GAS CHROMATOGRAPHY APPLIED TO THE STUDY
OF A FLAVOR DEFECT IN MILK CAUSED BY A. AEROGENES
AND THE CHARACTERIZATION OF SOME OTHER BACTERIA

by

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INTRODUCTION

Milk is a complex, highly unstable food, readily undergoing spontaneous changes, not only at room temperatures, but also at refrigeration temperatures. Psychrophilic bacteria as the cause of flavor defects in milk have become more important as improvement in refrigeration equipment has permitted longer holding periods on farms, at processing plants and in the consumers hands. Terms such as "bitter", "cowy", "putrid", and "unclean" have been used extensively to describe these bacterial flavor defects.

Comprehensive studies of the chemical nature of these off-flavors until recently, however, have been limited by lack of refined research equipment and methods. Current research at this and other stations utilizing extremely sensitive gas chromatographic instruments has demonstrated the capability of gas chromatography as a practical means of objective analysis of food flavors. Knowledge from these studies in flavor chemistry will undoubtedly be helpful in quality control work and in the definition and characterization of defects in milk and other dairy products.

Of the chemical substances involved in milk flavors, methyl sulfide has been shown recently to be of considerable importance. Although the presence of methyl sulfide in milk has been well established, the origin of this compound is not known. It has been suggested that the normal concentration of methyl sulfide in milk could result from the catabolism of methionine (24), and that this concentration together with the excess coming from particular feeds could give rise to feed flavor defects.

Within the past few years, organoleptic evaluation for quality control purposes at Kansas State University indicated that, at refrigeration temperatures, commercial pasteurized-homogenized milks sometimes developed an

abnormal flavor best described through its various stages of intensity
as cowy, feedy, chemical, and medicinal. In early stages, there was also
indication that the defect had an odor similar to that of methyl sulfide.
Bacteriological investigation resulted in the isolation and identification
of a strain of Aerobacter aerogenes. The typical defect, including the
feed-like, methyl sulfide odor could be reproduced by reinoculation of
this organism.

On the hypothesis that methyl sulfide might be involved in this flavor
defect of bacteriological origin, this study was undertaken to:

(a) Investigate the production of methyl sulfide in pasteurized-
homogenized milk by Aerobacter aerogenes.

(b) Determine the relationship between the methyl sulfide concen-
tration in pasteurized-homogenized milk and the abnormal flavor reproduced
by Aerobacter aerogenes.

(c) Attempt characterization of Aerobacter aerogenes and some
other organisms often encountered in milk and other dairy products on the
basis of methyl sulfide production as well as other volatile components.

REVIEW OF LITERATURE

Methyl Sulfide as a Flavor Substance in Dairy Products

Since 1956 when Patton, Forss and Day (44) made a preliminary report
on methyl sulfide and actually isolated it from milk, the presence and
importance of this compound in milk and other dairy products have been
adequately confirmed by many observers (16, 22, 23, 24, 32, 45, 46, 47,
57, 58, 59, 60, 61). Patton (46) in his discussion on the chemical aspects

of flavor research on milk and its products observed that methyl sulfide
may contribute to the normal flavor of milk. The compound was reported
to exhibit the flavor and aroma of milk at low concentration in water,
whereas a high concentration produced a cow's breath-like odor. And since
the sulfone derivative of methyl sulfide had been found in cow's blood, it
was believed that the route of methyl sulfide to milk was through the lungs
into the blood stream and then to the udder.

With the application of vacuum pasteurization techniques previously
reported as capable of removing up to 95% of the volatile substances from
milk (60), Wynn, Brunner and Trout (61) isolated volatile chemical compounds
from raw milk, from the vacuum line of a pasteurizer and from the milk
after vacuum pasteurization. The compounds partially identified included
acetaldehyde, methyl sulfide, acetone and water. Methyl sulfide was
also isolated by Jennings et al. (32) from copper-catalyzed-oxidized milk,
pasteurized-homogenized milk, milk exposed to fluorescent light, and from
milk with alfalfa feed flavor defect.

Day et al. (22), using low-temperature reduced-pressure distillation
techniques, found methyl sulfide as well as methyl disulfide among the
principal volatiles implicated in the flavor defect produced in skim milk
by gamma radiation. By a similar distillation technique using liquid
nitrogen, Wong and Patton in 1962 (57, 58) identified ethyl ether, methyl
sulfide, acetone, butanone, ethanol, chloroform, acetonitrile, and ethylene
chloride in the vapors separated from fresh milk and cream. One year later,
with the purpose of developing methods for detecting the adulteration of
foods, Wong (59) made another comparison of the volatile compounds from
fresh and decomposed cream. Although the results of his work are not

definitive for fresh and decomposed cream, the identification of methyl
sulfide and some other compounds have confirmed, in part, data previously
presented by Wong and Patton (57, 58).

The presence of methyl sulfide in some other dairy products and
its contribution to flavors have also been noted. Studies by Chou (16)
on the chemical nature of the characteristic flavor of 35 cultured butter-
milks of different flavor quality revealed that volatile compounds present
in all good buttermilks included propanal, acetone, pentanal, and either
formaldehyde and/or methyl sulfide. Cheddar cheese from six months to
one year old was found to contain in its volatile fractions methyl sulfide,
ethanol, acetone, and diacetyl (47). Furthermore, since methyl sulfide
has been detected in all good quality cheddar cheeses, it was considered
to be of possible importance to the cheese aroma. Day et al. (23) again
used low-temperature, reduced-pressure distillation techniques to isolate
volatile compounds from selected starter cultures and ripened cream
butter made with the starter cultures. Compounds tentatively identified
in ripened cream butter reflected most of those present in the starter
cultures and included methyl sulfide, acetone, and ethanol among others.
Recently, a cooperative research study between Oregon State University and
the Division of Dairy Research in Australia (24) on the flavor components of
butter has led to the conclusion that methyl sulfide may contribute signifi-
cantly to the flavor of butter. Bulk butter, cultured cream, cultured cream
butter, sweet cream butter, were listed as products from which methyl sulfide
was isolated. The compound was observed furthermore to have the capacity
of smoothing out the harsh flavor of diacetyl and acids associated with
culture flavor and was considered to be a desirable component of butter flavor.

The contribution of methyl sulfide and other chemical compounds to the flavor of some dairy products has been determined by a simple method developed by Patton and Josephson (45). Their approach was based on determining the threshold value of flavorful compounds psychometrically. Those compounds present in a food system at concentrations above their threshold levels were considered to make a direct contribution to flavor. If they existed at levels below thresholds, their flavor significance was doubtful. Using a spray technique to administer the sample and with five taste observers and five presentations per concentration per observer, the threshold of methyl sulfide at the 50% level of positive responses in water and skim milk was reported by Patton and Josephson (45) as 12 ppb and 21 ppb, respectively. With a flavor panel of nine members, Day et al. (24) found that the judges preferred a concentration of 40 ppb of methyl sulfide in butter oil, whereas the threshold of the compound in the oil was only 24 ppb. No threshold of methyl sulfide in whole or pasteurized-homogenized milk has been reported, however.

Psychrophilic Bacteria and Their Relation to Flavor Defects

According to Foster et al. (26), "Psychrophilic microorganisms are those which are able to develop comparatively rapidly at temperatures below 15° C (59° F)" but

> the term psychrophilic microorganisms, when used in relation to the dairy industry concerns those organisms capable of growth at refrigeration temperatures, even though their actual optimum temperatures may be more in the mesophilic range.

Many other definitions have been proposed, but none has been comprehensive enough to be universally accepted. These many definitions have

led to still more numerous, if not confusing, methods of enumerating psychrophilic microorganisms. An extensive review by Baumann and Reinbold (6) in 1963 on this subject revealed that there have been as many plate count incubation temperatures and times as there have been investigators. These temperature-and-time combinations ranged from -1.67^o C to -0.55^o C for six weeks to 25° C for three days. This wide range may justify to some extent the possibility of mesophilic bacteria that may be adapted to growth at lower temperatures and may become facultative psychrophiles as discussed by some workers (11, 26, 28, 33, 50), since facultative psychrophiles may need higher and broader ranges of temperatures and times for their growth. The standards recommended in Standard Methods for Examination of Dairy Products (6) also have changed from one edition to another, and are now 5° C to 7° C for seven to ten days (2). Nelson and Baker (6), and Foster et al. (26) recommended that plates be incubated at 21° C for four days or 25° C for three days for the detection of milk with a high bacterial count due to growth of microorganisms during refrigeration.

Hammer and Babel (28) reported that psychrophiles commonly grew rather slowly when fresh milk was first placed at low temperatures but the increase was quite rapid later. A sample of raw milk with an initial count of 28,000 organisms per ml has been shown to contain 36,000, 800,000, and 59,000,000 cells per ml after being held at 10° C for one, two and three days, respectively. Rogick and Burgwald (50) found that no psychrophiles were shown in 4.1 ml of pasteurized milk taken from vat (LTLT) or from HTST pasteurizing systems. Mesophilic counts were approximately three times greater than the psychrophilic counts in raw milk. But, at the end of one week storage at refrigeration temperatures, psychrophilic counts were obvious

in all samples and were higher than mesophilic counts. Studying the
relationship between psychrophilic bacterial content and flavor scores
of commercial pasteurized milk, Boyd et al. (9) reported that at 0.55° C
bacterial growth and flavor deterioration were markedly retarded and poor
flavor developed at a psychrophilic bacterial population of 2,500,000
per ml, whereas a population of 26,800,000 per ml was required for off-
flavor development when samples were stored at 4.44° C. Erickson and
Evans (25) inoculated sterile whole milk with pure cultures of eight
different organisms including A. aerogenes and stored the milk samples
at temperatures ranging from 5° C to 20° C. The bacterial population
at the time of initial flavor development ranged from 16,000 to more
than 30 millions per ml. Unclean, bitter, fruity, acid, nutty and cheesy
were among the flavors observed. Punch et al. (48), working with pure
cultures, reported a somewhat different result. Off-flavors were detected
at 5.2 millions to 200 millions organisms per ml for Pseudomonas, 2.5
millions to 14 millions for Alcaligenes, 8.3 millions to 120 millions
for Flavobacterium, and 2.7 millions to 150 millions for coliforms.

Although not considered as typical psychrophiles, there has been
evidence that species of the genera Escherichia and Aerobacter grow at
low temperatures and cause a wide variety of odor and flavor defects in
milk and other dairy products (18, 26, 28, 54).

By comparing the relative figures for individual growth at 5° and
10° C, Gyllenberg et al. (27) found Aerobacter among microorganisms that
appeared as "psychrophilic" types while Micrococcus, Alcaligenes visco-
lactis and Alcaligenes tolerans were less so. Marth and Frazier (35) reported
the genus Aerobacter as among the predominant bacteria in raw milk at 3.33° C.

With an initial count about 40,000 per ml, the counts would possibly
exceed 200,000 after 24 hours. With the same period of storage, Hammer
and Babel (28) observed that 59 samples of fresh milk averaging 52
Escherichia - Aerobacter organisms per ml showed an average of 964 and
60,762,000 cells at 15.55^{o} and 21.10^{o} C, respectively. Dahlberg (20)
investigated the relationship between growth of all bacteria and coliform
bacteria in pasteurized milk held at refrigeration temperatures (35° F -
40° F). He found a more rapid growth of coliform bacteria in warm weather
than in cool weather. Also, the coliform bacteria in pasteurized market
milk increased more rapidly in numbers than the total counts. On the
other hand Watrous et al. (55) concluded that, even though commercially
pasteurized milk, cream, and chocolate milk obtained from market sources
gave evidence of psychrophilic and coliform bacterial growth when held
at 5° C, coliform bacteria generally grew more slowly than other psychro-
philes.

That the development of Escherichia - Aerogenes in raw and pasteurized-
homogenized milks varies considerably is evident. This fact, in addition
to the various sources of these organisms, would be expected to contribute
to a wide variety of defects. As a group, Escherichia - Aerobacter
organisms may produce objectionable flavors and odors of various types,
but the Aerobacter and intermediate species cause a more serious and rapid
deterioration than the Escherichia species (28). Gas, acid formation,
"unclean", and souring are the most usual defects produced in milks
and milk products by these organisms (26, 28). Stark and Stark (53)
revealed in 1932 that the most common cause of ropy milk was Bacterium
aerogenes (Aerobacter aerogenes). In 1951 Claydon and Foltz (18) isolated

an Aerobacter species believed to be responsible for the development of a yeasty defect in farm-separated cream and in milk from grade-A milk-producing farms. The defect produced in cream, particularly when the isolated organism was inoculated together with Streptococcus lactis, ranged from a mild "bread dough" odor to a definite yeast-like defect. A medicinal flavor in market milk caused by Aerobacter aerogenes was reported by Claydon (17) as another possible modification of the bitter and unclean flavors often encountered in milk. The organism isolated from the defective milk reproduced the typical defect in sterile and pasteurized milk at room temperature of approximately 80° F, but more typical and intense at lower temperatures (about 50° F).

Gas Chromatographic Techniques in Milk Flavor Research

James and Martin (30) first described the analysis of mixtures of volatile fatty acids by gas chromatography in 1952. Since then this analytical method has developed rapidly and has been applied extensively to the dairy as well as non-dairy industries for flavor research. A review by Chou (16) indicated that more than 1600 publications on the application of gas chromatography were released during 1961. Also, gas chromatographic techniques have been part of the procedures used to detect methyl sulfide and other compounds in milk and milk products that have been previously mentioned in this paper.

It was with gas chromatographic techniques coupled with mass spectra analyses that Patton et al. (44) succeeded in detecting methyl sulfide in raw milk in 1956. These techniques have also been applied by a number of other workers (3, 16, 22, 23, 24, 47, 58, 59, 61) to the examination of milk.

In nearly all instances, low-temperature, reduced-pressure distillation techniques, together with different trapping procedures, were employed to recover the volatile compounds for chromatographic analyses. To increase the trapping efficiency, Wong and Patton (58) used as many as five traps after a cold water condenser. Mabbit and McKimon (34) suggested the possibility of contamination caused by this multiplicity of techniques.

Nawar et al. (38) applied gas chromatography to the study of volatiles formed in milk fat upon heating. They detected eight components from the volatile fraction of 500 g milk fat heated up to 120° C, whereas heating for 2 hr at 130° - 185° C produced up to 19 components of which eight were carbonyl. Nawar and Fagerson (39, 40) found in tests with Roquefort cheese that sampling head space organic volatile substances after concentration gave more detailed analyses than direct sampling. The effect of concentration, however, would vary for individual components and with the physical system, method and conditions used. Bavisotto et al. (7) used a dual-column, low-temperature operated gas chromatograph to study a number of volatiles of fermented milk products. Jennings et al. (32) also employed a dual column with flame ionization detector for the analyses of milk volatiles. Vapor samples were prepared by heating the milk 1 hr at 80° C in a stoppered Erlenmeyer flask. One ml of gas sample was then withdrawn through the stopper for chromatographic analysis. Bassette et al. (4) employed somewhat similar techniques by using serum vials of "5 ml capacity" with self-sealing rubber caps and saturating 2 ml of the aqueous test solution with sodium sulfate to increase volatiles in head space vapors to be analyzed. The sample was warmed to only 60° C prior to removing the head space gas sample for chromatographic analysis. These modifications,

in addition to a hydrogen flame detector and a modified electrometer, offered them the possibility of detecting some organic compounds at less than 0.1 ppm concentration in milk. These workers also reported (5) that, with volatile components present in biological fluids such as milk, blood, and urine at and below the part per million level, this technique proved successful for direct chromatographic analysis.

Gas Chromatography as a Means for Characterization of Bacteria

Although gas chromatography has been used extensively in other areas, little research has been published on the possibility of using gas chromatographic techniques as a means for the characterization of pure cultures of bacteria. Early works on bacterial characterization have been concerned mainly with paper chromatography. Cheeseman et al. (8, 12, 13, 14, 15, 31) 36, 51) conducted a series of investigations on the differentiation of bacterial species by this technique. Pure cultures of bacteria were grown in suitable media. Optical density of cell suspensions was determined and adjusted to a predetermined value before the cells were harvested. Centrifugation was then applied to the adjusted cells suspension. After being washed, cells of microorganisms were extracted with acetic acid and the extracts subjected to two dimensional paper partition chromatography. With certain species such as Bacillus and Lactobacillus, the method showed promise as an aid to differentiation, whereas the division was arbitrary and unsuccessful for others. Growth of psychrophilic bacteria that resulted in changes in the total protein content of pasteurized skim milk was studied by Skean and Overcast (52). Agar plate counts and paper electrophoresis were used to obtain the results.

Abel et al. (1) reported the feasibility of utilizing gas chroma-
tography for classification of microorganisms by analysis of chemical
composition of their cells. Again, extraction techniques were applied
to the preparation of samples for chromatographic analyses. Selected
bacterial species were grown from lyophilized cultures, harvested in the
accelerated death phase. In one instance, E. coli were harvested at
different growth phases to examine the relationship between reproduction
and lipid distribution of the organisms. Trans-esterification of the
lipids recovered was carried out by methyl alcohol in the presence of
boron trichloride. Diethyl ether was used to extract the methyl esters
which were then resolved by gas chromatography to provide distinctive
chromatographic elution patterns.

Recently, with the purpose of developing a method for the determina-
tion of the quality and flavor of milk before processing, Mabbit and
McKinnon (34) studied, by gas chromatographic patterns, the souring of
sterile raw milk due to growth of S. lactis. Their techniques for obtain-
ing vapor samples consisted of entraining the volatile compounds in a
nitrogen gas stream and trapping them in a syringe barrel cooled in liquid
nitrogen. After a suitable length of time, the syringe was removed from
the cooling agent, raised to room temperatures and about one ml of vapor
was injected into the gas chromatograph. Gas chromatography was considered
as a possible practical method of assessing the odor of milk before process-
ing.

MATERIALS AND EXPERIMENTAL PROCEDURE

There were two distinct phases of this research. First, a study was undertaken to determine the significance of A. aerogenes as a source of a unique off-flavor which preliminary studies in this laboratory attributed to methyl sulfide. This entailed using a medium relatively free initially of microorganisms and volatile substances. After inoculation with A. aerogenes and subsequent storage, comparisons were made among the development of off-flavors, titratable acidity, volatile substances, and increases in numbers of A. aerogenes. Quantitative measurements of methyl sulfide in experimental samples by gas chromatography compared with organoleptic threshold level of this material in the same media were designed to establish the role of methyl sulfide produced by A. aerogenes in the development of this off-flavor.

The second phase of this research was devoted to the characterization of bacteria by the profile of volatile materials that they produce. As a part of this study, special attention was given to development of methyl sulfide by the organisms studied.

Characterization of Flavor Defect Caused by A. aerogenes

Propagation of A. aerogenes Culture. The A. aerogenes strain was actually isolated from defective market milk having cowy, feed-like flavors. The culture was grown in litmus milk. To maintain the uniformity of bacterial populations, a loop (0.01 ml approximately) of culture was transferred aseptically into litmus milk (5 ml) every four days and incubated at 5° C. The numbers of bacteria present were determined by plating the culture daily on Standards Methods agar (2) and incubating for four days at 21° C.

Since the stock cultures of these organisms were transferred into new litmus milk every four days, no coagulation or gas formation occurred at 5° C during these periods; only a slight change in color of the cultures from blue to pale pink was observed.

Preparation of Milk Samples. Commercial pasteurized-homogenized milks were obtained from the University Creamery. Two quarts of milk were transferred into a two-neck round-bottom flask of 5 l. capacity, distilled at 63° C for 90 min at 26 cm Hg, then cooled to room temperature (20° - 25° C). All glassware and other equipment used in this study were previously auto-claved. The heat treatment was designed to eliminate most of the natural bacteria and assist in the removal of volatile materials from the milk. The amount of milk used for each batch and the 90-min period were determined experimentally as sufficient and necessary to reduce to a minimum the volatile materials present in the original milk. The pressure used was also considered as optimum, since a lower pressure induced vigorous agitation followed by foaming and entrainment of the milk into the cold water condenser and also lowered the milk temperature too much. Nitrogen gas was bubbled through the milk to displace the air in the system, minimize contamination, and assist in removing volatile materials. A round-bottom flask was cooled in an ice bath and connected to the cold water condenser of the distillation unit to collect water from the vacuum-heated milk. The amount of water collected was measured, and an equivalent volume of sterile dis-tilled water, that had been verified by gas chromatography as free of volatile materials, was added back to the processed milk to bring its volume back to normal.

After being cooled to room temperature, different batches of vacuum-heated milk were mixed and transferred to two sterile 2-1. Erlenmeyer flasks

(approximately 2 l. of milk in each flask). One of these flasks of milk served as control while the other was inoculated with a four-day old culture of A. aerogenes. Suitable dilutions had been made from the A. aerogenes culture to give an estimated 3000 to 4000 cells per ml of milk initially. A sufficient number of milk samples in screw-capped vials (40-ml capacity) were prepared from each of these two Erlenmeyer flasks and stored at $5°$ C so that two different vials, one control and one inoculated milk, could be removed daily for bacteriological and gas chromatographic analyses and other observation during each trial. To minimize the loss of developed volatile materials due to bacterial growth during the storage period, all the vials of prepared milk samples were covered with aluminum foil before screw caps were replaced. The remainders of the milks in the two 2-l. Erlenmeyer flasks were also stored at $5°$ C and removed every day during each trial for acidity test determination and for preparation of samples for organoleptic evaluations.

Bacteriological Analysis. Since only two vials of prepared milk samples (one control and one inoculated) were used for both bacteriological and gas chromatographic analyses, bacteriological work was performed first to avoid contamination.

Bacterial growth was followed by plating both the control and inoculated milks on Standard Methods agar in duplicate. Colony counts were made after four days of plate incubation at $21°$ C. In plating, dilutions of the inoculated milk were increased between consecutive daily analyses to keep up with bacterial growth.

Organoleptic Analysis. Detection and criticisms of the off-flavor produced as a result of bacterial growth in the milk samples were performed by at least two experienced judges.

Milk samples were prepared every day according to the triangle method described by Pangborn and Dunkley (43). Three coded milk samples, consisting of one inoculated and two control or vice versa, were considered as a test unit. Each judge was given three test units, i.e., a total of nine samples, and was asked to identify the sample believed to be different from the other two in each test unit and to describe the defect. The method was applied because it was thought that it might be used effectively with a limited number of experienced judges in testing for small variation between the control and inoculated milks.

Gas Chromatographic Analysis. Apparatus: The instrument used to obtain the chromatograms was an Aerograph 600-B with a hydrogen flame ionization detector and a 1.05 mv Brown-Honeywell recorder. A 10 ft x 1/8 in. stainless steel column packed with 20% carbowax 20 M on 60 to 80 mesh acid washed firebrick was employed. With nitrogen as carrier gas, the operating conditions were:

                Column temperature (°C)   100
                Nitrogen outflow (ml/min)   15.4
                Nitrogen input (psig)   18
                Hydrogen outflow (ml/min)   20
                Chart speed: 1/3 in./min

Sampling bottles. serum vials, 15 mm diameter x 52 mm, of 5 ml capacity with self-sealing rubber caps.

Syringe. 1 ml gas tight syringe, Hamilton No. 1001.

Mechanical shaker. A Fisher Clinical shaker equipped with a Kahn tube rack and operated at a rate of 275 to 285 oscillations per min was used.

Reagents: Acidic and basic hydroxylamine solutions prepared according to the procedure of Bassette et al. (5).

Sodium sulfate, anhydrous, ACS grade.

Mercuric chloride, anhydrous, ACS grade.

Samples of control and inoculated milks were analyzed as head space gas prepared according to the procedure of Bassette et al. (4, 5) with some modifications. Two ml of milk was saturated with 1.2 g of sodium sulfate in a serum vial. After heating to 60° C for 2 min in a water bath, mixing on the shaker for 5 min, introduction of a new dry serum cap, and heating again to 60° C for 8 min, 1 ml of head space vapors was taken from above the milk and injected into the chromatograph. Gas chromatographic analyses for both control and inoculated milks were made in duplicate.

Identification of methyl sulfide as well as carbonyl and ester was accomplished by the simple prechromatographic reaction technique of Bassette, Ozeris and Whitnah (5).

The methyl sulfide peak could be eliminated by treating 2 ml of inoculated milk with 0.2 g of mercuric chloride in a self-sealing rubber-capped serum vial on the shaker for 1 hr, after which samples of head space gas for chromatographic analyses were obtained by the foregoing procedure.

To establish a quantitative analysis for the methyl sulfide produced, peak heights of this compound, as shown by chromatograms obtained from the analyses of the inoculated milk, were compared with a standard curve prepared from the chromatographic analyses of methyl sulfide added in pasteurized homogenized milk that had been subjected to exactly the same treatment as the inoculated milk. Dilutions of methyl sulfide in pasteurized homogenized milk and in water were prepared on a weight basis.

Elimination of carbonyl and ester peaks was accomplished by treating the analyzed milk samples with 0.1 ml of acidic and basic hydroxylamine

solutions, respectively. After the 1-hr reaction period on the mechanical shaker, head space vapors for chromatographic analyses were prepared as previously described.

Determination of Taste Thresholds of Methyl Sulfide. The method developed by Patton and Josephson (45) for determining significance of volatile flavor compounds in foods was employed to establish the taste threshold of methyl sulfide in distilled water and in commercially pasteurized-homogenized milk that had been treated in the same way as the milks used for bacteriological, chromatographic and organoleptic analyses.

There were from eight to nine tasters who were students and staff members of the University. Tasting was conducted in the product evaluation room at the Dairy Science Department, with one taster at a time. The tasters were told that methyl sulfide was the taste substance under investigation. A dilution well above the expected threshold, and a control sample were first presented for familiarization and orientation, after which control samples and different dilutions of methyl sulfide prepared from a stock solution on a weight basis were presented at random to the judges in 3/4 oz cups. The panel was asked to indicate, without guessing, whether or not methyl sulfide could be detected in a given sample by saying "yes" or "no." With an eight- to nine-member panel and with five replications per concentration, a total of 40 to 45 judgments were obtained for a given concentration. The threshold level was, as previously defined, the 50 per cent correct response level.

Titratable Acidity. Changes in acidity of both control and inoculated milk were determined at daily intervals and at approximately the same time the other analyses were performed. All titrations were made in duplicate and titratable acidity was expressed as per cent of lactic acid.

Characterization of Some Bacterial Species by Gas Chromatographic Patterns

Propagation of Bacterial Cultures. Three bacteria were used in this second phase of the study: Lactobacillus casei, Streptococcus lactis, and Pseudomonas fragi. Stock cultures were grown, transferred every four days as previously described for the A. aerogenes culture, but incubated at 15° C. Standard Methods agar plate counts were used to determine the number of organisms present in the cultures. Plates of Ps. fragi culture were incubated at 21° C, those of L. casei and S. lactis at 35° C, for four days.

Preparation of Growth Media. Commercially pasteurized homogenized milks were used as growth media for all cultures. The preparation of the milks was as described previously with the following modifications: after being vacuum treated at 63° C for 90 min, the milks were transferred into four 250-ml Erlenmeyer flasks, steamed for 1-hr at 88° C, then cooled to room temperature. Steaming after the distillation procedure was determined experimentally as necessary to provide a medium reasonably free of undesirable bacteria during a period of incubation of seven days at 15° C. One of the flasks of milk so prepared was used as control, while the other three were inoculated respectively with four-day old cultures of L. casei, Ps. fragi, and S. lactis. After inoculation, the milks were again transferred to screw-cap vials and incubated at 15° C until bacterio-logical and gas chromatographic analyses were carried out at daily intervals.

Bacteriological Analysis. Plate counts for both control and inoculated milks were prepared as described for the A. aerogenes study above. Colony counts were made after four-days plate incubation at 21° C for Ps.

<u>fragi</u> and at 35° C for <u>L. casei</u> and <u>S. lactis</u>. Plates from control milks were incubated at both 21° C and 35° C.

<u>Gas Chromatographic Analysis</u>. The apparatus used to obtain the chromatograms and the preparation of the milk samples for qualitative and quantitative analyses were as previously described. Operating conditions were as previously described.

RESULTS

#### Characterization of Flavor Defect Caused by <u>A. aerogenes</u>

<u>Bacteriological Analysis</u>. Bacterial counts obtained at 24-hr intervals during growth of <u>A. aerogenes</u> in vacuum-distilled pasteurized homogenized milks from three trials are presented in Fig. 1. Attempts were made to prepare milks with initial counts of 3000 to 4000 cells per ml.

In presentation of data in Fig. 1, an adjustment was made in the locations of the growth curves. Two of the three trials represented in the figure had initial counts well below the 3000 to 4000 organisms per ml sought. The curves as drawn in Fig. 1 represent the growth from the time the counts reached 3000 to 4000 cells per ml in each trial. The purpose of this shift in the curves along the abscissa of the figure was to illustrate the growth from approximately the same initial count.

Examination of growth curves of <u>A. aerogenes</u> at 5° C during these trials revealed two distinct phases. A near linear logarithmic growth rate occurred during the first four days, with count changing from 3000 - 4000 organisms to 90 - 110 millions organisms per ml. The second phase, a negative accel-eration stage, showed an increase at a decreasing rate, with counts changing

Figure 1. Growth curves of <u>A. aerogenes</u> in three trials with adjustments made to bring initial numbers of microorganisms in between 3000 and 4000 cells per milliliter.
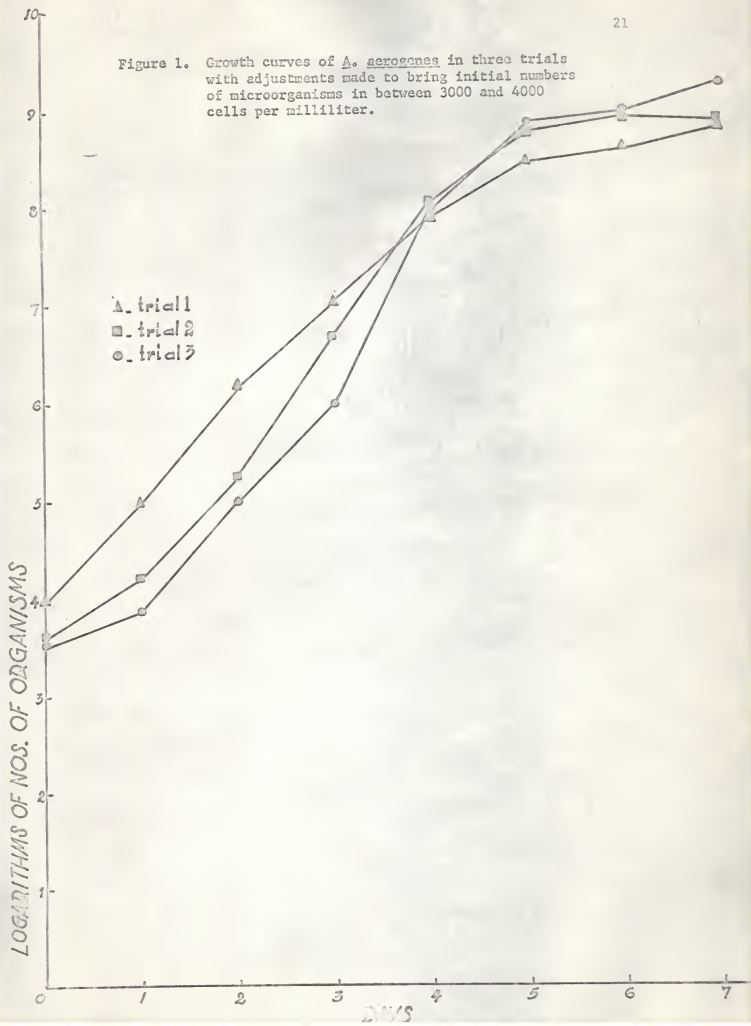
from 90 - 110 million to 750 million - 2.2 billion organisms per ml, from
the fourth to the seventh day.

Throughout this part of the study, bacterial counts in the control
milks changed from 50 - 200/ml at the time of inoculation to 500 - 9000/ml
at the end of the trials.

Organoleptic Analysis. Organoleptic analyses from three trials are
presented in Table 1. The results indicated that the bacterial induced
off-flavor could not be definitely detected during the first three days
of every trial. By the fourth day, the off-flavor and odor were pronounced.
A cooked or caramelized flavor was apparent in both control and inoculated
milks before the bacterial induced flavor appeared. Terms such as "molasses
like", "methyl sulfide like", "cowy", "acid", and "putrid" were among
criticisms given to the inoculated samples, with the two latter criticisms
being used in the later stages of defect development. The control milk
maintained a predominant cooked flavor. The off-flavors and odors were
first in evidence when bacterial counts were between 12.5 and 110 million
organisms per ml. In the early stage of flavor development, a comparison
of dilutions of methyl sulfide in vacuum-distilled pasteurized homogenized
milk with the inoculated milk suggested the same flavor and aroma. However,
the criticisms as reported here were subjective and appeared to reflect
previous experiences of the judges in regard to the typical odor of methyl
sulfide in milk.

Gas Chromatographic Analysis. Chromatograms prepared from milks
inoculated with A. aerogenes contained six easily recognized peaks by the
end of every trial that have been designated in this study as A, B, C, D,
E, and F in the respective order of their emergence from the column at 2.5,

Table 1. Organoleptic analysis by the triangular taste test (43) of vacuum-distilled pasteurized homogenized milk inoculated with A. aerogenes and incubated at 5° C.

Days	First trial			Second trial			Third trial		
	Number of judges	Number of test units observed[1]	% Correct responses[2]	Number of judges	Number of test units observed	% Correct responses	Number of judges	Number of test units observed	% Correct responses
0	4	12	0	3	9	0	2	6	0
1	4	12	25	3	9	0	2	6	0
2	4	12	8.33	3	9	44.44	3	9	0
3	3	9	66.66	3	9	66.66	2	6	0
4	2	6	100	3	9	100	2	6	100
5	2	6	100	2	6	100	2	6	100
6	2	6	100	2	6	100	2	6	100
7	2	6	100	2	6	100	2	6	100

1 Test unit = set of three coded samples consisting of two inoculated and one control milks or vice versa.

2 Correct response = correct identification of the three coded samples of one test unit.

3.0, 4.0, 6.3, 9.0, and 12.0 min. One of these chromatograms is reproduced in Fig. 2. A typical chromatogram from control samples is also reproduced in this figure. Throughout the study, chromatograms from control milk contained only peak C, and sometimes peak D, and these peaks remained unchanged, or increased only slightly by the end of a trial.

Evidence of changes in the inoculated milk was shown in almost all instances first by a slight increase of peak D on the third day, then by the appearance of peaks A and B in the following analysis on the next day. Peak C, and sometimes peak D, were observed in all analyses and from the beginning to the end of each trial because part of these peaks were contributed by volatiles that could not be completely removed from the original milk by the vacuum steam distillation procedure previously described. Peaks E and F usually were observed one or two days after the other peaks had developed with peak F showing considerable increase by the end of trials 2 and 3 (see Figs. 4 and 5).

By reaction with mercuric chloride and by comparison with the retention time of the authentic compound in distilled water and in vacuum-distilled pasteurized homogenized milk, peak B in the inoculated milk was identified as methyl sulfide.

Peaks A and C were shown to be carbonyl by a prechromatographic reaction of the analyzed milk samples with acidic hydroxylamine. They were later identified by comparing with retention times of authentic compounds as acetaldehyde and acetone, respectively.

Retention times of peak D agree with that of ethanol, a compound commonly found among fermentation products of microorganisms and reported by others to be present in milk (16, 47, 57, 58, 59).
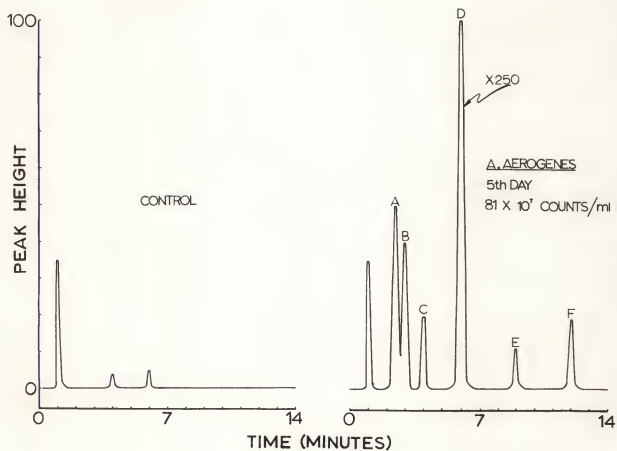
Figure 2. Comparison of chromatographic patterns of control milk and milk inoculated and incubated at 5° C with A. aerogenes. All peaks have an attenuation factor of 4 unless indicated.

Reaction with basic hydroxylamine indicated that chromatograms prepared from A. aerogenes milks did not contain any esters. The nature of peak E and F awaits further investigation. Prechromatographic reactions that resulted in the elimination of different peaks from chromatograms of two selected milk samples inoculated with A. aerogenes on the seventh day of a trial are included in Table 2. The overall results of this identification work are summarized in Table 3. Peaks D, E, F, although not identified, were proven not to be sulfide, carbonyl or ester.

The similarities of the volatile chemical compounds produced by A. aerogenes in three trials are shown in Figs. 3 to 5. All peak heights have been corrected for the slight amounts of volatile components present in control samples.

One striking feature in this study was the tremendous increase of peak D toward the end of each trial. On the basis of chromatograph peak heights (% of full scale deflection of the recorder multiplied by the attenuation factor), this peak alone accounted for more than 95% (95.23% - 95.51%) of the total peak heights measured. Less than 5% of the total peak heights measured on the same basis was divided among five other components in the following descending order: peak A (1.39% - 1.74%); peak B (1.04% - 1.33%); peak F (0.81% - 0.32%); peak C (0.43% - 0.62%); and peak E (0.04% - 0.32%). A preliminary study of this A. aerogenes culture during which milk samples were incubated at 12° C instead of 5° C revealed the same numbers of peaks and the same characteristic regarding peak D.

The relationship between peak heights and actual concentration must be considered carefully. Although chromatographic peak heights have been used for quantitative analysis (5), it is necessary to make such an analysis

Table 2. Effect of selective reactions on peak heights of different volatile chemical compounds on chromatograms of _A. aerogenes_ milk.

| Peak | Peak heights[1] before reaction | Peak heights after reaction of the milk with | | |
		mercuric chloride	acid hydroxylamine	basic hydroxylamine
			Sample 1[2]	
A	252	240	0	
B	116	0	112	
C	64	80	0	
D	19625	16250	19250	
E	72	72	68	
F	140	140	128	
			Sample 2	
A	288		0	0
B	144		92	80
C	136		0	24
D	23000		24000	22500
E	92		92	84
F	524		600	580

1 % of full scale recorder deflection x attenuation factor.
2 Average results from duplicate analysis.

Table 3. Summary of gas chromatographic analysis of the
          volatile compounds produced by A. aerogenes in
          vacuum distilled pasteurized homogenized milks.

Peak	Retention time in minutes	Chemical compounds	
		Identified	Partially identified
A	2.5	acetaldehyde	
B	3.0	methyl sulfide	
C	4.0	acetone	
D	6.3		ethyl alcohol
E	9.0	unknown	
F	12.0	unknown	

with the aid of standard curves of the known materials. For example,
0.1 ppm methyl sulfide produced a peak height of 240 (see Fig. 7) while
ethyl alcohol at that same concentration gave a peak height of less than
10 (42). This means if peak D is ethanol, it would represent over 20 times
as much material as would occur from the same size peak of methyl sulfide.
Although most neutral chemical compounds give about the same response in
this head space gas chromatographic analysis, there are individual differences
due to the specific nature of each chemical compound.

Determination of threshold concentrations of methyl sulfide. The average
results of taste threshold determinations of methyl sulfide in distilled
water and in vacuum-distilled pasteurized homogenized milk are presented
in Fig. 6. Threshold concentrations of approximately 9 parts per billion
in distilled water and 115 parts per billion in the milk were deduced
respectively from the two cruves shown in the figure.

Figure 3. Changes in volatile components produced by A. aerogenes in vacuum-distilled pasteurized homogenized milk incubated at 5° C. First trial.

Figure 4. Changes in volatile components produced by A. aerogenes in vacuum-distilled pasteurized homogenized milk incubated at 5° C. Second trial.

Figure 5. Changes in volatile components produced by <u>A. aerogenes</u> in vacuum-distilled pasteurized homogenized milk incubated at 5° C. Third trial.
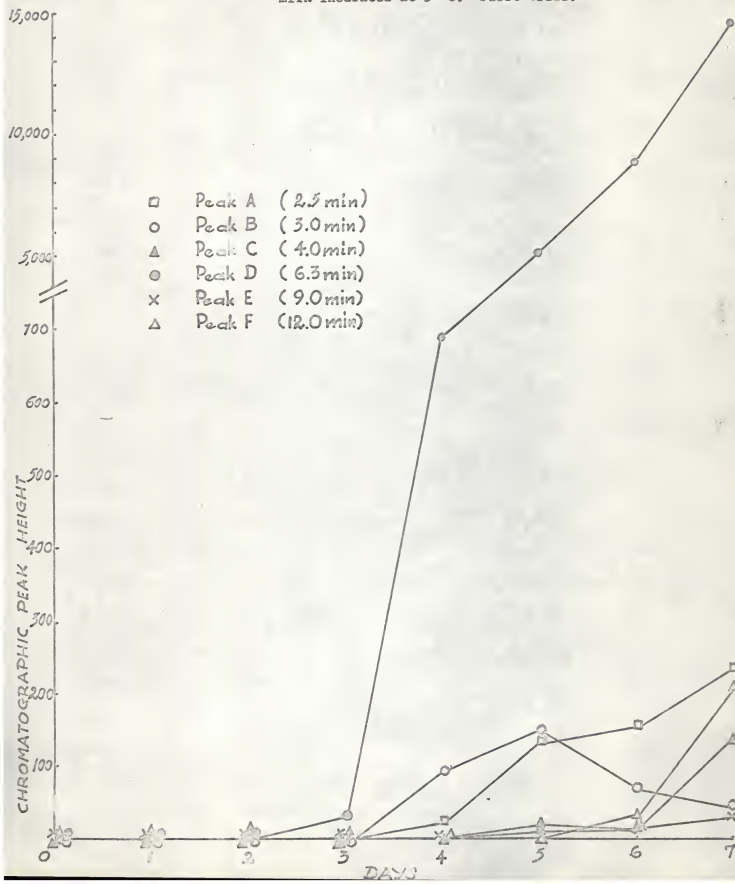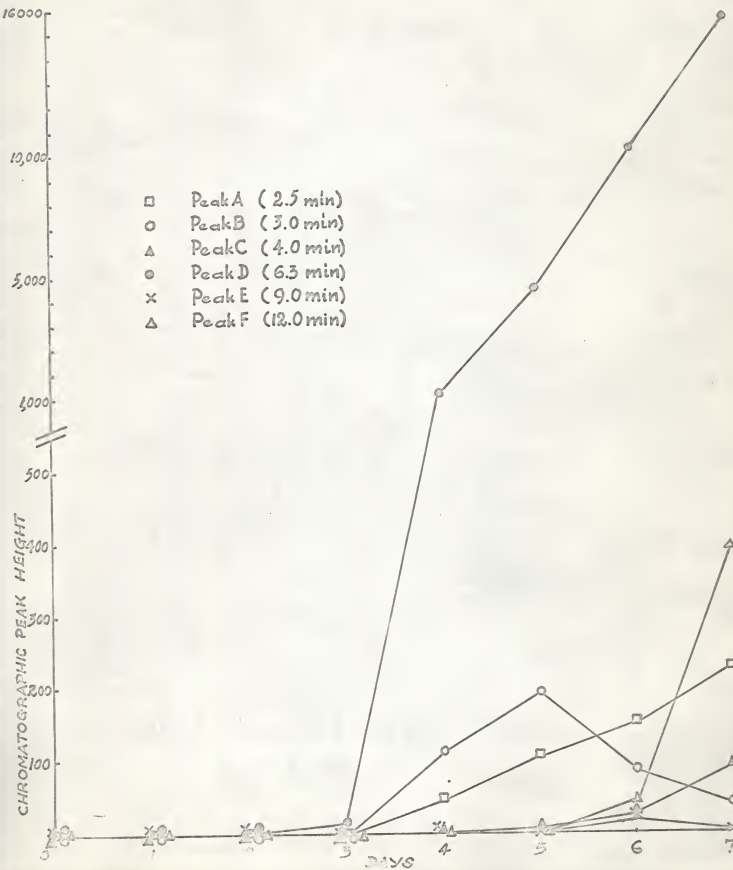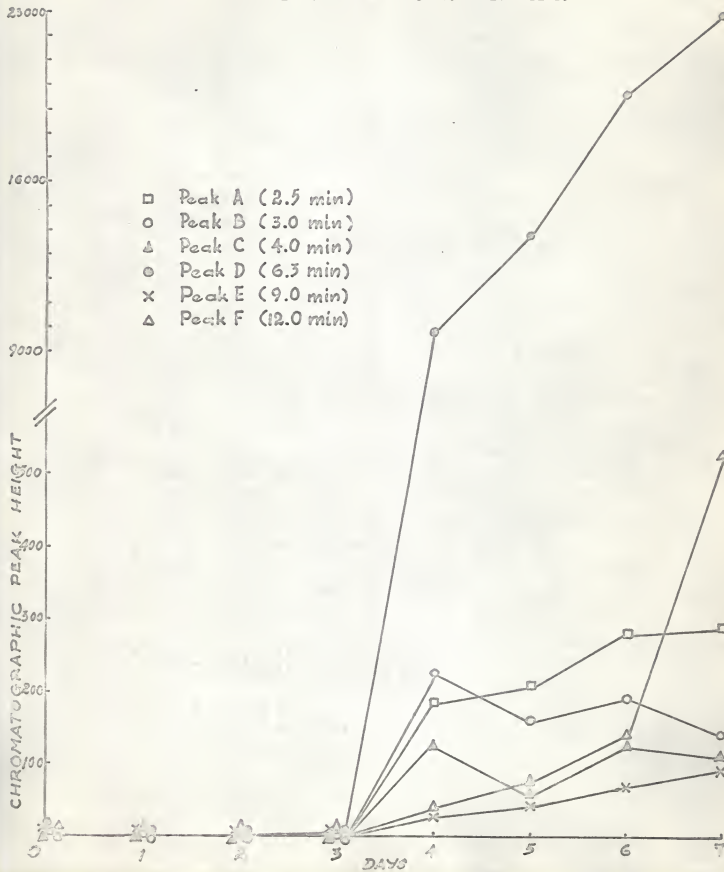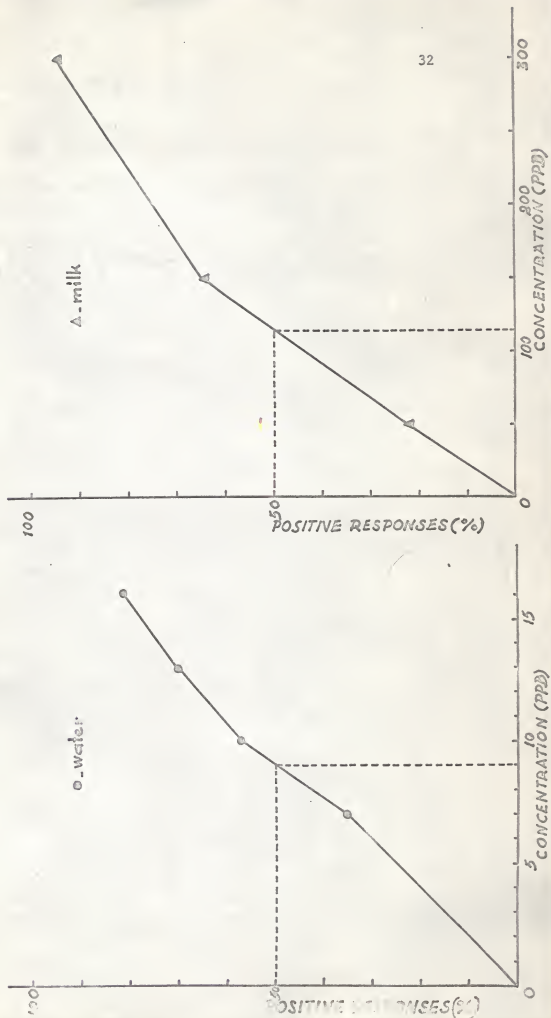
Figure 6. Threshold concentrations of methyl sulfide in distilled water and in vacuum-distilled pasteurized homogenized milk. Averaged data from 9 observers for water and 8 observers for milk using 5 presentations per concentration per observer.

All the chromatographic peak heights representing the changes of methyl sulfide in A. aerogenes milk in the three trials previously reported were converted to parts per billion from an extrapolated standard curve of methyl sulfide in vacuum-distilled pasteurized homogenized milk. A standard curve of methyl sulfide in distilled water was also determined for purpose of comparison. These standard curves were prepared from the chromatographic analyses of different prepared solutions of added methyl sulfide, ranging from 1 ppb to 100 ppb (10 dilutions) in distilled water and 50 ppb to 100 ppb (5 dilutions) in the milk. The curves are shown in Fig. 7. Each point on these standard curves in the figure represents a duplicate determination with variation in peak heights being less than 10%. This line was extrapolated in order to encompass the necessary range to give concentration of methyl sulfide reported.

Curves representing the amounts of methyl sulfide, in parts per billion, produced by A. aerogenes in vacuum-distilled pasteurized homogenized milk during three trials are compared in Fig. 8 with the line of threshold concentration obtained as described above in the milk. It is evident from these graphs that the amounts of methyl sulfide produced by A. aerogenes, though below 1 ppm in all instances, were more than sufficient to influence the flavor of the inoculated milk.

Titratable Acidity. Results of all analyses in three trials are included in Table 4.

Relationships of Results. The interrelationship among the changes in bacterial count, flavor, total peak heights, methyl sulfide concentration and titratable acidity production during growth of A. aerogenes in milk from three trials is illustrated in Figs. 9, 10 and 11. Data used to prepare

Figure 7. Standard curves of methyl sulfide in water and in vacuum-distilled pasteurized homogenized milk.
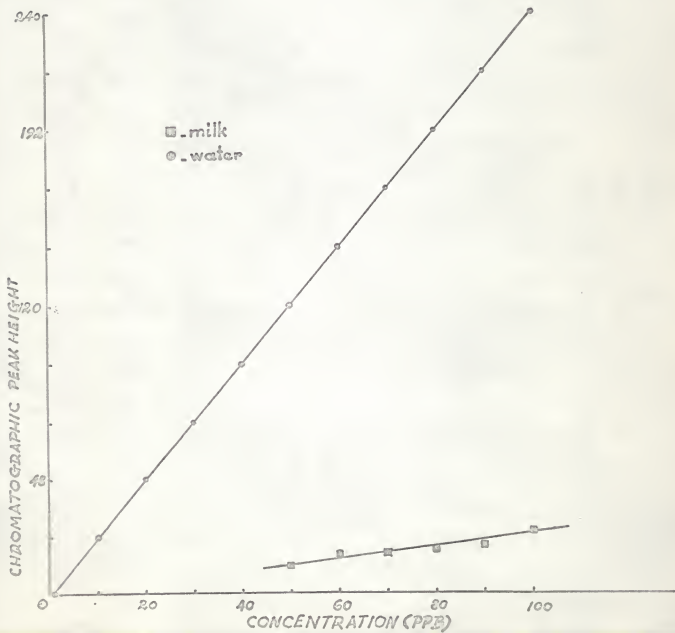
Figure 8. Concentration of methyl sulfide produced by
A. aerogenes compared with the threshold level
of methyl sulfide in vacuum-distilled pasteurized
homogenized milk.
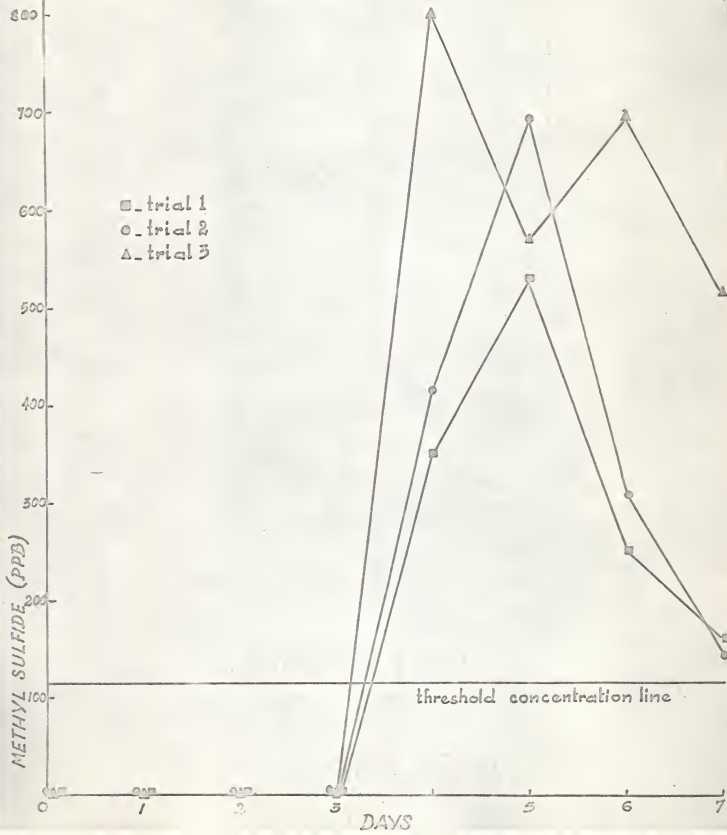
Table 4. Titratable acidity[1] produced by A. aerogenes in vacuum-distilled pasteurized homogenized milk incubated at 5° C.

Days	First Trial			Second Trial			Third Trial		
	Inoculated milk	Control milk	Difference	Inoculated milk	Control milk	Difference	Inoculated milk	Control milk	Difference
0	0.165	0.165	0	0.160	0.160	0	0.160	0.160	0
1	0.165	0.165	0	0.160	0.160	0	0.160	0.160	0
2	0.165	0.165	0	0.160	0.160	0	0.160	0.160	0
3	0.165	0.165	0	0.160	0.160	0	0.160	0.160	0
4	0.195	0.165	0.030	0.190	0.165	0.025	0.250	0.160	0.090
5	0.265	0.175	0.090	0.225	0.165	0.060	0.280	0.165	0.115
6	0.270	0.175	0.095	0.260	0.170	0.090	0.325	0.165	0.160
7	0.315	0.175	0.140	0.300	0.170	0.130	0.355	0.165	0.190

1 Expressed as % lactic acid.

the curves in the figures had been corrected for the values of the respective characteristics in control milks (bacterial counts, total peak heights, methyl sulfide concentration, and titratable acidity).

It is of interest to note that after adjustments had been made for bacteriological data (adjustment of initial counts to 3000 - 4000 cells/ml), and thence for those of gas chromatographic analysis, organoleptic evaluation and titratable acidity, all the changes were obvious or could be detected on the fourth day of each trial.

Bacterial counts during the 24-hr period between the third and fourth day changed from 960,000 to 110 million organisms per ml (overall result). The other data being measured showed the following changes on the fourth day for trials 1, 2, and 3, respectively:

(a) methyl sulfide concentration: 350 ppb, 414 ppb, 800 ppb.

(b) increase in total measurable volatile material (peak heights): 812, 1246, and 10,436, (summation of all peak heights).

(c) increase in titratable acidity: 0.025%, 0.030%, 0.090%.

(d) organoleptic analysis: off-flavor 100% detected in all three trials.

These results in Figs. 9-11 show relatively small differences between trials 1 and 2 (Figs. 9 and 10). However, with the exception of organoleptic analyses, large differences appeared between data of the first two trials and the third one, though adjusted bacteriological data (Fig. 1, 9-11) implied approximately the same counts for three trials. No explanation for this is available.

Figure 9. Changes in bacterial counts, methyl sulfide concentration, titratable acidity and total peak heights during growth of *A. aerogenes* in vacuum-distilled pasteurized homogenized milk incubated at 5° C. Trial 1.

○ bacterial counts
△ Me₂S concentration
□ titratable acidity
× total peak heights

Figure 10. Changes in bacterial counts, methyl sulfide concentration, titratable acidity and total peak heights during growth of A. aerogenes in vacuum-distilled pasteurized homogenized milk incubated at 5° C. Trial 2.

o Bacterial counts
▲ Me₂S concentration
□ titratable acidity
x total peak heights
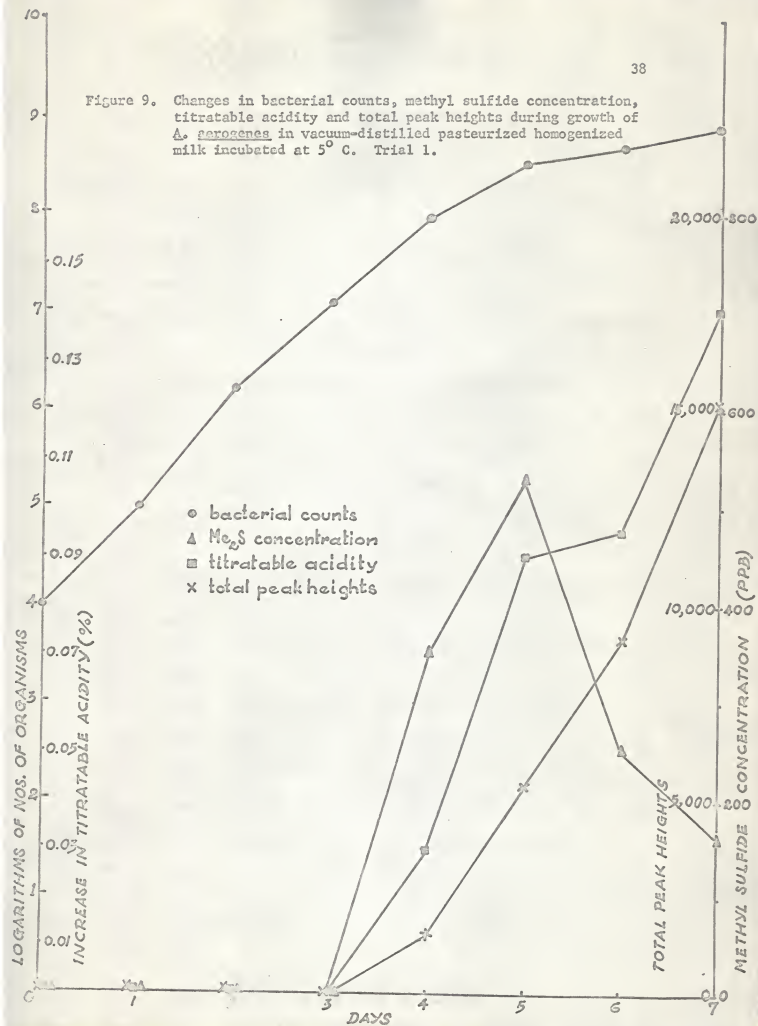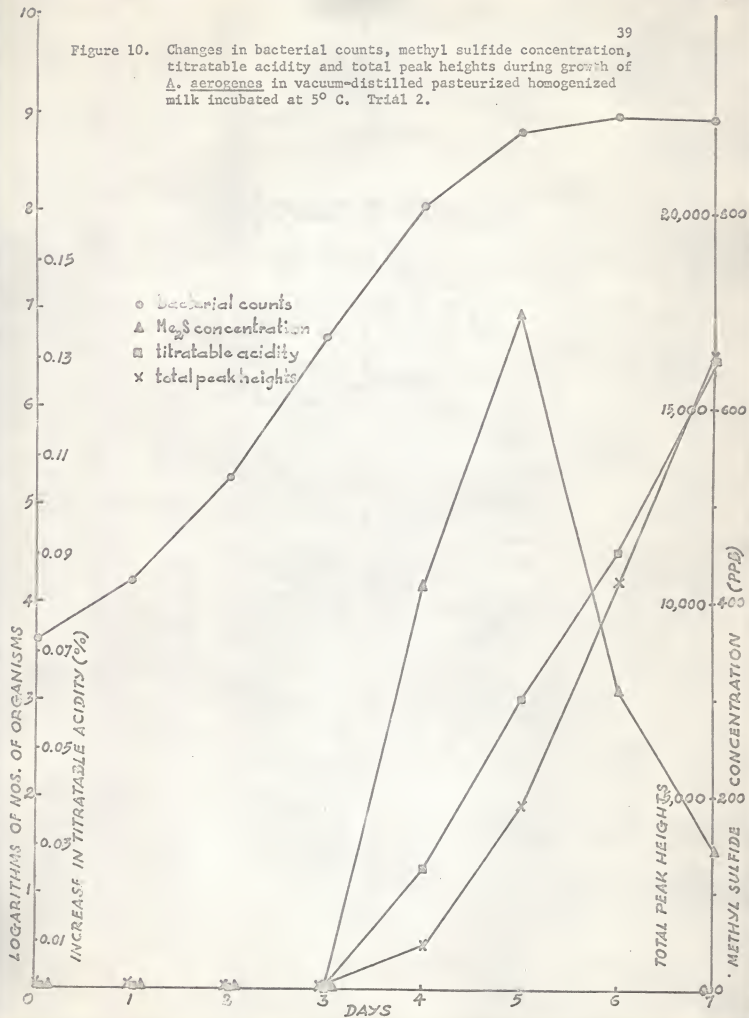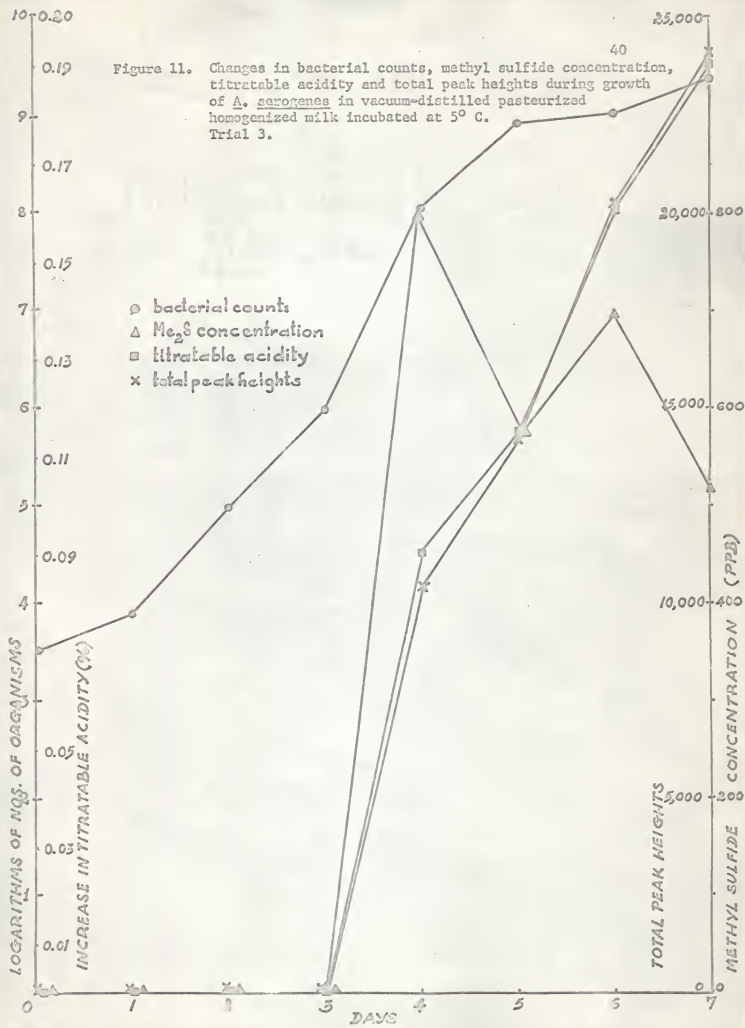
Figure 11. Changes in bacterial counts, methyl sulfide concentration, titratable acidity and total peak heights during growth of A. aerogenes in vacuum-distilled pasteurized homogenized milk incubated at 5° C. Trial 3.

ρ   bacterial counts
△   Me₂S concentration
□   titratable acidity
✗   total peak heights

The methyl sulfide level increased rapidly in the early stage of bacteria growth, then decreased toward the end of the trial, on the seventh day. Its greatest concentration (Figs. 8, 9-11) appeared on either the fourth or the fifth day. Since chromatograms were prepared on a daily basis, and since great changes in the inoculated milk might be expected during this period, the highest point on a methyl sulfide curve (Figs. 8, 9-11) may actually represent the highest concentration or it may only be in the vicinity of its maximum concentration.

The reduction of methyl sulfide toward the end of each trial was first believed to result from losses of the volatiles during the incubation period and preparation of milk samples for chromatographic analyses. However, a comparison of chromatograms prepared simultaneously from milk samples incubated in screw-capped air-tight vials and in Erlenmeyer flasks stoppered only with sponge-like porous corks indicated that these were not the causes of the decrease. The water bath temperature used during the preparation of samples was approximately 60° C, but serum vials containing analyzed milk samples were not in any case opened after the warming period. Consequently, losses during this preparation, if they were significant, should vary randomly from one sample to another and from one trial to the next. They could not result in such a definite decreasing trend in all instances as evidenced by chromatograms obtained. There is a possibility that methyl sulfide was utilized by competing organisms or that a chemical change took place in the molecule.

There was a definite increase in the total peak heights after the third day of every trial with peak D in all cases accounting for more than 95% of the increase. This feature can be reaffirmed by comparing in the

trends and the peak heights of the curves representing changes of peak D
in Figs. 3 to 5 with the corresponding curves representing changes of
total peak heights in Figs. 9-11, respectively.

Titratable acidity expressed as percentage of lactic acid in the
control milk varied from .160% to .175%, while the inoculated milks changed
from .160% or .165% up to .355% at the end of the trials, on the seventh
day. Titratable acidity and total volatile productions followed a similar
pattern, particularly in trial 3 (see Figs. 9-11). The decrease in methyl
sulfide production was observed when titratable acidity production changed
by 0.06% - 0.09% and when the highest bacterial counts among three
trials averaged 630 x 10^6 organisms per ml.

It was expected that, based on its selectivity and sensitivity,
gas chromatography could detect the changes in the inoculated milks before
organoleptic evaluation, or even titratable acidity. The advantage of
gas chromatographic method over the changes of pH in following the
process of natural souring of milk has been reported by Mabbit and McKinnon
(34). Results of this study show a critical period between the third and
fourth day of every trial. Thus, subtle differences among chromatographic
method, organoleptic evaluation and titratable acidity could not be
determined with analyses performed only at the beginning and the end of
24-hr intervals.

Characterization of Some Bacterial Species by Gas Chromatography

Bacteriological Analysis. Attempts were first made to prepare milks
with initial inocula to give 3000 to 4000 cells per ml as in the case of
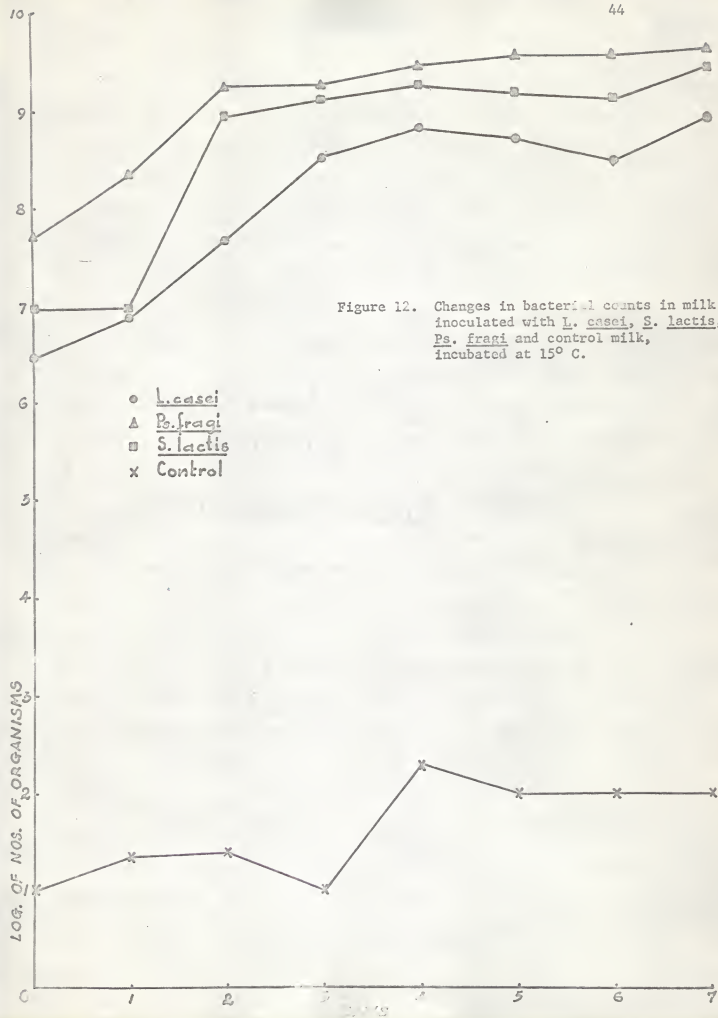A. aerogenes study. However, the vacuum-steam distillation procedure

previously employed proved insufficient when the incubation temperature
was increased from 5 to 15° C. Bacterial counts increased rapidly in the
control milk by the third day. A high initial inoculation to accelerate
changes in experimental milk and thus reduce the length of the holding
period for control milk and a 1-hr steaming period at 88° after the
distillation procedure were finally adopted. The longer steaming time
provided a reasonably low bacterial count in control milk during a seven-
day period needed for chromatographic analysis.

Curves representing the growth of *L. casei*, *S. lactis*, *Ps. fragi*,
in vacuum-distilled pasteurized homogenized milks in one selected trial,
each are presented in Fig. 12. The initial counts after inoculations were
respectively, 2.8×10^6, 9.1×10^6, and 49×10^6 organisms per ml.

As mentioned above, milks prepared utilizing the less severe heat
treatment and the lower initial inoculum had considerable bacterial growth
in the control milk in four days. Although the results of these trials
are not included herein, the patterns of chromatographic peaks that
were obtained from the samples were similar to those shown in Fig. 13,
if allowance is made for peaks contributed by the control milk.

As shown in Fig. 12, only the phase of negative acceleration was
observed on the three growth curves. The high initial inoculation led to
an earlier development of volatile chemical compounds than occurred in the
A. aerogenes study.

Growth of organisms present in control milk, changing from less than
ten to more than 100 cells per ml, was insignificant compared to the numbers
in the inoculated milks.

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Figure 12. Changes in bacterial counts in milk inoculated with _L. casei_, _S. lactis_, _Ps. fragi_ and control milk, incubated at 15° C.

- ⊙ _L. casei_
- △ _Ps. fragi_
- ▢ _S. lactis_
- × Control

LOG. OF NOS. OF ORGANISMS

Chromatographic Analysis. Changes in chromatograms from different
inoculated milks were obvious from the first to the third day of the
trial. Chromatograms of milk from Ps. fragi changed after one day with
counts increasing from 49 millions to 214 million organisms/ml; S. lactis,
after 2 days with counts increasing from 9.4 millions to 900 million
organisms/ml; and L. casei, after 3 days with counts increasing from 44
millions to 320 million organisms/ml. Determination of the exact numbers
of organisms at which changes in chromatographic patterns could first be
detected was again impossible with these 24-hr periods between analyses.

The additional heat treatment necessary to reduce bacterial numbers
in milk to be used in this phase of the study resulted in the formation
of a number of volatile materials. This was evidenced by chromatographic
peaks that appeared in chromatograms of the control and inoculated milks
prior to bacterial growth. Chromatograms of each of the cultured milks
as well as freshly prepared and aged control milks are presented in
Fig. 13. Chromatograms of the inoculated milks represent lots with
approximately the same number of organisms.

All chromatograms from control milk contained four easily recognized
peaks that appeared at 2.5, 3.0, 4.0, and 8.7 min. A comparison of
chromatograms from control milks in Figs. 2 and 13 indicates that the
increase of the 4.0 min peak and the presence of the other three peaks,
over that observed in the A. aerogenes study, were due to the steam-
sterilization process. All these peaks, however, remained unchanged
throughout the trial.

Chromatograms from milk with L. casei, Ps. fragi or S. lactis (see
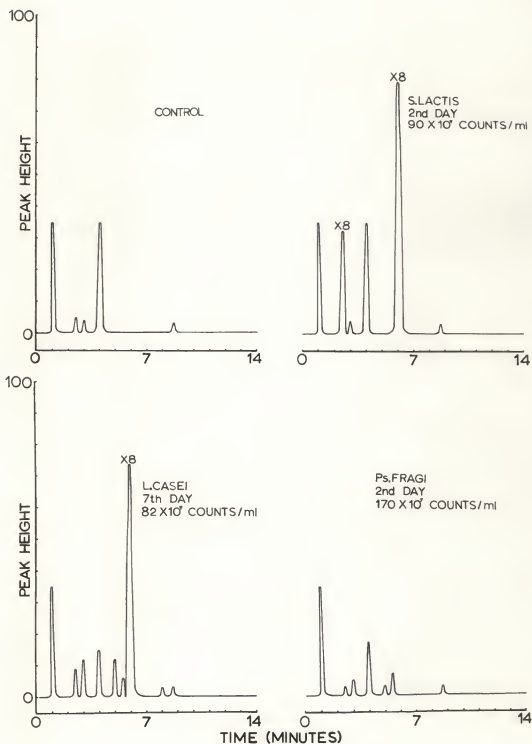Fig. 13) contained various recognizable peaks in addition to those from

Figure 13. Comparison of chromatographic patterns from control milk and
milk inoculated and incubated at 15° C with S. lactis, L.
casei, and Ps. fragi to approximately the same bacterial
populations. Numbers above the peaks are attenuation factors;
peaks with no numbers have an attenuation factor of 4.

control milk that have been named after their retention times in min. The utilization and/or the production of the volatile chemical compounds represented by the peaks developed during the course of the growth of each of the bacterial species in these inoculated milks are summarized in Table 5.

Table 5. Volatile compounds utilized and/or produced by L. casei, Ps. fragi and S. lactis in vacuum-distilled pasteurized homogenized milk.

Peaks (RT)[1]	Production	No production	Utilization
2.5	S.-lactis (1)[2] L. casei (2)		Ps. fragi
3.0	Ps. fragi (1) L. casei (2)	S. lactis	
4.0		S. lactis	Ps. fragi (1) L. casei (2)
5.0	L. casei (1) Ps. fragi (2)	S. lactis	
5.5	Ps. fragi (1) L. casei (2)	S. lactis	
6.3	S. lactis (1) L. casei (2)	Ps. fragi	
8.0	L. casei S. lactis	Ps. fragi	
8.7		L. casei Ps. fragi S. lactis	

1 RT: retention time in minutes.
2 The rate of production or utilization on the basis of chromatographic peak heights during a seven-day period.

The changes in each of the different volatile components in the control and three inoculated milks are all shown in Figs. 14-21. In these figures, to facilitate comparison and characterization of the organisms studied, the absence of any peak on chromatograms from any particular milk has been replaced by a line drawn on the axis of abscissas.

The 2.5, 3.0, 4.0 min peaks were identified by methods previously described as acetaldehyde, methyl sulfide and acetone, respectively. The 6.3 min peak agreed in retention time with ethyl alcohol.

Even though most of the peaks observed in this study were common to all chromatograms obtained from the three bacterial species studied, their peak heights differ markedly, and a general observation of the chromatographic patterns produced has served to differentiate these different organisms. Detailed analysis of the results indicated that Ps. fragi and L. casei could be distinguished from S. lactis by their utilization of acetone as evidenced by the reduction of the 4.0 min peak, especially in the case of Ps. fragi. Also S. lactis can be characterized by its production of acetaldehyde and the sharp increase of the 6.3 min peak (probably ethyl alcohol).

Approximately 24 hr after inoculation, chromatograms from Ps. fragi milk exhibited a sharp decrease in acetone, while a decrease of this compound was much more gradual in case of L. casei. Ps. fragi also appeared to utilize some of the acetaldehyde present in the milk after the steam-sterilization process. These features, in addition to the absence of the 6.3 min peak and the greater production of methyl sulfide helped to differentiate it from L. casei. The different patterns of the 5.0 and 5.5 min peaks could also help differentiate these two bacterial species.

The curves in Fig. 15 show a gradual production of methyl sulfide by Ps. fragi and L. casei. On the fourth day, the chromatographic peak heights on chromatograms from these milks reached a value of 43 and 34 respectively, i.e., approximately 150 ppb and 120 ppb of methyl sulfide. These quantities and those produced on subsequent days could therefore influence the flavor and aroma of the milks based on threshold studies previously reported.

Although the A. aerogenes study was carried out at 5° C instead of 15° C, results from a preliminary investigation on this bacterial species at 12° C showed promise that chromatograms of the milk inoculated with this culture could also be differentiated from those of L. casei, Ps. fragi and S. lactis by the presence of the two typical 9.0 and 12.0 min peaks. Furthermore, the sharp increase of the methyl sulfide in the early stage, the tremendous increase of the 6.3 min peak (approximately 10 times as high as the one of S. lactis at the end of the trial), and the productions of acetaldehyde and acetone also provided an aid for its characterization. Harvey (29) reported the production of acetone and acetaldehyde in sterilized skim milk by certain strains of S. lactis and S. cremoris. The strain of S. lactis used in this study did not produce any acetone. But even if acetone were produced by S. lactis, the differentiation between these organisms and A. aerogenes would still be possible by the general chromatographic patterns and the characteristics of the peak heights developed.

DISCUSSION

References to methyl sulfide in milk and dairy products are numerous. Some studies have implicated methyl sulfide as characteristic of normal milk

Figure 14. Comparison of the 2.5 min peak heights among _L. casei_, _Ps. fragi_, _S. lactis_ and control milk during a seven-day period.

Figure 15. Comparison of the 3.0 min peak heights among *L. casei*, *Ps. fragi*, *S. lactis*, and control milk during an eight-day period.

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Figure 16. Comparison of the 4.0 min peak heights among L. casei, Ps. fragi, S. lactis, and control milk during a seven-day period.

Figure 17. Comparison of the 5.0 min peak heights among
L. casei, Ps. fragi, S. lactis and control milk
during a seven-day period.

Figure 18. Comparison of the 5.5 min peak heights among <u>L. casei</u>, <u>Ps. fragi</u>, <u>S. lactis</u>, and control milk during a seven-day period.

x   Control
o   L.casei
△   Ps.fragi
▣   S.lactis

CHROMATOGRAPHIC PEAK HEIGHT

DAYS

Figure 19. Comparison of the 6.3 min peak heights among *L. casei*, *Ps. fragi*, *S. lactis* and control milk during a seven-day period.

Figure 20. Comparison of the 8.0 min peak heights among L. casei, Ps. fragi, S. lactis and control milk during a seven-day period.

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Figure 21. Comparison of the 8.7 min peak heights among L. casei, Ps. fragi, S. lactis and control milk during a seven-day period.

x Control
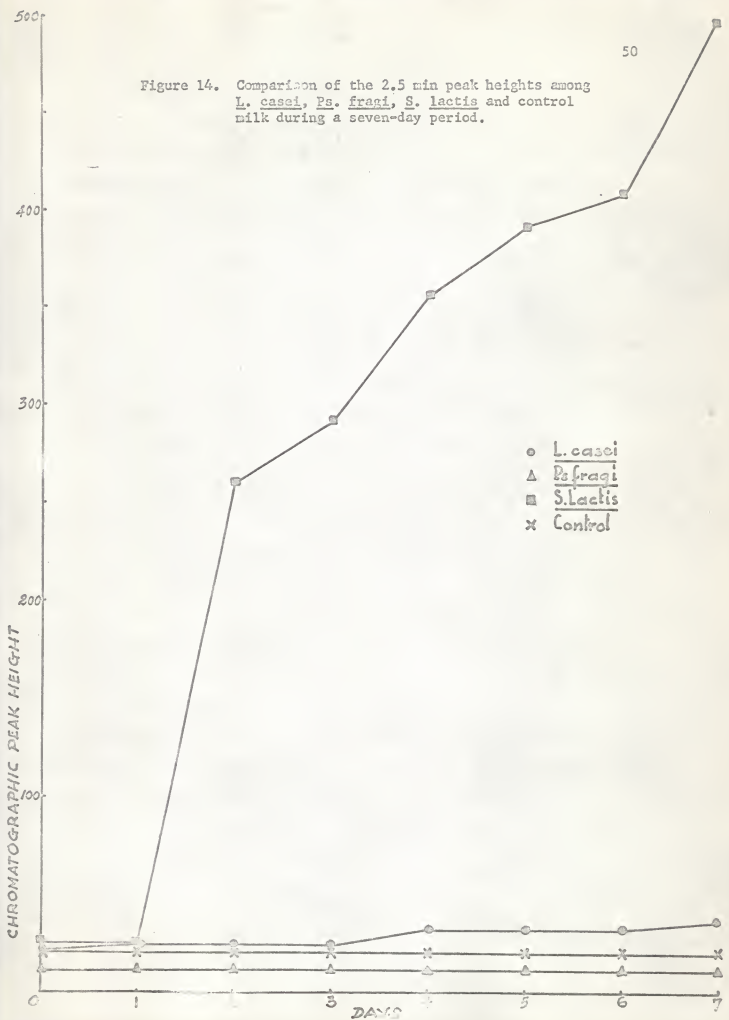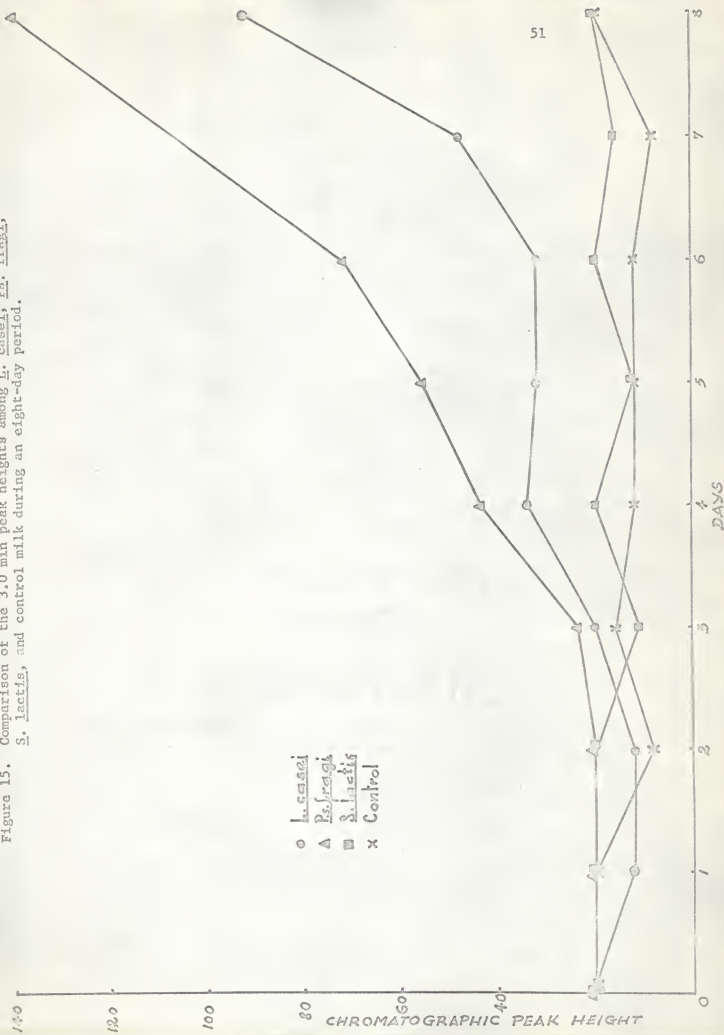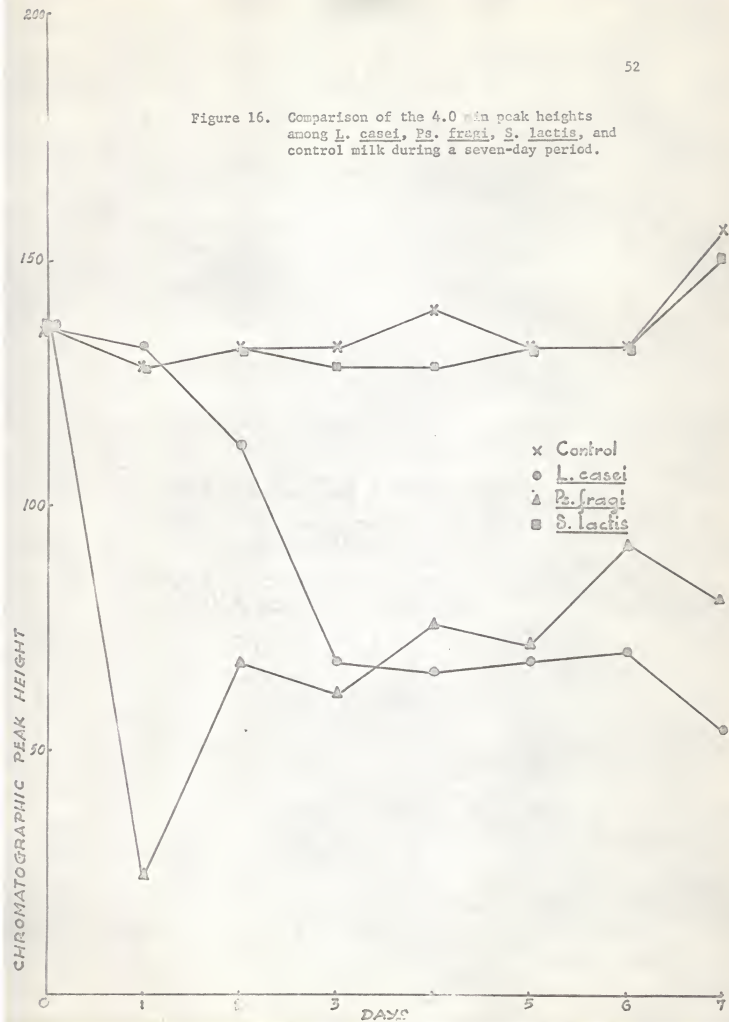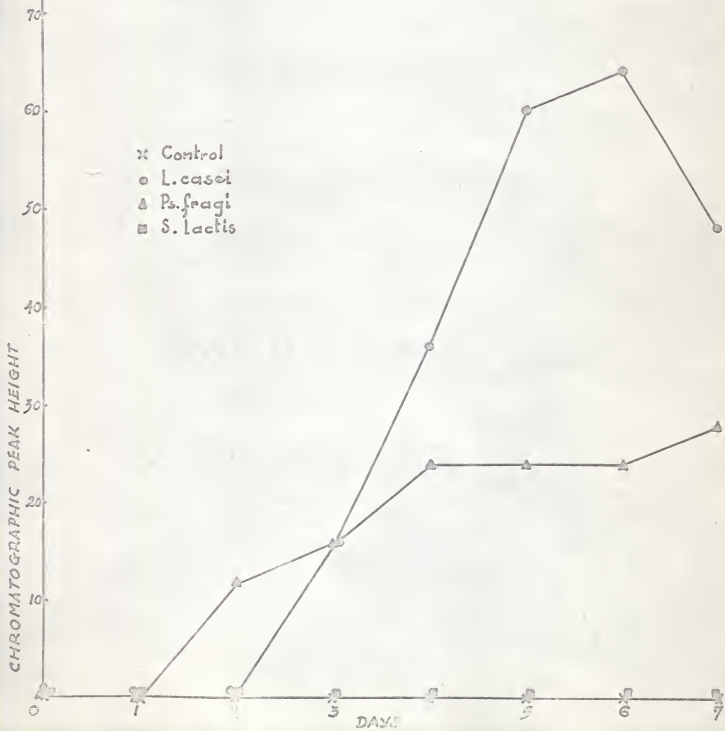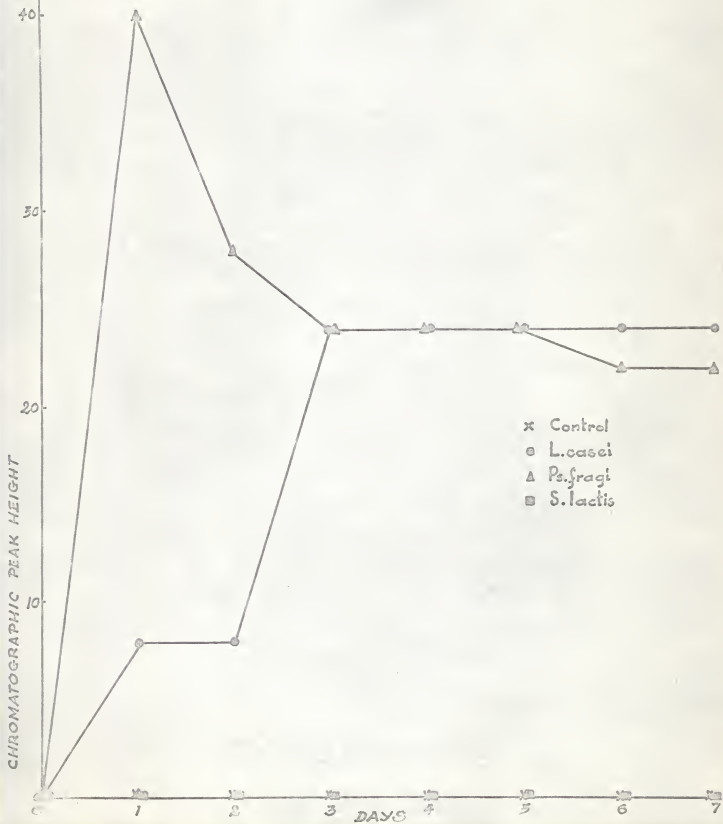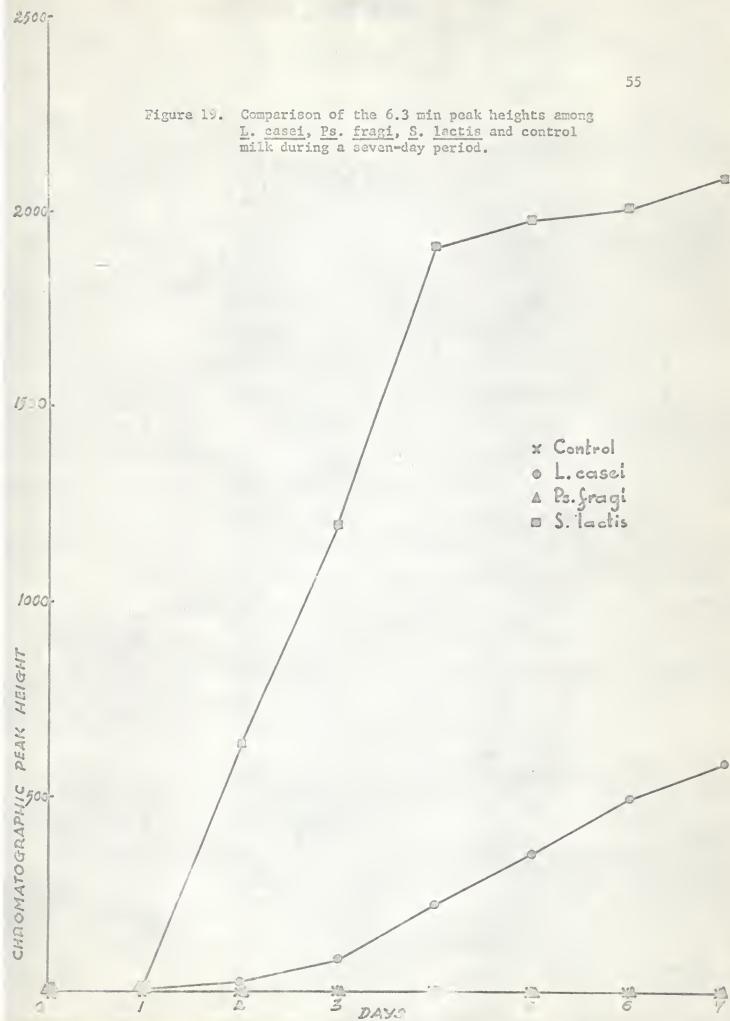⊙ L.casei
△ Ps.fragi
□ S.lactis

CHROMATOGRAPHIC PEAK HEIGHT

DAYS

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and good quality dairy products while others have suggested that it is associated with the feed flavor. Little attention has been directed to bacteria as a possible source of the compound. The findings of this study provide evidence that microorganisms often encountered in milk can be a source of methyl sulfide, and thereby the cause of some milk flavors. The quantities of methyl sulfide so produced may be high enough to influence significantly the flavor and aroma of the milk.

The normal flavor of milk that results from the blending of many compounds has been best defined in A Dictionary of Dairying by Davis (21) as cowy. Patton et al. (44) found that at low dilution in water, methyl sulfide suggested a "milk-like" flavor, while at higher concentrations it was described as malty or cowy. Claydon (17) reported an incidence of medicinal flavor caused by A. aerogenes in market milk. The sequence of the flavor changes was described by Claydon as from cowy through slightly bitter, to strong medicinal which later became intensely unclean. The observations noted in the literature together with the finding of this investigation suggested that methyl sulfide concentration in A. aerogenes milk culture greatly increased in the early stage of bacterial growth and contributed to off-flavor development.

Methyl sulfide production by A. aerogenes and the finding that Ps. fragi and L. casei studied also produced this compound suggest that its production may be rather general by microorganisms encountered in milk and dairy products. The possibility exists that the so-called normal flavor of mixed milk may be influenced by the presence of certain numbers of microorganisms which, when increased considerably, would induce some off-flavors to the milk. Methyl sulfide itself may not enhance a typical flavor and odor

encountered. Though the quantities of methyl sulfide produced by cultures
of Ps. fragi and L. casei studied were enough to produce a significant
effect on flavor and odor, milk samples inoculated with Ps. fragi exhibited a
typical fruity odor, whereas L. casei milk culture smelled predominantly sour.
Certain balance and interrelationship between methyl sulfide and other volatile
components produced may be necessary to bring about the characteristic
"methyl sulfide-like" off-flavor in A. aerogenes milk samples.

The confirmation of the production of methyl sulfide by microorganisms
raises the question of its origin. Dimethyl-B-propiethetin and methyl-
methionine sulphonium salt originating in plant materials have been
suggested as possible precursors of this compound (24). Different
organisms may arrive at methyl sulfide through unique pathways of metabolism
for bacteria. As pointed out by Patton (46), however, a barrier to progress
in the knowledge of the accumulation of methyl sulfide in milk is the
relative scarcity of references on sulfur compounds and the obscurity of
their chemistry.

The resemblance between the large 6.3 min peak observed from A. aerogenes,
L. casei and S. lactis and similar chromatographic peaks reported in the
literature appears to merit some consideration. Wong (59) found a pre-
dominant peak on chromatograms of fresh cream that greatly increased in
decomposed cream. This peak was identified by comparison with the retention
time of authentic compounds as ethanol. Mabbit and McKinnon (34) observed
a peak of similar nature on chromatograms from S. lactis milk culture, but
their conclusion was that, since only about 1% by weight of the total
fermentation products of S. lactis excluding lactic acid are available for
detection, this peak could represent neither ethanol nor any of the common

fermentation products of S. lactis including diacetyl, acetoin, 2,3-butanediol, acetone and acetaldehyde. Evidence that the 6.3 min peak in this study was in fact ethanol was provided by demonstrating it to be neutral, non carbonyl, ester or sulfide and possessing retention time identical with ethanol. The positive identification of the 6.3 min peak, however, remains to be determined. The fact that this peak was produced by three of the four bacterial species studied, and that it represented at least 95% of the detectable volatile components on the basis of chromatographic peak heights was interesting. Its change (and therefore the change of the total volatile materials) following the same pattern as the change in titratable acidity of the A. aerogenes milks emphasizes its importance. Future work on the identification of bacteria and characterization of off-flavor by direct analysis of chromatograms will involve this peak.

Future research in characterization of bacteria by this technique will require that a better growth medium be developed. This medium should be relatively free from volatile materials, sterile or able to demonstrate no growth when incubated at 15° C and not subject to physical or chemical changes during processing. The heat treated milk employed in this study did not satisfy these requirements. It was necessary to use massive inoculations of test organisms to obtain changes within four days before changes occurred in the control milk. Also, the steaming treatment necessary to obtain even this degree of sterility altered the control milk by producing more volatile substances than were obtained by the less severe heat treatment in the A. aerogenes study (compare controls in Figs. 2 and 13).

In the A. aerogenes study, the 24-hr period between the third and fourth day of each trial was critical with respect to changes that took place.

Therefore analyses of a similar nature should be carried out at shorter time intervals in future studies. Analyses performed at hourly intervals would assist in detecting any subtle changes among the various observations being made. Such procedure also would help in minimizing the strenuous effort needed in preparation of sterile growth medium.

Even though scores of judges showing unreasonable inconsistency during the threshold determination of methyl sulfide were eliminated, the threshold concentration of methyl sulfide in distilled water obtained during this study (9 ppb) differed slightly from the 12 ppb reported by Patton (45). Threshold concentration in vacuum-distilled pasteurized homogenized milk were approximately ten times as high as those in distilled water. This fact illustrates the importance of the medium in detection of volatiles by organoleptic analysis. Sensory testing is of basic importance in flavor identification research. However, with the highly subjective nature of human behavior, the reliability of the organoleptic evaluation and threshold determination as again revealed in this study should be approached with care and understanding.

The difference among threshold levels of methyl sulfide in water and pasteurized homogenized milk found in this study, and in skim milk as reported by Patton and Josephson (45) reaffirms the observation by Bassette et al. (5) on the effect of solubility characteristics on the release of different volatile chemical compounds into the vapor phase. Chromatographic peak heights of methyl sulfide in the whole milk were relatively small compared with those obtained in water (Fig. 6). This fact together with the relative insensitivity of judges to methyl sulfide in milk compared with that in water may reflect the solubility of methyl sulfide in whole milk,

particularly in the fat phase. This would be coincident with the reluctant release of this compound into vapors for organoleptic evaluation.

That chromatograms of control milk and milks of pure bacterial cultures may differ qualitatively and quantitatively have been demonstrated in this study. These results are contrary to the conclusion by Wong (59) that differences among the volatile compounds of fresh and decomposed cream regardless of type of organism involved are only of a quantitative nature. These qualitative and quantitative differences, together with the identification of the volatile compounds separated would provide a practical means for objective analysis of bacterial species.

A comparison of the chromatographic patterns of A. aerogenes, L. casei, Ps. fragi and S. lactis with similar bacterial populations shows that the extent of volatiles produced is not directly proportional to the aromatic properties of the fermentation. Ps. fragi which is well known for its fruity odor was almost void of volatile substances (chromatographic peaks). The peaks that are obtained are not proportional to numbers. All of the chromatograms from the test organisms shown in Fig. 13 are from milk with approximately the same bacterial counts.

Although the results from characterization of bacteria by gas chromatography reported in this thesis are based on only one trial, preliminary experiments, that were hampered by growth in control milk, yielded identical patterns when compensation was made for the influence of control milk. It is clear from these results that if chromatograms can be consistently obtained, one could identify any of these bacteria in the presence of the other three once isolated.

To what extent this method may be used for characterization of organisms in general must be established. Even if it is only useful to

distinguish between certain organisms, the author feels the technique merits consideration.

CONCLUSIONS

In addition to the confirmation of the significance of A. aerogenes organisms as a source of a unique off-flavor which preliminary studies in this laboratory attributed to methyl sulfide, this investigation has established the following:

1. The bacterial population of A. aerogenes at which methyl sulfide could be detected chromatographically and organoleptically at a level between 960,000 and 110×10^6 organisms per ml.

2. Methyl sulfide exhibited a threshold concentration of approximately 9 ppb in distilled water and 115 ppb in vacuum-distilled pasteurized homogenized milk.

3. The production of methyl sulfide by A. aerogenes (143 ppb to 800 ppb) was sufficient to produce a significant effect on the flavor and aroma of the milk.

4. The changes in titratable acidity and the total chromatographic peak heights of volatile components in the A. aerogenes study showed a close relationship.

5. The differences among volatile chemical compounds from milks inoculated with A. aerogenes, S. lactis, Ps. fragi and L. casei were both qualitative and quantitative in nature.

6. In this trial chromatographic patterns could be used to distinguish the organisms studied.

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GAS CHROMATOGRAPHY APPLIED TO THE STUDY
OF A FLAVOR DEFECT IN MILK CAUSED BY A. AEROGENES
AND THE CHARACTERIZATION OF SOME OTHER BACTERIA

by

TOAN TRONG TRAN

B. S., Kansas State University, 1963

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AN ABSTRACT OF A MASTER'S THESIS

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requirements for the degree

MASTER OF SCIENCE

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Manhattan, Kansas

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The purpose of this study was (a) to determine the presence and significance of methyl sulfide in a flavor defect caused by A. aerogenes in pasteurized homogenized milk, and (b) to attempt an objective characterization of some other bacterial species encountered in milk and dairy products on the basis of volatile chemical compounds produced during their growth. Special attention was directed to the use of gas chromatography to elucidate the chemical nature of the flavor defect and the characterization of bacterial species.

The procedure for the first part of this study consisted of preparation of milk samples reasonably free of bacteria and volatile materials. Pasteurized homogenized milk was distilled at 63° C, under a reduced pressure of 26 cm Hg for 90 min, then cooled to room temperature. Nitrogen gas was bubbled through the milk during the distillation procedure to help remove the volatile materials and to minimize air contamination. Sterile glassware and equipment were used throughout the study. Three separate trials were conducted each employing two quarts of milk which were subsequently inoculated with predetermined numbers of A. aerogenes to give initial counts between 3000 and 4000 cells/ml. Two quarts of milk were also prepared and maintained as control in each trial. Control and inoculated milks were then transferred into two series of sterile, screw-capped vials (40 ml capacity) and incubated at 5° C. Growth of the organisms present was followed during a seven-day period of incubation by plating both control and inoculated milks on Standard Methods Plate Count agar and the plates incubated at 21° C for four days. Changes in control and inoculated milks were also followed daily by organoleptic evaluation, direct gas chromatographic analysis, and titratable acidity. Threshold

determinations of methyl sulfide in pasteurized homogenized milk, prepared as indicated by the vacuum-heat treatment, were conducted later to establish the significance of methyl sulfide in the induced off-flavor.

Gas chromatographic analysis of the _A. aerogenes_ milk together with respective prechromatographic reactions of this milk with mercuric chloride, acidic and basic hydroxylamines, and comparisons with retention time of authentic compounds revealed the presence of methyl sulfide and five other volatile compounds including acetone and acetaldehyde. Methyl sulfide concentration in the abnormal milk ranged from 143 ppb to 800 ppb. Since the threshold concentration of methyl sulfide in vacuum-distilled pasteurized homogenized milk was found to be 115 ppb, the concentrations occurring in the _A. aerogenes_ milk were high enough to provide an abnormal flavor in the early stage of bacterial growth. This was confirmed by organoleptic evaluation. The bacterial induced off-flavor could be detected chromatographically and organoleptically at a bacterial population between 96×10^4 and 110×10^6 organisms per ml. Changes in titratable acidity and total chromatographic peak heights of the volatile compounds produced showed a close relationship.

In the second phase of the study, attempts were made to characterize _L. casei_, _Ps. fragi_ and _S. lactis_ by gas chromatographic analysis of volatile materials produced during their growth. The preparation of growth medium was as described in the _A. aerogenes_ study with the exception that the vacuum-heat treated milk was steamed for an additional hour at 88° C. Since milk samples were incubated at 15° C instead of 5° C, steaming of the milk was found to be necessary to destroy the organisms present which would otherwise grow during a seven-day period. Bacteriological and gas chromatographic

analyses were carried out at daily intervals for the purpose of character-
ization. Plate counts were incubated for four days at 21° C for Ps. fragi,
and at 35° C for L. casei and S. lactis. Identification of peaks on
chromatograms was as previously described.

The steaming procedure resulted in the appearance of four peaks on
chromatograms from control milk, three of which were identified as methyl
sulfide, acetone, and acetaldehyde. Excluding those contributed by the
control milk samples, chromatograms from L. casei showed six chromatographic
peaks whereas those from Ps. fragi and S. lactis yielded only three. The
three peaks from S. lactis milk differed in retention time from those of
Ps. fragi milk. L. casei and Ps. fragi were found to produce methyl sulfide
at concentrations above threshold levels. These bacterial species appeared
to use the acetone present in the control milk. Chromatographic patterns
also showed the utilization of small amounts of acetaldehyde from the
control milk by Ps. fragi. The differences among chromatograms from L. casei,
Ps. fragi, S. lactis milks in the one trial reported herein and in preliminary
trials were of both qualitative and quantitative natures. The indications
from this research are that, once isolated and grown in a suitable medium
under controlled conditions, L. casei, Ps. fragi, S. lactis and A. aerogenes
could be differentiated by their chromatographic patterns.