acetaldehyde in the following three systems were determined; (a) 2%-fat milk, (b) 2%-fat milk plus 8% sucrose, (c) 2%-fat milk plus 8% sucrose plus 0.4%

strawberry flavor.[2] A stock solution of acetaldehyde (600 ppm) was prepared in distilled water and dilutions were made for appropriate ranges for AFT determination. Concentrations of acetaldehyde tested for the systems (a) and (b) were 0.2, 0.4, 0.8, 1.6 and 3.2 ppm and those for system (c) were 5, 10, 15, 20 and 25 ppm.

AFTs were determined by the method described by Wyatt and Day (1965). As references, milk without added acetaldehyde and milk containing the highest concentration were served at the same time. All samples were warmed to room temperature before being served. About 30 ml of each sample was served to 8 experienced judges seated in individual booths. A yes or no type ballot was used and the 50% level of positive response was taken as the AFT.

[2] Florasynth Inc., Chicago.

RESULTS AND DISCUSSION

Acetaldehyde Production by Commercial Mixed Cultures

Since acetaldehyde is the most important flavor component of yogurt, the examination of the effect of added sucrose on acetaldehyde production in yogurt bases by mixed cultures is of importance.

Sucrose retards the growth of yogurt bacteria, according to some authorities, so that high acid production, which results in a less palatable product, can be prevented (Stocklin, 1969). Also, sucrose imparts sweetness to yogurt which is complimentary to the other added flavorings. So far, a quantitative analysis of acetaldehyde in yogurt with added sucrose has not been reported in the literature. Commercial mixed cultures were examined for acetaldehyde production in yogurt bases containing 0, 4, 8, 12 and 16% sucrose.

Table 2 shows the acetaldehyde production, pH, titratable acidity, direct microscopic counts of each species and the ratio between the two species in four commercial mixed cultures. It appeared that acetaldehyde production was lower in mixed cultures grown in media containing 8% or more sucrose. The lower levels of acetaldehyde produced at higher sucrose levels paralleled the decreases in lactic acid production and the lower cell counts of both species. These observations suggest a general inhibition of growth,

Table 2. Acetaldehyde production by commercial mixed cultures incubation at 43-44°C for 3 hr 30 min. [a/]

Sucrose (%)	Acetaldehyde (ppm)	pH	Titratable Acidity % lactic acid	Direct Microscopic Count ($X10^6$) L. bulg.	Direct Microscopic Count ($X10^6$) S. ther.	Ratio S. ther. / L. bulg.
			Mixed Culture "A"			
0	27.7	4.41	1.00	53.50	44.5	0.83
4	27.7	4.46	0.91	40.00	72.0	1.30
8	24.9	4.49	0.87	38.00	55.5	1.50
12	14.0	5.12	0.64	20.00	50.0	2.50
16	1.0	6.25	0.18	0.10	5.1	50.00
			Mixed Culture "B" [b/]			
0	36.5	4.29	1.05	375.00	185.0	0.50
4	32.1	4.42	1.00	270.00	115.0	0.42
8	30.6	4.43	0.91	135.00	70.0	0.87
12	22.5	4.68	0.75	55.00	73.0	1.30
16	6.0	5.41	0.45	16.50	90.0	5.50

Table 2. (Cont.)

Sucrose (%)	Acetaldehyde (ppm)	pH	Titratable Acidity % lactic acid	Direct Microscopic Count ($X10^6$) L. bulg.	S. ther.	Ratio S. ther. / L. bulg.
			Mixed Culture "C-a"			
0	31.4	4.27	1.13	121.00	83.0	0.68
4	31.0	4.28	1.10	70.50	75.0	1.10
8	28.5	4.30	1.05	54.00	65.0	1.20
12	8.0	5.48	0.55	1.00	28.0	28.50
16	1.7	6.72	0.16	0.14	3.4	31.30
			Mixed Culture "C-b"			
0	32.7	4.26	1.15	111.00	95.0	0.85
4	32.7	4.28	1.11	103.00	92.0	0.89
8	29.1	4.30	1.05	88.00	77.0	0.87
12	8.9	5.39	0.54	3.00	15.0	5.00
16	1.5	6.80	0.15	0.10	2.5	23.00

a/ Held at room temperature for 1 hr and at 5°C for 1 hr.

b/ Incubated for 2 hr 45 min at 47-48°C.

with L. bulgaricus being somewhat more sensitive than S. thermophilus.

Even though 4% or 8% sucrose slightly inhibited the mixed cultures in most cases, the concentration of acetaldehyde produced by all mixed cultures at 0% to 8% sucrose was high enough to give a good flavor, considering the range of acetaldehyde which resulted in an acceptable flavor in plain yogurt as reported by Görner et al. (1968).

Acid production by all mixed cultures appeared to be inhibited by 4% sucrose and decreased further as the sucrose content increased. Sucrose concentrations of 12% strongly retarded acid production in all mixed cultures.

As the sucrose content increased, direct microscopic count revealed that the multiplication of strains of L. bulgaricus was more depressed than that of strains of S. thermophilus. Thus, the ratio of S. thermophilus to L. bulgaricus changed with sucrose content. For example, the ratio of S. thermophilus "A" to L. bulgaricus "A" changed from 0.83:1 at 0% sucrose to 1.3:1 at 4% sucrose and 50:1 at 16% sucrose.

Acetaldehyde Production by Single Strains of S. thermophilus and L. bulgaricus

The acetaldehyde production in yogurt bases containing sucrose by three single strains of S. thermophilus and three single strains of

L. bulgaricus was determined. Incubation was continued until coagulation first became evident and the culture was then analyzed. As indicated in Tables 3 and 4, the effect of sucrose on acetaldehyde production in the strains of S. thermophilus varied from strain to strain. Decreased acetaldehyde production was first detected for S. thermophilus strains "B" and C3 at the concentrations of 12% and 16% sucrose, respectively, while the production of acetaldehyde by S. thermophilus "N" was inhibited by as little as 4% sucrose.

In contrast to acetaldehyde production, all strains of S. thermophilus were detectably inhibited at concentrations of 4% sucrose in terms of acid production and microbial population. As sucrose contents increased, less acid was produced and plate counts were lower.

All strains of L. bulgaricus were stimulated by 4, 8, and 12% sucrose so that a higher production of acetaldehyde and acid and a higher microbial population occurred, compared with 0% sucrose. An exception was L. bulgaricus "B" which produced 5.6 ppm at 0% sucrose and 4.2 ppm at 12% sucrose. It appeared that 8% sucrose was most effective. For example, L. bulgaricus "N" produced 4.1 ppm acetaldehyde at 0% sucrose, while producing 5.2 ppm at 8% sucrose. Microbial population and pH were 80×10^5 and 5.66 at 0% sucrose in contrast to 165×10^5 and 5.52 at 8% sucrose. Each strain of L. bulgaricus was inhibited to some degree by 16% sucrose but not as much as strains of S. thermophilus. The acetaldehyde and acid

Table 3. Acetaldehyde production by strains of S. thermophilus incubated at 37-38°C until coagulation occurred.

Sucrose (%)	Acetaldehyde (ppm)	pH	Titratable Acidity % lactic acid	Plate Count ($\times 10^5$)
		S. thermophilus "N" [a/b/]		
0	6.1	5.55	0.47	300[c/]
4	5.7	5.61	0.46	110
8	5.5	5.63	0.42	65
12	4.9	5.70	0.37	50
16	1.3	6.49	0.15	7.5
		S. thermophilus "B" [d/e/]		
0	8.5	5.61	0.54	890
4	8.4	5.71	0.48	790
8	8.5	5.81	0.42	820
12	5.9	6.21	0.31	480
16	2.1	6.80	0.18	108
		S. thermophilus C3 [f/g/]		
0	8.5	5.40	0.63	720
4	8.5	5.41	0.56	570
8	8.4	5.42	0.52	110
12	8.5	5.73	0.40	42
16	0.45	6.70	0.17	0.21

[a/] Incubated for 4 hr 20 min.

[b/] Original plate count 343 x 10^6.

[c/] Direct microscopic count.

[d/] Incubated for 5 hr 5 min.

[e/] Original plate count 730 x 10^6.

[f/] Incubated for 5 hr 15 min.

[g/] Original plate count 390 x 10^6.

Table 4. Acetaldehyde production by strains of L. bulgaricus incubated at 43-44°C until coagulation occurred.

Sucrose (%)	Acetaldehyde (ppm)	pH	Titratable Acidity % lactic acid	Plate Count ($\times 10^5$)
		L. bulgaricus "N"[a/b/]		
0	4.1	5.66	0.46	80[c/]
4	4.7	5.59	0.48	100
8	5.2	5.52	0.50	165
12	4.7	5.56	0.47	155
16	2.5	5.99	0.29	145
		L. bulgaricus "B"[d/e/]		
0	5.6	5.63	0.48	145
4	7.4	5.45	0.53	190
8	8.1	5.47	0.54	950
12	4.2	5.67	0.42	300
16	3.8	5.69	0.40	9.6
		L. bulgaricus ATCC 7993[f/g/]		
0	3.7	6.11	0.26	33.5[c/]
4	6.8	5.53	0.50	55
8	7.3	5.51	0.52	115
12	5.1	5.69	0.42	50
16	2.0	6.12	0.25	17

a/ Incubated for 4 hr 5 min.

b/ Original plate count 410 x 10^5.

c/ Direct microscopic count.

d/ Incubated for 4 hr 5 min.

e/ Original plate count 341 x 10^5

f/ Incubated for 4 hr.

g/ Original plate count 380 x 10^5.

production parallel the microbial population.

Acetaldehyde Production by a Single Strain of S. thermophilus and L. bulgaricus and by the Mixed Culture as a Function of Incubation Time

The production of acetaldehyde over time by single strains of S. thermophilus "A" and L. bulgaricus "A" and by the mixed culture in yogurt bases containing 0% and 8% sucrose are presented in Tables 5 and 6. These tables also show bacterial populations and the ratio of two species in the mixed culture. Figure 4 shows graphically the comparison of acetaldehyde concentrations.

At 0% sucrose, S. thermophilus "A" produced a slightly higher amount of acetaldehyde than at 8% sucrose over time, demonstrating sucrose inhibition, except at 7-1/2 to 10-1/2 hr when more acetaldehyde was produced at 8% sucrose. The production of this compound at 0% sucrose increased to 3.7 ppm for the first 6 hours incubation, decreased to 1.8 ppm for further incubation and then increased to 3.4 ppm at 24 hr. On the other hand, the concentration at 8% sucrose increased to 2.7 ppm for 7-1/2 hr and levelled off for 24 hr. Görner et al. (1968) reported that the acetaldehyde produced by S. thermophilus in nonfat milk was three or four times as great at the end of 23 hours incubation as for 5 or 9 hr.

It appeared that acetaldehyde production by S. thermophilus

Table 5. Acetaldehyde production by a single strain of S. thermophilus "A" and L. bulgaricus "A" as a function of incubation time.

Incubation (hr.)	Acetaldehyde (ppm)		Plate count (x 10^6)	
	0% Sucrose	8% Sucrose	0% Sucrose	8% Sucrose
	S. thermophilus "A" [a]			
1-1/2	2.6	1.7	128	25
3	3.1	1.8	158	55
4-1/2	3.2	2.1	173	80
6	3.7	2.5	217	91
7-1/2	2.6	2.7	310	104
9	1.8	2.4	380	630
10-1/2	2.2	2.4	200	77
12	3.1	2.6	230	70
24	3.4	2.8	208	121
	L. bulgaricus "A" [b]			
1-1/2	1.1	1.6	13	60
3	4.4	5.5	76	78
4-1/2	6.3	7.0	85	89
6	7.3	8.0	71	64
7-1/2	9.4	10.0	75	91
9	12.8	12.0	26	39
10-1/2	12.4	11.7	33	89
12	12.3	11.6	78	104
24	16.8	15.7	1.6	5.2

[a] Original plate count was 289 x 10^6.

[b] Original plate count was 489 x 10^6.

Table 6. Acetaldehyde production by the mixed culture "A" incubation at 43-44°C as a function of incubation time.[a/]

Incubation (hr.)	Acetaldehyde (ppm)		Direct Microscopic Count ($X10^6$)				Ratio. S. ther. / L. bulg.[b/]	
			0% Sucrose		8% Sucrose			
	0% Sucrose	8% Sucrose	L. bulg.	S. ther.	L. bulg.	S. ther.	0% Sucrose	8% Sucrose
1	0.6	0.5	6	43	2.1	20	7.300	9.500
2	1.5	0.7	130	288	80.0	38	2.140	4.700
3	17.4	16.9	281	390	120.0	410	1.400	3.400
4	25.8	19.2	433	402	160.0	220	0.930	1.500
6	34.8	28.9	442	283	310.0	150	0.640	0.500
9	28.4	28.1	510	220	380.0	87	0.440	0.230
15[b/]	21.2	20.7	240	29	146.0	5	0.120	0.030
24[b/]	20.7	20.1	173	1	125.0	1	0.006	0.008

[a/] Held at room temperature for 1 hr and at 5°C for 1 hr.

[b/] For 15 and 24 hr incubations, plate counts were made counting colonies having rough-edges as rods and those having smooth-edges as cocci.

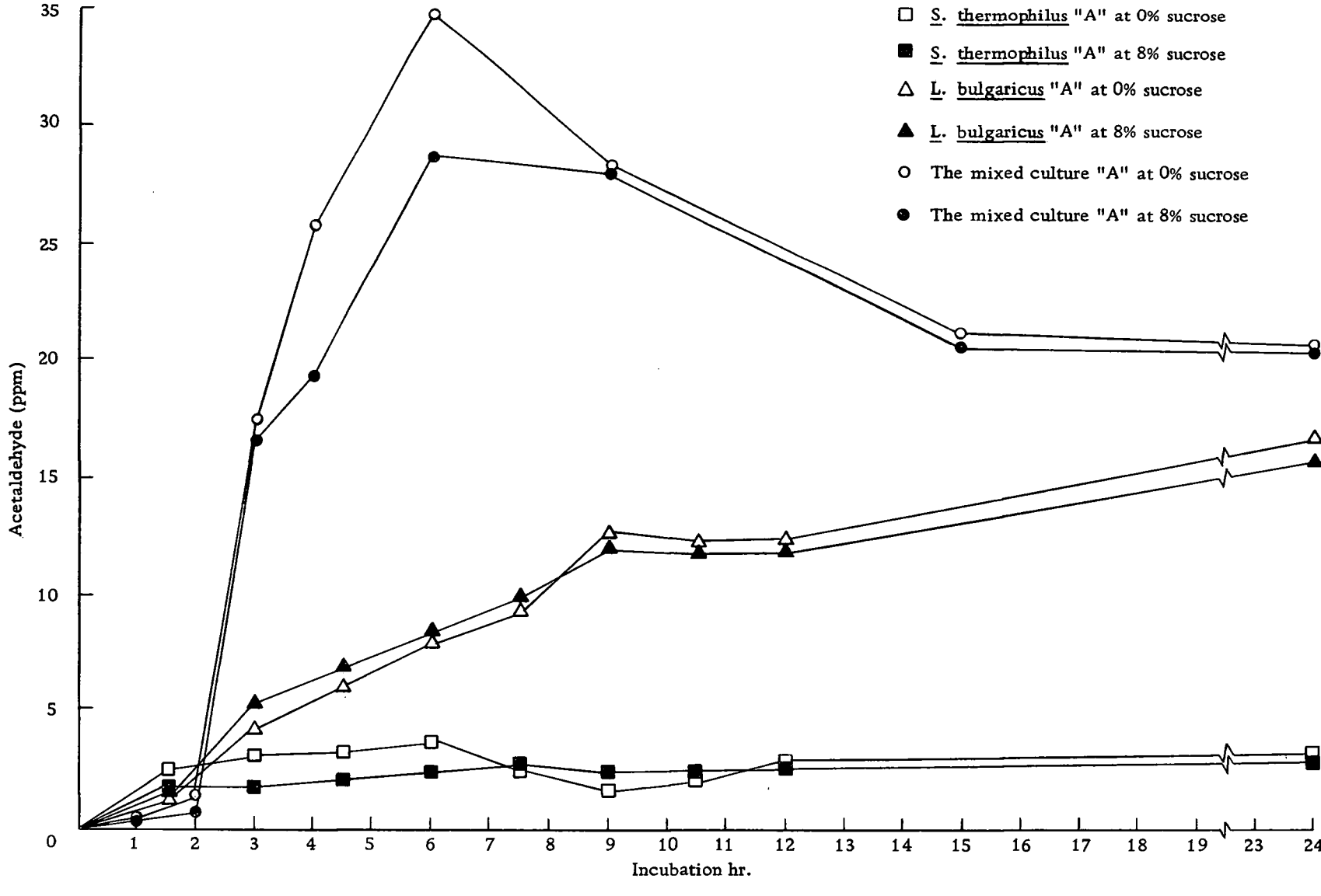

Figure 4. Acetaldehyde production by a single strain of S. thermophilus "A" and L. bulgaricus "A" and by the mixed culture as a function of incubation time.

also paralleled the microbial population. Plate counts made at equivalent incubation times were higher for yogurt bases containing 0% sucrose than for 8% sucrose, except at 9 hr.

The general pattern of acetaldehyde production by L. bulgaricus "A" was comparable to that of S. thermophilus "A". The rod produced increasing amounts of acetaldehyde as the incubation time increased. Compared at equivalent incubation times, L. bulgaricus "A" produced much higher amounts of acetaldehyde than S. thermophilus "A". The conclusion can be drawn from this that L. bulgaricus is mainly responsible for acetaldehyde production in yogurt. The quantities of acetaldehyde found in the cultures of lactobacillus without added sucrose are in good agreement with those reported by Görner et al. (1968). It appeared that sucrose stimulated the production of acetaldehyde by L. bulgaricus "A" for the first 7-1/2 hr, then inhibited it during further incubation to 24 hr.

The acetaldehyde production by the rod also appeared to parallel the microbial population. Plate counts were higher at 8% sucrose than at 0%.

In the mixed culture, rapid production of acetaldehyde occurred between 2 to 6 hr incubation. Sucrose seemed to have an inhibitory effect on the mixed cultures, which produced less acetaldehyde in the presence of 8% sucrose than 0% sucrose. The highest concentration of acetaldehyde in the media containing 0% sucrose was 34.8 ppm,

while that in media containing 8% sucrose was only 28.9 ppm. With incubation of up to 15 hr, the levels of acetaldehyde in media containing 0% and 8% sucrose decreased to 21.2 and 20.7 ppm, respectively, and then levelled off. Without added sucrose, Görner et al. (1968) also obtained a similar result of 56.1 ppm acetaldehyde following 6 to 8 hr incubation and then a decrease to 19.8 ppm following 23 hr incubation. This decrease was assigned to alcohol dehydrogenase activity of S. thermophilus (Lundstedt, 1969).

The levels of acetaldehyde produced by L. bulgaricus or the summation of the levels produced independently by both species did not reach those produced by the mixed culture. The symbiotic action exerted by both bacteria in the mixed culture appears to be important to the production of high concentrations of acetaldehyde. Pette and Lolkema (1950b) and Bautista et al. (1968) reported that the high acid production in the mixed culture is due to S. thermophilus stimulated by valine, histidine and glycine produced by L. bulgaricus. Galesloot et al (1968) and Verigna et al. (1968) found that formic acid produced by S. thermophilus stimulates L. bulgaricus resulting in the high acid production in the mixed culture. In yogurt bases containing both 0% and 8% sucrose, the symbiotic action seemed also to apply to acetaldehyde production.

The microbial populations of S. thermophilus "A" and L. bulgaricus "A" in the mixed culture were higher in the medium

containing 0% sucrose than in that containing 8% sucrose. Both species in the mixed culture at 0% and 8% sucrose multiplied rapidly attaining higher numbers at equivalent incubation times as compared with single strain cultures. At the earlier incubation times in 0% sucrose medium, the coccus multiplied more rapidly than the rod, resulting in ratios of 7.3:1 for 1 hr and 2.14:1 for 2 hr, respectively. As the incubation time increased, the ratio became 1.4:1 for 3 hr and 0.006:1 for 24 hr. It can be seen that the rod was favored over the coccus during a long incubation. This trend has been reported by Pette and Lolkema (1951), Davis (1956b) and Görner et al. (1968).

Quantitation of Acetaldehyde during Refrigerated Storage

In an analysis of several samples of commercially manufactured plain and fruit flavored yogurt obtained in markets, it was found that there were great differences in the content of acetaldehyde. Plain yogurt samples contained 30.6, 27.9, 17.3, and 16.5 ppm acetaldehyde. Cherry or strawberry flavored yogurts contained 18.8, 16.9, 16.3, 15.2, 12.8, 10.0 and 6.9 ppm acetaldehyde. No yogurts in the previous experimental data or in reports in the literature contained such low quantities of acetaldehyde as the latter three samples.

In Table 7 it can be noticed that a considerable decrease occurred in the quantity of acetaldehyde in media containing both 0% and 8%

sucrose at 5°C as time passed.

Table 7. Quantitation of acetaldehyde in yogurt made with the mixed culture "A" during refrigerated storage at 5°C.

Refrigerated Storage (days)	Acetaldehyde (ppm)	
	0% sucrose	8% sucrose
1	24.8	21.9
2	21.7	19.5
4	19.3	16.9
7	18.4	14.0
10	15.0	10.8
14	12.7	8.7

This phenomenon also was observed by Rašić and Milanović (1966) who observed a decrease in acetaldehyde content from about 16.5 ppm to 15 ppm at 4-7°C after 6 days.

This limited data suggests that yogurt containing added sucrose will be likely to contain a lower level of acetaldehyde following incubation and that the level of acetaldehyde will continue to decline during refrigerated storage.

Determination of Volatile Acidity

Volatile acidity seems to be a good indicator of flavor in some cultured dairy products (Keenan and Bills, 1968b). Table 8 shows

the volatile acidity of yogurts containing 0% and 8% sucrose.

Table 8. Volatile acidity of yogurt.[a/]

	Volatile Acidity	
Name of Culture	0% Sucrose	8% Sucrose
Mixed Culture "A"	0.98	0.94
Mixed Culture "C-a"	1.10	1.05
Mixed Culture "C-b"	1.10	1.10

[a/] Expressed as ml of 0.1 N NaOH required to neutralize 100 ml distillate.

As can be seen, volatile acidities of cultures in 8% sucrose were only slightly lower than, or the same as, those in 0% sucrose. These values were quite low, compared with cultured butter milk which had values of 4.98 to 7.54 as determined by Keenan et al. (1968a).

Determination of Average Flavor Threshold of Acetaldehyde

The AFT values for acetaldehyde were determined since fruit flavored yogurt organoleptically seemed to have lower levels of this compound than plain yogurts even when concentrations were similar as determined by GLC. It can be appreciated that the AFT will vary depending upon the medium in which acetaldehyde is dissolved or upon the presence of other flavor components which mask the acetaldehyde

flavor note. Table 9 shows AFTs of acetaldehyde as determined in three different systems.

Table 9. Average flavor threshold of acetaldehyde.

System	AFT (ppm)
2%-fat milk	0.8
2%-fat + 8% sucrose	1.0
2%-fat + 8% sucrose + 0.4% strawberry flavor	11.7

The AFT of acetaldehyde in 2%-fat milk was determined as 0.8 ppm which is in good agreement with the results of Harvey (1960) and Patton and Josephson (1957) who reported that the AFT values in skimmilk were 0.4 ppm and 1.3 ppm, respectively. Eight percent sucrose appeared to have a slight masking effect resulting in an AFT of 1.0 ppm. Strawberry flavor imparted a strong masking effect and resulted in an AFT value of 11.7 ppm. These observations seem to indicate that equivalent concentrations of acetaldehyde will not yield equivalent flavor sensations in plain yogurt and yogurt containing fruit or fruit flavor and sucrose.

SUMMARY AND CONCLUSIONS

Single strains and mixed cultures of yogurt bacteria were investigated for acetaldehyde production in yogurt bases with and without added sucrose. The yogurt base was composed of 2%-fat homogenized milk fortified with 2% nonfat dry milk. The volatile compounds produced by yogurt culture bacteria were trapped and chromatographed by a gas entrainment on-column trapping GLC technique. Acetaldehyde was identified by coincidence of retention time with that of the authentic compound and quantitated with the use of methyl acetate as an internal standard.

Production of acetaldehyde by four mixed cultures, three single strains of S. thermophilus and three single strains of L. bulgaricus was compared in media containing 0% to 16% sucrose. Lactic acid production, microbial numbers and the ratio between the two species in the mixed cultures were determined. Acetaldehyde production by single strains of S. thermophilus and L. bulgaricus and mixed culture of these in yogurt bases containing 0% and 8% sucrose was determined as a function of incubation time.

Changes in acetaldehyde concentration during refrigerated storage of 1 to 14 days were determined.

The volatile acidity developed in yogurts containing 0% and 8% sucrose was determined.

Average flavor threshold values of acetaldehyde were determined in 2%-fat milk, 2%-fat milk plus 8% sucrose and 2%-fat milk plus 8% sucrose plus strawberry flavor.

The following results and conclusions were obtained from the investigation:

1. Acetaldehyde production by mixed cultures was detectably inhibited by 8% sucrose. However, acid production and cell counts of both species were detectably inhibited by 4% sucrose. A rapid production of acetaldehyde in media containing 0% and 8% sucrose occurred between 2 and 6 hours incubation. The level of this compound then decreased up to 15 hr of incubation and leveled off with continued incubation up to 24 hr.
2. L. bulgaricus was stimulated by 4%, 8% and 12% sucrose, 8% being most effective, resulting in the production of higher levels of acetaldehyde and acid and increased cell numbers in comparison with cultures grown without added sucrose. The rod continuously produced acetaldehyde up to 24 hr suggesting that it is primarily responsible for the production of the high concentrations of acetaldehyde in yogurt.
3. The acetaldehyde production by S. thermophilus was somewhat variable from strain to strain. In general however, less acid was produced and microbial numbers were lower, as sucrose content increased. The coccus produced much lower amounts

of acetaldehyde at equivalent incubation times than the rod.

4. During refrigerated storage of from 1 to 14 days, the acetaldehyde concentrations decreased considerably in yogurts containing both 0% and 8% sucrose.

5. Volatile acidity of yogurts containing both 0% and 8% sucrose was found to be low. There was little or no difference in volatile acidities between yogurts containing 0% and 8% sucrose.

6. The AFT value for acetaldehyde in 2%-fat milk with 8% sucrose was slightly higher than the control milk and was much higher in 2%-fat milk containing strawberry flavor and 8% sucrose.

7. Although 8% added sucrose (the approximate amount added to flavored yogurt) detectably inhibited acetaldehyde production by mixed cultures, the differences between the levels of acetaldehyde at 0% and 8% sucrose were not great. It is suggested that the strong masking effect exerted by fruit, fruit flavor and sucrose is largely responsible for the organoleptic sensation of lower levels of acetaldehyde in flavored yogurt.

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