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Non-sporulating obligately anaerobic bacteria in dairy products

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NON-SPORULATING OBLIGATELY ANAEROBIC BACTERIA IN DAIRY PRODUCTS

by

Glenn Alan Claybaugh

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INTRODUCTION

The non-sporulating anaerobic bacteria found in dairy products have received very little attention, since only aerobic technics usually are used for examination. It commonly is believed that these organisms will produce changes very slowly, if they can initiate growth at all. Therefore the work on anaerobic bacteria in milk and dairy products has been concerned primarily with the sporulating types, chiefly with those types which may survive pasteurization conditions and which may cause abnormal fermentation in products such as milk, cheese and condensed products.

This study has been undertaken in order to determine what types of non-sporulating anaerobic bacteria may be found in dairy products. Since new methods have been developed for isolation of anaerobic bacteria resulting in new concepts of microenvironment, it was presumed that they could be used successfully on the non-sporulating anaerobes of dairy products.

STATEMENT OF PROBLEM

The purpose of this study was to determine the presence and possible importance of non-sporulating anaerobic bacteria in dairy products. For that purpose, the study was divided into the following phases:

Establishment of an isolation procedure for the non-sporulating anaerobes from dairy products.

Determination of their biochemical and other relationships to known species.

Investigation of the effect of these organisms on various dairy products.

REVIEW OF LITERATURE

Non-sporulating anaerobic bacteria have not been reported in the literature as being associated with dairy products. A survey of the literature revealed extensive but varied research concerned with the isolation and study of non-sporulating anaerobic bacteria from the normal human intestinal and respiratory tracts, and from lesions of the human and animal body (Halle, 1898; Veillon and Zuber, 1898; Guillemot, 1899; Cottet, 1899; Tunnicliff, 1913; Olitsky and Gates, 1921, 1922, 1923; Olitsky and McCartney, 1923; Teisser et al., 1929; Noble and Brainard, 1932; Dack et al., 1935; Henthorne et al., 1936; Weinberg et al., 1937; Weiss and Rettger, 1938; McCoy and McClung, 1939; Lewis and Rettger, 1940; and Dack, 1940). Hall (1929) discussed 207 references in an excellent review of the early literature on the cultivation of obligate anaerobes and the physical and chemical principles involved in production and maintenance of anaerobiosis. Reports of the early research were difficult to evaluate because of differences in methods, inconsistencies in nomenclature and inherent differences in the organisms studied.

Apparently Castellani and Chalmers (1919) were the

first to group non-sporulating anaerobic rods into a new genus, Bacteroides. This genus was included in the first editions of Bergey's Manual (1923, 1925, 1930 and 1934). Later a more elaborate classification of the non-sporulating anaerobes was given by Prévot (1938); this classification has not been used extensively by other workers.

Eggerth and Gagnon (1933) were the first to review the literature concerning obligate anaerobes from the intestinal tract of man. A large majority of their isolates from human feces were obligate anaerobes. These findings were confirmed later by Weiss and Rettger (1937). Eggerth and Gagnon found 91 per cent of the 60 adult stools studied contained non-sporulating obligate anaerobes. They divided the isolated anaerobes into 18 species, two of which had been described previously by Distaso (1912). Eggerth, in a later study (1935), dealt only with the gram-positive strains of the non-sporulating anaerobic bacilli from human feces. He grouped the 130 strains into 11 new species, chiefly on the basis of differences in carbohydrate fermentation. In the earlier study, Eggerth and Gagnon (1933) placed both gram-positive and gram-negative strains under the generic term Bacteroides only as a temporary designation. However, in the second article, Eggerth suggested placing the gram-positive strains in the genus Lactobacillus or in a new genus in the order Actinomycetales.

Agreeing with Eggerth's work, Weiss and Rettger (1937, 1938) placed their gram-negative strains in the genus Bacteroides; they emphasized the relationship of the gram-positive strains to the genus Lactobacillus. They isolated 87 strains from human feces and found the numbers of non-sporulating anaerobes to be similar to those found by Eggerth and obtained 22 of Eggerth's isolates. They divided the 36 gram-positive and 73 gram-negative strains into four groups, based primarily on agglutination and secondarily on morphology. In 1938, they reported that half of their gram-positive cultures were similar to Eggerth's Bacteroides bifidus. After further testing, they showed that this species should be placed in the genus Lactobacillus, and suggested the name L. bifidus type II. The differences between types I and II of L. bifidus were based on fermentation of mannose, xylose, melizitose and arabinose, oxygen requirement, ability to branch, serological relationships, and amounts and types of acids formed from glucose.

In the fifth edition of Bergey's Manual (Breed et al., 1939) the gram-positive strains were excluded from the genus Bacteroides and some strains were regarded as being closely related to L. bifidus.

Lewis and Rettger (1940) studied the occurrence and taxonomic relationships of non-sporulating anaerobes from the intestinal tracts of humans and rats. From 550 colonies

picked from human feces and 841 from rat feces, 40 per cent and 21 per cent, respectively, were non-sporulating anaerobes. In their studies, they selected 76 strains from their own isolates and 19 strains from other laboratories. These cultures were divided into two main groups. The first of these was gram-positive consisting of three different types, A-1 (13 strains), A-2 (nine strains) and A-3 (four strains). The second group was gram-negative and was composed of two types, B-1 (four strains) and B-2 (46 strains). Types A-1 and A-2 were regarded as anaerobic members of the genus Lactobacillus. Type A-3 closely resembled Eggerth's (1935) Bacteroides bifidus. Type B-1 resembled Bacteroides funduliformis described by Henthorne et al. (1936) and type B-2 was designated as Bacteroides vulgatus, previously described by Eggerth and Gagnon (1933).

King and Rettger (1942) were interested in the intra- and extra-group relationships of the gram-positive, non-sporulating anaerobes of the intestinal tract. They concluded that all of the gram-positive cultures were so intimately related to each other, that they should be regarded as one species, L. bifidus type II. Agglutination, agglutinin absorption, precipitation and complement fixation tests were conducted. The nature of the respiratory mechanism of the anaerobes and certain lactobacilli was investigated. A very definite cyanide-sensitive system was

found in both the anaerobic organisms and in L. bifidus type I. Weak solutions of potassium cyanide had no effect on these strains; however, the higher concentrations caused inhibition.

Barker and Haas (1944) studied the fermentation by the gram-positive strains of Lewis and Rettger (1940) and found that eight strains of the A-1 and A-3 types produced little or no volatile acids. Five strains of the A-2 type produced large amounts of volatile acid, primarily butyric, and utilized lactates. The authors proposed the new genus Butyribacterium for this organism type. This genus has been accepted by the sixth edition of Bergey's Manual (Breed et al., 1948) and placed in the family Lactobacteriaceae.

A year later, Pederson (1945) obtained ten of Eggerth's (1935) gram-positive strains and studied the fermentation end products. As a result of these studies he combined Eggerth's 11 groups into four. One of these strains was related to the genus Lactobacillus; two strains formed butyric acid and were classified as Butyribacterium; two strains were related to Bacterium bifidus of Orla-Jensen et al. (1936); and the remaining gram-positive forms were intermediates between the non-proteolytic Lactobacillus types and the proteolytic types found in pathological conditions.

Sijpesteijn (1948) and Hungate (1950) have written

excellent reviews of the literature on the anaerobic rumen organisms. In these articles many of the techniques of isolation and propagation of anaerobic rumen organisms are discussed. A recent review of the bacteriology of the bovine rumen has been published by Doetsch and Robinson (1953). They reviewed 133 articles published since 1946 and divided the material into sections on bacterial studies of the bovine rumen, functions of rumen bacteria and factors which modify flora in the rumen.

Weiss and Spaulding (1937) and Brewer (1942) reviewed the literature on techniques of obtaining anaerobiosis. Weiss and Spaulding suggested a simplified procedure which used a Cenco Hy-vac pump to remove much of the air from the anaerobic chamber, replacing it with carbon dioxide and hydrogen. Palladinized asbestos served as the catalyst for removing residual oxygen by combination with hydrogen. Miller et al. (1938), after working with three procedures, found that the method of Weiss and Spaulding was the most suitable. Spaulding (1939) and Spaulding and Goode (1939) later improved this procedure.

Valley and Rettger (1927) were the first to show that obligate anaerobes require carbon dioxide for development and continued existence. Later Bedell and Lewis (1938) confirmed these findings. They found that complete exclusion of carbon dioxide inhibited the growth of obligate anaerobes.

Eggerth and Gagnon (1933) noted that some of their strains of Bacteroides were favored by addition of carbon dioxide but presented no data to support this observation. Lewis et al. (1940) found that non-sporulating anaerobes of intestinal origin were favored by 10 per cent carbon dioxide.

A variety of media have been used for culturing non-sporulating anaerobes. Only those media most widely used will be considered here. Eggerth and Gagnon (1933), Weiss and Rettger (1934, 1937) and Lewis and Rettger (1940) used a beef-infusion medium, while Eggerth (1935) and Bedell and Lewis (1938) used a liver-infusion medium. The glucose-cysteine medium developed and used by Lewis et al. (1940) was used by King and Rettger (1942) and Barker and Haas (1944). Many of those studying rumen microorganisms have used a medium quite similar to the one described by Hungate (1950) and Sijpesteijn (1948).

EXPERIMENTAL METHODS

Source of Samples

The raw milk samples used throughout the course of the study were secured from the Market Milk Laboratory of the Department of Dairy Industry, Iowa State College. Twelve patron samples were taken from the milk cans of individual patrons; eight vat samples were taken from the raw milk storage tanks. The volume of the milk samples was 1 or 2 liters.

All of the raw cream samples used in this study were secured from the Slater Cooperative Creamery, Slater, Iowa. These samples likewise were obtained from the cream cans of individual patrons or from pasteurization vats before heating. The samples were taken by means of a stainless steel dipper, placed in sterile 2 oz. screw-cap jars and transported back to the laboratory.

Ten pasteurized-milk and raw-milk Cheddar cheese samples in 1 lb. blocks were obtained from the Kraft Foods Company, Chicago, Ill. Two samples of pasteurized milk cheddar and one sample of raw milk Iowa blue were obtained from the Cheese Laboratory of the Department of Dairy Industry, Iowa State College.

The rumen-liquid samples were taken from a rumen-fistulated 3 year old cow, No. 3052, of the Department of Dairy Husbandry herd, Iowa State College. The samples were taken by means of a 18 ml. plastic-acid-dipper, and placed in a sterile 250 ml. erlenmeyer flask which had been flooded with carbon dioxide prior to taking the sample. The sample was taken so that as little solid material as possible was placed in the flask. As soon as the flask was filled, it was stoppered.

Dilutions

Approximately 30 glass beads 6 mm. in diameter were placed in a 125 ml. dilution bottle, which was then partially filled with distilled water and sterilized for 17 minutes at 15 lb. pressure. The 1.25 ml. sample was added to the dilution bottle and it was filled aseptically with sterile distilled water. The diluted sample was mixed by the glass beads when the bottle was shaken vigorously. Wilkowske (1948) showed that additions of sodium thiosulfate, glucose or thioglycollic acid to distilled water was not advantageous and sterile skim milk was not suitable as a diluent. He used sterile distilled water as he considered it the most satisfactory diluent.

Anaerobic Conditions

To secure anaerobic conditions for the plates and/or test tubes, the method of Weiss and Spaulding (1937) with later improvements of Spaulding (1939) was used. This method consists of placing either test tubes or plates with porcelain tops in a vacuum desiccator, evacuating to 720 mm. mercury as determined by manometric methods, refilling with carbon dioxide, evacuating 90 per cent of the added carbon dioxide and slowly filling with molecular hydrogen until atmospheric pressure is restored. Ten per cent carbon dioxide was left in the desiccator, as the work of Lewis et al. (1940) has shown this amount is necessary for the growth of non-sporulating anaerobic bacteria. Approximately one-half gram of palladinized asbestos was placed in the jar for the catalyst. This catalyst was heated over a bunsen burner in a porcelain cup for a few minutes between runs to restore its activity. An alkaline glucose indicator solution, as suggested by Ulrich and Larsen (1948), was placed in the desiccator in test tubes to test for anaerobiosis. After the incubation period was completed, the hydrogen was evacuated and the desiccator refilled with carbon dioxide to prevent accidents.

Media

Three media were used in the beginning phases of the experiments, to determine which might be best for isolation of non-sporulating anaerobes. The first of these was Wilkowske's (1948) modification of the medium used by King and Rettger (1942). It contained 20 g. tryptone, 10 g. beef extract, 5 g. glucose, 5 g. yeast extract, 4 g. monobasic sodium phosphate, 0.5 g. cysteine hydrochloride, 500 ml. water and 500 ml. V-8 Juice[1]. Prior to sterilization the medium was adjusted to pH 6.8-7.0.

The second medium used was a modification of B.B.L. Eugonagar (Baltimore Biological Laboratory, 1951). It was modified by replacing l-cystine with cysteine hydrochloride. The third medium used was a modification of one used by Hungate (1950) and consisted of 15 g. trypticase, 5 g. phytone, 5 g. glucose, 0.259 g. dibasic potassium phosphate, 0.25 g. monobasic potassium phosphate, 0.1 g. sodium chloride, 0.05 g. ammonium sulfate, 0.05 g. cysteine hydrochloride, 0.005 g. calcium chloride, 0.005 g. magnesium sulfate and 1000 ml. water. This medium will be known as Trypticase-soy-glucose-plain (T.S.G.P.) throughout the experiments. Prior to sterilization the medium was adjusted to pH 6.8-7.0.

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[1] V-8 Juice, a product of Campbell Soup Co., Camden, N. J.

In later work, this medium was modified by adding 10 per cent V-8 Juice to replace an equal amount of water and 0.05 per cent Tween 80[1]. This medium was used throughout the majority of the work and will be known as Trypticase-soy-glucose (T.S.G.). In most instances this medium required no pH adjustment as the reaction was held at pH 6.3-6.5 by the buffer. In some cases the T.S.G.P. and Eugonagar were modified by replacing the glucose with 10 g. of a 50 per cent solution of sodium lactate. In all cases a solid medium was made up with the addition of 1.8 per cent agar and a semi-solid medium with a 0.13 per cent agar. Some lots of agar varied and the semi-solid medium did not give the desired results. In those instances, the agar content of the medium was increased 0.01-0.02 per cent. In all cases, the media were sterilized for 17 minutes at 15 lb. pressure. The resulting pH of the T.S.G. medium was found to be between 6.3 and 6.5, while the Eh varied somewhat but usually was found to be about -102 mv. Solid or semi-solid media were melted in a steam chest and allowed to cool to 40-45° C. before using.

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[1]Tween 80, a polyoxyethylene derivative of sorbitan monooleate.

Determination of pH and Eh

A Beckman glass electrode potentiometer was used throughout the study for the measurement of pH in growth media and in milk. A platinum electrode was used for the measurement of Eh in the growth media.

Colony Isolation from Shake Tubes

To facilitate the picking of colonies from deep-agar shake tubes, 18 x 120 mm. soft glass test tubes were used. When colonies had formed, the bottoms of these tubes were cracked by using a Castaloy clamp holder, regular model (Fisher catalogue No. 5-756). The tip of a thumb screw was filed down to a dull point and after the tube was placed in the clamp, pressure was applied to the bottom of the test tube until the bottom of the tube cracked off in a smooth straight edge. In later trials, old pyrex test tubes of the same size were found to be suitable. After the bottom of the tube was broken, the bottom of the solid plug was cut off with a sterile wire and colonies picked as desired. In some instances where colonies appeared toward the top of the plug, the plug was pushed down and cut off again where desired.

Methods Used for Characterization

Colony characteristics

Colony characteristics were observed on T.S.G. agar plates and tubes after incubation at 35-37° C. under anaerobic conditions for 2 and 7 days.

Growth characteristics in semi-solid T.S.G. medium

To denote the type of growth in semi-solid T.S.G. medium, cultures were inoculated so they would form from 10 to 20 colonies after incubation at 35-37° C. After 7 to 10 days, the appearance of the colonies was observed. Colony growth appeared as "clusters" or as "strings" (Figure 3).

Gram stain

The gram stain was made on young and old cells of each culture following the recommended procedures of the Manual of Methods for Pure Culture Study of Bacteria (Committee on Bacteriological Technic, 1951).

Electron micrographs

Electron micrographs were taken from preparations made of 2 day-old cultures in semi-solid medium. The culture tubes were centrifuged and after the supernatant was poured off, the cells were washed several times in sterile distilled

water, re-centrifuged and resuspended. Mounts were made on collodion membranes prepared on the surface of water and stretched over a 200-mesh wire screen. Evaporation was controlled so that shadow casts 1:4 with gold were made on some preparations. A R.C.A. type E.M.U. electron microscope was used, and a magnification of approximately 4,200x was employed.

Photomicrographs

Preparations for the optical microscope photomicrographs were made of 2 day-old cultures grown in T.S.G. broth under anaerobic conditions, which were stained for one minute with Hucker's crystal violet. A magnification of 1,700x was employed.

Motility

Motility of the cultures was tested by use of semi-solid T.S.G. medium following the procedure of Tittsler and Sandholzer (1936).

Carbohydrate fermentation

For the determination of carbohydrate fermentation, a modification of the basal medium of Tittsler (1947) was used. The modification was made by the addition of 0.5 g. of cysteine hydrochloride and 1.3 g. of agar per liter of broth.

The medium was sterilized without the sugars for 17 minutes at 15 lb. pressure. The sugars used were arabinose, cellobiose, galactose, glucose, inulin, lactose, levulose, maltose, mannose, raffinose, sucrose and xylose. Mannitol and sorbitol were the alcohols used, while salicin was the only glucoside. These were sterilized in 10 per cent solutions by filtration. They were added aseptically by pipette to the basal medium and the completed medium was incubated prior to inoculation to assure sterility. Each culture was tested in the basal medium to make certain there were no slight changes which would be interpreted incorrectly.

Catalase

Presence of catalase was determined by placing a few drops of an actively growing culture in a depression of a spot plate and adding a few drops of a 30 per cent hydrogen peroxide solution. A positive reaction was recorded when evolution of gas was seen from the plate.

Hemolysis

To test for hemolysis a special medium recommended by Packer (1952) was used. This medium consist of 15 g. tryptose, 5 g. yeast extract, 3 g. beef extract, 5 g. sodium chloride, 0.5 g. cysteine hydrochloride, 18 g. agar and 1000 ml. water. The pH of the medium was not adjusted and was

used as prepared. The medium was sterilized for 17 minutes at 15 lb. pressure. To this medium, 5 per cent citrated bovine, sheep, rabbit or human blood was added to the medium after it had been melted and cooled to 45° C. and the bottom layer of 10-20 ml. was poured immediately and allowed to harden. Then the cultures were streaked-out and the top layer of 10 ml. was poured. Plates were incubated under anaerobic conditions for 7 days at 35-37° C.

Proteolysis and lipolysis

Proteolytic action was determined by adding 5 per cent of sterile skim milk to the T.S.G. medium. The plates were incubated under anaerobic conditions for a period of time which gave good colony growth, usually a period of 7 days. Proteolysis was observed by the clear zone around separate colonies on the opaque medium. Further proteolytic tests were made by determining the soluble nitrogen in 5 g. of milk after incubation for 7 days. The method of Collins and Nelson (1949) was followed in the preparation and digestion of the samples.

Lipolysis was determined by plating the cultures with T.S.G. medium to which 5 ml. of a sterile 0.2 per cent Nile blue sulfate solution had been added to 100 ml. of medium. To each plate, 0.5 ml. of coconut oil emulsion was added just prior to the pouring of the plate (Jensen and Grettie, 1937).

Incubation occurred under anaerobic conditions at 35-37° C. for a period of 7 days. After incubation the plates were examined using a low-power microscope. Lipolytic colonies showed nearby fat globules of an intense blue color, as contrasted to the normal light pink color of unhydrolysed fat in the other areas of the plate.

Nitrate reduction

In testing for presence of nitrite, the sulfanilic acid-dimethyl alpha-naphthylamine test was used. The cultures were grown in the semi-solid T.S.G. medium to which had been added 0.1 per cent potassium nitrate. After the incubation period of 7 and 14 days at 35-37° C., small amounts of the culture were placed in a spot plate for testing. Negative nitrite tests were followed by a test for presence of nitrate by use of zinc dust. Positive nitrate reduction tests were recorded when a brick-red color appeared in the spot plate.

Indole and skatole formation

The vanillin test method of Roessler and McClung (1943) and Kovac's reagent (Committee of Bacteriological Technic, 1951) were used to test for the production of indole. The medium used was the semi-solid T.S.G. medium minus the glucose, phytone, V-8 Juice and Tween 80. The cultures grew very slowly; growth appeared only after incubation for 7-10

days. Cultures were tested after 7 and 14 days incubation at 35-37° C. by placing two drops of the culture in a spot plate followed by two drops of a 5 per cent alcoholic solution of vanillin, followed by three drops of concentrated hydrochloric acid. A brick-red color reaction indicated a positive test. After the reaction had been recorded, 0.1 per cent sodium nitrite was added for the skatole test. A positive test for skatole was a deep purple color reaction. The second test for indole was conducted by adding two drops of Kovac's reagent to two drops of culture in a spot plate. A brick-red color reaction indicated a positive test.

Ammonia formation

Nessler's reagent was used to test for the presence of ammonia. After cultures were grown in semi-solid T.S.G. medium for 7 days at 35-37° C., two drops of Nessler's reagent were added to two drops of each culture in a spot plate. A positive test was indicated when a deep rust-red precipitate was formed.

Hydrogen sulfide formation

The production of hydrogen sulfide was tested by placing cultures in the semi-solid T.S.G. medium to which had been added 0.05 per cent ferric ammonium citrate. Positive tests were indicated when the entire medium turned black. Tubes

were incubated for 14 days at 35-37° C.

Growth temperature range

The growth temperature range was determined by incubation of five identical semi-solid T.S.G. medium cultures at 10, 21, 30, 37 and 45° C. A few selected cultures also were incubated at 5° C. Those cultures which were incubated at 21° C. or below were cooled in ice water after inoculation with one drop of an active culture. All cultures were incubated for 30 days unless growth appeared sooner.

Thermal resistance

To test the thermal resistance of the cultures, one drop of an actively growing culture was added to 9 ml. of semi-solid T.S.G. medium in 18 x 120 mm. screw cap test tubes. After inoculation the medium was mixed and placed in the agitated water bath so that the water completely covered the tubes when they were immersed. The temperatures used were 60±0.01° C. and 80±0.01° C. The periods of holding were 10, 20 and 30 minutes at 60° C. and 10 minutes at 80° C. A thermometer was placed in a control tube fitted with a rubber stopper to determine the come-up time of the tubes. When the desired temperature was reached, timing began and as soon as the time interval had elapsed the tubes were cooled in ice water. The tubes then were incubated at 35-37° C. for 14

days and observed for growth. If growth was observed in the tube, it was considered that the culture survived that temperature and time tested. The relative number of organisms surviving also was noted approximately.

Growth in milk

In order to observe the pH changes caused in milk by the anaerobes, skim milk was treated as follows: One per cent non-fat dry milk solids, previously tested for absence of inhibitory substances were dispersed in some of the skim milk with the aid of a Waring Blendor. After dispersing the dry milk solids, the remainder of the milk was added along with 0.05 per cent cysteine hydrochloride. The milk was sterilized in 100 ml. quantities in 6 oz. ovals for 17 minutes at 15 lb. pressure. The milk then was inoculated as soon as it had cooled to 40° C. The pH determinations were made on the milk after 7 and 14 days of incubation at 35-37° C.

Litmus milk was made up from the above mixture, by adding a sufficient aqueous solution of litmus for a lavender color. The litmus milk was dispensed in the large-size test tubes, and sterilized for 17 minutes at 15 lb. pressure.

For determination of the total developed acid, volatile acid and lactic acid produced in milk, skim milk with 1 per cent non-fat dry milk solids added was used. The milk was sterilized for 17 minutes at 15 lb. pressure, and inoculated

with 1 per cent of an active culture.

Volatile acids

To determine the amounts and types of volatile acid produced by the anaerobes, the method of Ramsey and Patterson (1945) was used. The cultures were grown in 6 oz. ovals containing 100 ml. of semi-solid T.S.G. medium minus the phytone, V-8 Juice and Tween 80; however, the glucose was increased to 1 per cent. The incubation periods were determined by the amount of growth showing in the culture bottles. This period normally was 7-10 days at 35-37° C. The above medium without the glucose was included as the control. One modification was made in the procedure. After the 200 ml. of distillate had been collected and titrated, it was evaporated to dryness on a hot plate regulated so the temperature of the liquid never exceeded 95° C. This was used instead of evaporating to dryness on a steam bath. The distillation apparatus was standardized according to procedure outlinedby the Association of Official Agriculture Chemists (1950).

Lactic acid determinations and utilization of lactate

All lactic acid determinations were made following the procedure of Barker and Summerson (1941). The cultures were grown 7 days at 35-37° C. in semi-solid T.S.G. medium. A

standard lactic acid curve was prepared by using lithium lactate prepared according to the procedure of Hawk et al. (1951).

To determine whether the cultures utilized lactates, they were inoculated into a medium consisting of 2 per cent Difco peptone, 1 per cent sodium lactate, 1 per cent yeast extract, and 0.5 per cent cysteine hydrochloride. After incubation at 35-37° C. growth was observed after 7 and 14 days. The cultures also were inoculated into the semi-solid T.S.G. medium in which sodium lactate replaced the glucose. After suitable incubation, lactic acid determinations were made to determine the loss of lactate.

Optical activity of lactic acid

To determine the optical activity of the lactic acid produced by some strains of anaerobes, a modification of the procedure described by Hyde and Hammer (1927) was used. The cultures were grown in sterile skim milk at 35-37° C. for 7 days. The coagulated milk was centrifuged in a high-speed centrifuge and the whey collected. The whey proteins were precipitated by a saturated solution of ammonium sulfate and separated by centrifugation. The whey supernatant was decanted and the pH adjusted to 2.0. The lactic acid was extracted by several washings with ether, and treated by the procedure described by Hyde and Hammer (1927).

Pathogenicity

Pathogenicity tests were conducted on mice by injecting subcutaneously 0.5 ml. of centrifuged cells re-suspended in sterile saline solution. The mice were observed for a period of 7 days.

Flavor Changes Produced in Milk

The anaerobes were inoculated into plain pasteurized homogenized milk to observe the possible changes in flavor. This milk, obtained from the Market Milk Laboratory, Iowa State College, was steamed for 45 minutes on three consecutive days, with incubation at room temperature between steamings. When the milk had cooled to 40° C. after the last steaming, it was dispensed in 100 ml. and 1000 ml. quantities, and inoculated with the cultures. As soon as the milk had coagulated, or after 14 days, the milk was judged for flavor by an experienced judge.

Method of Cheddar Cheese Manufacture

The method described by Van Slyke and Price (1949) was used for the manufacture of pasteurized milk Cheddar cheese. The cheese was made in small 50 gallon experimental vats, using 100 lb. of pasteurized milk. The S. lactis starter, FL,

was added at the rate of 2.5 per cent, and the anaerobic cultures were added at the rate of 0.05 per cent. Anaerobic cultures 18, 42, 62, 63, 64, and 103 were used. A total of eight vats of cheese were made on two days, six experimental vats and two control. Two five-pound cheese were made from each vat; one was cured at 10° C., while the other was cured at 10° C. for 2 weeks, and then placed at 15.5° C. for 2 weeks, and then placed back at 10° C. for the remainder of the curing period.

EXPERIMENTAL RESULTS

Establishment of an Isolation Procedure

Milk and rumen liquid were used as source material in the early phase of this study, and isolations were accomplished by plating and by using deep-agar shake tubes.

Three media, Wilkowske's, Eugonagar and Eugonagar with sodium lactate replacing the glucose, were used. The milk used as a source material was held at room temperature (24-28° C.) for enrichment until coagulation took place. In these studies, the three media were compared to determine the one best suited for further investigations. Counts were determined and a record kept on the number of anaerobic cultures isolated from each of the three media. It soon became apparent that Wilkowske's medium became dark upon prolonged incubation; therefore, it was quite difficult to pick colonies from either shake tubes or plates. However, it did support the growth of the anaerobes studied. The glucose in the Eugonagar medium was replaced by sodium lactate in some trials to determine if some anaerobes were not being isolated because glucose was the only carbohydrate used. Although some cultures were isolated by use of this latter medium, more were isolated when the glucose was present. It was

found later that one group of anaerobes isolated did not use lactate and would not grow in the lactate medium. In a few instances, cultures were lost when glucose was used as the sole source of carbohydrate.

In a few early trials, a melted vaspar mixture consisting of 50 per cent vaseline and 50 per cent paraffin, was used to cover the semi-solid and solid shake tubes. A 3 per cent agar also was tested. When the sealing preparations had hardened, sterile mineral oil was added to the surface to prevent drying. These sealing preparations were difficult to handle as a routine procedure and were found to be unnecessary in later work.

In all cases colonies were picked into tubes of the melted and cooled medium from which deep-agar shake cultures were prepared. Those cultures that showed localized growth in the bottom of the tube with no growth on the surface or in the top 8-10 mm. of the medium were selected as anaerobic bacteria. It also was noticed that the anaerobic cultures grew very slowly and that the facultative and aerobic cultures grew faster, which aided in the selection of the anaerobes. Very few aerobes were observed throughout the study, while a large majority of the cultures picked were either facultative types or failed to initiate growth. In picking colonies, usually a very small colony was selected; however, in later studies it was found that under anaerobic

plating a large winged or oval colony usually proved to be anaerobic. The samples were diluted into shake cultures. After incubation, the region from which a colony was picked was observed, but there was no direct evidence that anaerobes grew solely on or near the bottom of the tubes. Some anaerobes were isolated near the middle and 8-10 mm. from the surface of the medium. Observations made during the study showed that, with pure cultures, the first signs of anaerobic growth usually occurred in the region 8-10 mm. from the surface. This phenomenon was observed in both solid and semi-solid medium. Evidently the optimum conditions for growth were found in this region.

Effect of pH on the Growth of the Anaerobes

A medium pH of 7.0-7.4 was used in early trials, because many of those working with rumen and intestinal anaerobes had used this pH. Smith (1941) showed that the *in vivo* pH readings of rumen contents were 6.0-6.2, whereas *in vitro* pH readings of 7.0-7.4 were reported by other workers. Sijpesteijn (1948) found by using a medium with a lower pH (6.5-6.8) anaerobic cultures from the rumen grew without difficulty. It was believed that the cultures isolated in this study should be tested to determine the pH best suited for growth in the T.S.G. medium. Ten representative

anaerobic cultures were inoculated into the semi-solid T.S.G. medium, and incubated for 48 hours at 35-37° C. The sterilized medium was adjusted prior to inoculation to pH levels from 7.6 to 4.6 in 0.2 intervals, using sterile N/1 sodium hydroxide or N/1 hydrochloric acid. At the end of the incubation period, the relative rapidity and abundance of multiplication was observed. All cultures grew quite well in the range of pH 5.4-7.0; however, most rapid multiplication occurred in the pH range of 6.4-6.8. This trial was repeated and the above results were substantiated. It then was decided to adjust the phosphate buffer so that the pH of the sterilized T.S.G. medium was 6.3-6.5. This pH was used for this medium throughout the remainder of the study.

Effect of Temperature

The first anaerobes isolated in this study were incubated at a temperature of 35-37° C., the temperature used by those studying rumen and intestinal anaerobes. To make certain that the long incubation periods encountered were not due to a non-optimum incubation temperature, five growth temperatures were selected for study with ten representative anaerobic cultures. The temperatures selected were 10, 21, 30, 35-37 and 45° C. The cultures incubated at 30 and 35-37° C. grew well in 48-72 hours, but cultures incubated at the

other test temperatures grew very poorly. The work of Lewis et al. (1940) showed that the optimum incubation temperature for the non-sporulating anaerobes from the intestinal tract was 35-40° C. Therefore an incubation temperature of 35-37° C. was used for the remainder of this study.

Effect of Reducing Agent

Various reducing agents have been used by those working with anaerobes for addition to the medium for lowering the oxidation-reduction potential. Sijpesteijn (1948) tested sodium sulfide, ascorbic acid and thioglycollic acid and found all three produced equal results. The following concentrations were used: 0.01 and 0.03 per cent sodium sulfide, 0.1 per cent thioglycollic acid and 2 per cent ascorbic acid. In this country, cysteine hydrochloride and sodium thioglycolate have been used predominately. To test differences between reducing agents, a study was made of cysteine hydrochloride, sodium thioglycolate and thioglycollic acid neutralized with sodium hydroxide. Varying concentrations up to 1 per cent were added to T.S.G.P. medium, and the decrease in the pH was recorded after the cultures were incubated for 24 and 31 hours at 35-37° C. The results indicated that any of three reducing agents could be used effectively. If no reducing agent was added, growth would take place, but at a slower rate. This also was true when the larger quantities

were added. This effect was not pronounced when a large inoculum was used; however, sometimes with a low inoculum the anaerobes would not initiate growth. The results of these trials with cysteine hydrochloride agree with those of Valley (1929) and Lewis et al. (1940); therefore, cysteine hydrochloride was used and the concentration in the T.S.G. medium increased from 0.02 to 0.05 per cent.

Use of V-8 Juice and Rumen Liquid

Because it has been the common practice of those working with rumen bacteria to employ rumen liquid in the medium, it was decided to add it to the medium with the thought that perhaps some reducing agent or possibly some growth factor might be present in the liquid that would stimulate the growth of the anaerobes from dairy products. Strained V-8 Juice was tested in the same trial because Bedell and Lewis (1938) had observed the stimulatory effects of tomato juice upon the growth of intestinal anaerobes. The V-8 Juice and rumen liquid replaced 10 per cent of the liquid of the medium. The T.S.G.P. medium served as the control. Plates and deep-agar shake tubes were made with the above three media, using a milk and a rumen-liquid sample. Approximately 50 colonies were picked from each medium. Eight isolates were obtained, five from the milk sample and three from the rumen-liquid sample. The eight anaerobes were isolated from

either the medium with the added V-8 Juice or the medium with the added rumen liquid. No anaerobes were isolated from the T.S.G.P. medium. The V-8 Juice had an advantage over the rumen liquid because it was believed that it would be more uniform and always available, while it was not certain that the rumen-fistulated cow would be available.

Use of Cell-Free Extract of S. lactis

The enrichment procedure used with the majority of the milk and cream samples was to allow the milk or cream to sour naturally when incubated at room temperature. The lactic streptococci probably played the most important role in the enrichment procedure. It was assumed that after the milk had coagulated, the anaerobes were able to initiate growth and to increase their numbers sufficiently for isolation purposes. It was thought possible that these lactic streptococci might play another role by producing some factor or factors which might aid anaerobic growth. To test this possibility and to determine what effect the cell-free extract of S. lactis might have on the growth of the anaerobes, a preliminary study was made. S.lactis culture No. 33 of McDowell (1951) was inoculated into T.S.G.P. broth and incubated for 24 hours at 21° C. After incubation the medium was divided, one-half of the broth was sterilized at 15 lb. pressure for 10

minutes, the other was centrifuged in a high-speed centrifuge to harvest the cells. The supernatant (No. 1) also was sterilized as above, while the harvested cells were ground in pyrex-glass powder, according to the method of Utter and Werkman (1942). The wet cells were mixed with the powdered glass and sufficient distilled water was added to make a batter. After grinding, the mass was centrifuged again to separate the cell debris and glass from the supernatant (No. 2). These three preparations were added to semi-solid T.S.G.P. medium by adding 1 ml. to 9 ml. of medium. Two anaerobic cultures were inoculated by a wire loop into the above three media and also into T.S.G. minus the Tween 80, which served as the control. The tubes were incubated at 35-37° C. and inspected every 2-3 hours to denote the time the first colony appearance was made. Colonies were noticed in all tubes at the same time (48 hours) and the counts were approximately the same. After incubation for 5 days, the colonies in the tubes where supernatant No. 2 had been added were somewhat larger than the ones in the control tubes. It appeared that the cell-free extract of S. lactis might be beneficial, but not to the extent that it should be used as a routine procedure. The T.S.G. medium seemed to give comparable results.

Effect of Adding Different Lower Fatty Acids and Pyruvic Acid

The work of West et al. (1942) has shown the beneficial effects of pyruvic acid on the non-sporulating anaerobic bacteria of intestinal origin; therefore, pyruvic and other short chain fatty acids which might be useful or stimulatory for the anaerobes of dairy origin were tested. In a preliminary trial, pyruvic, lactic, propionic and butyric acids were added to semi-solid T.S.G.P. medium at the rate of 0.25 per cent. They also were added to the same medium when glucose was absent, leaving the acids as the sole source of carbon. After incubation at 35-37° C. for a suitable length of time, the tubes were examined for the relative rapidity and abundance of multiplication. The media containing pyruvic and lactic acids in addition to the glucose seemed to stimulate the growth of the 11 anaerobic cultures tested. Butyric and propionic acids gave no such effect. When these acids were added to the medium without glucose, very little growth occurred.

In another series, varying amounts of pyruvic acid and sodium lactate were added to varying amounts of glucose containing medium. Concentrations of acids used were 0.05, 0.1 and 0.15 per cent, while concentrations of glucose used were 0.1, 0.25 and 0.5 per cent. In the study 18 different

combinations of acids and glucose were used, with the three different levels of glucose acting as controls. The pyruvic acid and sodium lactate showed some stimulatory effect in the 0.05 and 0.1 per cent levels when 0.5 per cent glucose was used. This effect was not great but was definitely noticeable. When the lower concentrations of glucose were used, sodium lactate seemed to stimulate the cultures more than did pyruvic acid.

Effect of Sodium Lactate, Pyruvic Acid, Sodium Acetate and Tween 80

After the preliminary trials were concluded, further studies were made to confirm the stimulatory effect observed. In place of the relative turbidity readings, the cultures were tested for developed acidity by measuring the pH after periods of incubation. The semi-solid T.S.G. medium was used and the acids and Tween 80 were added at the rate of 0.05, 0.1, and 0.15 per cent. The results of the trial with the 0.15 per cent level are shown in Table 1.

All concentrations of Tween 80 added to the medium allowed the pH to drop faster and the growth was increased over the control tubes. Approximately the same results were obtained with the 0.05 and 0.15 per cent levels, while 0.1 per cent gave a slightly faster decrease in pH. The addition of sodium acetate or sodium lactate gave some stimulatory

Table 1. Effect on growth of anaerobes of additions to T.S.G. medium of acids and Tween 80 at the rate of 0.15 per cent

Culture	Incubation time (hr.)	pH after incubation with additions to T.S.G. medium of:				
		Control	Acetate	Pyruvate	Lactate	Tween 80
3.	0	6.32	6.28	6.28	6.35	6.34
	24	5.80	5.89	5.94	6.01	6.07
	36	5.32	5.29	5.56	5.34	4.91
	48	5.22	5.04	5.19	5.18	4.92
4.	0	6.34	6.29	6.28	6.37	6.35
	24	5.78	5.88	5.93	6.18	5.91
	36	5.47	5.31	5.54	5.32	4.97
	48	5.19	5.05	5.20	5.13	4.96
5.	0	6.32	6.29	6.28	6.37	6.35
	24	5.59	5.81	5.93	6.06	5.71
	36	5.29	5.21	5.68	5.21	5.08
	48	5.14	5.08	5.21	5.05	5.09
6.	0	6.32	6.29	6.28	6.37	6.35
	24	6.18	6.21	6.11	6.38	6.25
	36	5.98	5.89	6.01	6.07	5.49
	48	5.50	5.32	5.80	5.32	5.19

effect at the 0.05 and 0.1 per cent levels, but none was observed with the 0.15 per cent level. Addition of pyruvic acid did not increase the acid production, but caused a lag when compared with controls. This was contrary to the findings of West et al. (1942) with the intestinal types and to the preliminary observations of this study.

To make certain that combinations of the above acids with and without the Tween 80 did not increase the stimulation over and beyond that which Tween 80 produced alone, several trials were run with nine representative anaerobic cultures with the following combinations.

Tween 80	Lactate	Acetate	Pyruvic
0.05%	0.05%	%	%
0.05		0.05	
0.05			0.05
0.05	0.05	0.05	
0.05	0.05	0.05	0.05
0.05	0.05	0.10	
0.05	0.10	0.05	
0.10	0.05		
0.10		0.05	
0.10			0.05
0.10	0.05	0.05	
0.10	0.10	0.05	

With these combinations, generally no increased stimulation was noticed over that which Tween 80 could produce alone. In a few instances the combination of 0.10 per cent Tween 80 and 0.05 per cent sodium lactate permitted slightly faster decrease in pH; however, the difference was only 0.05-0.10 pH unit. This difference was believed to be of little significance.

Use of Sodium Azide, alpha-Bromopropionic Acid, and Iodoacetic Acid to Eliminate Facultative Anaerobes

In the early as well as the later phases of this study, most colonies picked were found to be facultative anaerobes. The gram-positive cocci, presumably lactic streptococci, or gram-negative rods, presumably of the coliform group, were encountered most often. In an effort to reduce the number of these two types, limited studies were made using three bacteriostatic agents and four antibiotics.

Sodium azide, alpha-bromopropionic acid and iodoacetic acid were tested to determine if they might be utilized in an isolation procedure to inhibit facultative anaerobes and allow obligate anaerobes to grow. Four strains of S. lactis isolated and typed by McDowell (1951) and six strains of anaerobes were tested. Proper dilutions of the agents were added to T.S.G.P. semi-solid medium. The findings in one series of experiments are summarized in Table 2. Of the three bacteriostatic agents tested, only sodium azide held any promise for inhibiting the facultative anaerobes without restraining the obligate anaerobes. However, the concentration differences were not great enough for actual use. Iodoacetic acid had different effects upon the strains of S. lactis used, and the high concentration tolerated by these strains prevented its use.

Table 2. Comparison of bacteriostatic agents for inhibition of S. lactis and anaerobes after incubation for 72 hours at 35-37° C.

Organism	Molar concentrations of the agents		
	Sodium azide	α-bromopropionic acid	Iodoacetic acid
S. lactis[a]			
Growth	1×10^{-3}	1×10^{-2}	$6\text{-}10 \times 10^{-5}$
Inhibition	2×10^{-3}	2×10^{-2}	$8\text{-}20 \times 10^{-5}$
Anaerobes[b]			
Growth	4×10^{-3}	1×10^{-3}	4×10^{-5}
Inhibition	6×10^{-3}	1×10^{-2}	6×10^{-5}

[a]Four cultures

[b]Six cultures

Effect of Penicillin, Streptomycin, Aureomycin and Terramycin on Elimination of Facultative Anaerobes

These four antibiotics were tested against three S. lactis cultures and nine anaerobic cultures to determine their relative sensitivity. If enough differences were found in the sensitivity pattern, then the antibiotics might be used effectively in a procedure for the isolation of obligate anaerobes. The semi-solid T.S.G.P. medium was used in these studies. The results of several studies are summarized in Table 3.

The anaerobes were more sensitive to penicillin and streptomycin than were the S. lactis cultures. Thus, neither could be used to inhibit the facultative organisms without first inhibiting the anaerobes. The reverse is true in the case of aureomycin and terramycin. Although growth was obvious in the tubes containing from 0.00060-0.00100 mg. of terramycin per ml. of medium the best growth was evident when quantities up to 0.00040 mg. were used. The anaerobic cultures showed a different sensitivity pattern to the aureomycin than to terramycin; four cultures showed abundant growth in the tubes containing up to 0.00060 mg., one culture up to 0.00100 mg. and another culture up to 0.00035 mg. of aureomycin per ml. However, growth was still observed with all anaerobic cultures when up to 0.00100 mg. of aureomycin

Table 3. Concentrations of four antibiotics necessary for inhibition of *S. lactis* and anaerobes after incubation for 216 hours at 35-37° C.

Cultures	Concentration of Antibiotic			
	Terramycin (mg./ml.)	Aureomycin (mg./ml.)	Penicillin (units/ml.)	Streptomycin (units/ml.)
S. lactis[a]				
Growth	0.00030-40	0.00010-15	0.1	30
Inhibition	0.00050	0.00020	0.5	not determined
Anaerobes[b]				
Growth	0.00060-100	0.00070-100	0.01	15
Inhibition	0.00125	0.00125	0.05	20

[a] Three cultures

[b] Nine cultures

was present per ml. All the S. lactis cultures were inhibited at a very definite level, whereas no definite division could be made for the anaerobes. Abundant growth was observed up to a certain level and then from that level up to 0.00100 mg. per ml. growth was not as abundant. After incubation for 108-128 hours, growth was not shown at the 0.00125 mg. level with either terramycin or aureomycin. Further tests utilizing this information will be reported in other studies.

Effect of Potassium Cyanide on Facultative Anaerobes

Since direct evidence was not obtained on whether facultative anaerobes concerned in this study contained a cyanide-sensitive system, it was decided to study the effect of blocking such a system by the use of potassium cyanide (Werkman and Wilson, 1952). Several pure cultures of coliform bacteria and S. lactis and three anaerobic cultures were plated out with 0.1, 1.0 and 3 per cent potassium cyanide added to T.S.G.P. medium and incubated under anaerobic conditions. After 2 days only the coliform types showed growth in all concentrations, and after 4 days all cultures tested were growing as well as they were in the control plate. These results with the dairy anaerobes were not the same as those of King and Rettger (1942). Thus, this possible means of eliminating the facultative anaerobes was not pursued further.

Use of Nile Blue Sulfate as an Indicator

On some platings of anaerobes for detection of lipolysis, the Nile blue sulfate (N.B.S.) used as an indicator was decolorized immediately surrounding the colonies. This phenomenon has been observed by other workers. Possibly anaerobes reduced the oxidation-reduction potential enough to reduce the indicator while other facultative organisms would not. To test this theory, known species of S. lactis and coliform bacteria were streaked on N.B.S. plates using T.S.G. medium and after anaerobic incubation no discoloration was noticed. One vat sample of milk was tested by plating the milk with T.S.G. medium with and without the addition of N.B.S. After enrichment of the milk for 3 days, platings were made, but no anaerobes were isolated from the 54 colonies picked. After enrichment for 7 days, 34 anaerobes were isolated, 19 of which came from colonies on the N.B.S. medium. Some colonies that reduced the indicator were not anaerobes. This procedure may be a means of selection of obligate anaerobes from facultative types. Incubation times might be extended somewhat, as very small colonies were not able to reduce the indicator. Perhaps the concentration of the dye may be a factor for proper selection. Other indicators which would function at the proper oxidation-reduction potential might be utilized more effectively, but this

theory was not explored further.

Use of Deep-Agar Plates

After a few samples had been plated, it appeared that anaerobic platings were inefficient: the long incubation period dried some of the agar in the plates and the growth of spreader types on the surface of the plate interfered in picking and transfering pure cultures. To overcome both of these difficulties, plates were poured deeper and with an overlay. In most instances, 50-60 ml. of medium was poured with the sample, and immediately after it had hardened, 10 ml. of overlay was poured. This method proved very effective and was used in the latter part of the study for all the cream and cheese samples and for some milk samples. It was presumed that the anaerobes grew better in the deeper plates and could initiate growth faster. The spreader trouble ceased when overlays were poured.

Period of Incubation of Plates

In the majority of cases, the plates were incubated for 5-7 days before colonies were picked. After the deep plates with an overlay were found successful, it was thought that a longer incubation period might be effective in increasing the efficiency of anaerobe isolation. A vat sample of milk was

plated in duplicate in T.S.G. medium and incubated at 35-37° C. for periods of 5 days and 10 days. In each instance, approximately 40 colonies were picked from the plates of each series. Platings of the same sample were made after 2, 4, 6 and 7 days of enrichment at room temperature. Plates were incubated under anaerobic conditions. A total of 299 colonies was picked, 150 of which were picked from the plates incubated for 5 days. The results of these platings are shown in Table 4.

Although the results probably are not conclusive, eight anaerobes were isolated from the plates incubated for 10 days, while only three anaerobes were isolated from the plates incubated for 5 days. Even though isolates were found after incubation for 5 days, it would appear that the 10 day incubation time or longer would be more desirable.

Enrichment Methods of Milk for Isolation Purposes

Several enrichment methods were tested, other than the usual procedure of allowing the milk to sour naturally at room temperature with the flora that was present in the milk. After varying lengths of time and/or after coagulation of the milk, appropriate dilutions were placed in deep-agar shake tubes or were plated. The purpose of this procedure was to increase the population before proceding to isolate the

Table 4. Plate counts and numbers of isolates from plates incubated under anaerobic conditions after varying days of enrichment at room temperature of one milk sample.

Days of enrichment	pH of sample	Plate counts per ml. and number of isolates after incubation for: 5 days	10 days
2	5.18	40×10^7 (1)[a]	67×10^7 (1)[a]
4	4.24	89×10^7 (1)	59×10^7 (4)
6	4.27	59×10^7 (1)	47×10^7 (2)
7	4.27	141×10^7 (0)	121×10^7 (1)

[a]Indicates number of anaerobes isolated from approximately 40 colonies picked.

anaerobes. No attempt was made to determine the number of anaerobes in the product or to determine the percentage of the total population that existed as anaerobes. A large majority of the anaerobic isolations were made from the higher dilutions (10^7-10^8). Those working with intestinal and rumen anaerobes likewise found that their most successful isolations were made from these dilutions.

In light of previous experiments, where S. lactis cultures were shown to be more sensitive to aureomycin and terramycin than were the anaerobes, it was decided that perhaps this would be a means of using the antibiotics as an aid for the enrichment procedure. In one of the first experiments, a patron sample of milk was divided and to one portion, 0.00050 mg. of aureomycin was added per ml. of milk while the other portion without antibiotic served as the control. After incubation for 3 and 7 days at room temperature, each portion was tube diluted into shake cultures of T.S.G.P. and T.S.G. media with and without the addition of 0.00020 and 0.00050 mg. of aureomycin per ml. of medium. The portion of the sample that contained the added aureomycin contained a large majority of spore-formers and gas producers; when placed in any of the above media isolation of any colonies was impossible. The agar plugs were broken and spreader colonies were observed throughout the plugs. In the normal enrichment procedure the acid producers could produce enough

acid to control the gas producers, but when they were inhibited by the aureomycin, the gas producers were not inhibited and were able to grow. As a result of this trial, the aureomycin was never added directly to the milk but added to the medium.

Four other samples of milk were tested by additions of aureomycin to the medium. Three were patron samples and one was a vat sample. Appropriate dilutions of one patron sample enriched for 3 days at room temperature were placed into deep-agar shake tubes of T.S.G.P. and T.S.G. media with 0.00020 and 0.00050 mg. of aureomycin per ml. of medium. From a total of 174 colonies, only two anaerobes were isolated: these were picked from shake tubes that contained 0.00020 mg. aureomycin per ml. of T.S.G. medium. In another patron sample, the milk was plated into T.S.G., T.S.G. plus 0.00020 mg. aureomycin per ml., and T.S.G. plus the aureomycin and 1 per cent potassium cyanide. Two anaerobes were isolated from 69 colonies picked from the T.S.G. medium. No anaerobes were found with the additions of aureomycin or aureomycin plus potassium cyanide. For the last patron sample, the milk was plated into T.S.G., T.S.G. plus 0.1 per cent potassium cyanide, T.S.G. plus 0.00050 mg. aureomycin per ml., and T.S.G. plus 0.1 per cent potassium cyanide and 0.00050 mg. aureomycin per ml. No anaerobes were isolated from any of the above media.

To determine if a more rapid development of the acid production might eliminate some of the difficulties that the gas producers caused in a previous experiment, a 1 per cent inoculum of S. lactis was added to a vat sample of milk prior to the enrichment at room temperature. After enrichment for 3 days the milk was plated in T.S.G. medium with 0.00050 mg. of aureomycin added per ml. Anaerobes were isolated from media with and without the added aureomycin; however, the isolates from the aureomycin medium failed to continue growth after two transfers and were lost before any characteristic tests could be run. Although anaerobes were isolated when the aureomycin was added to the medium, they also were found in the same medium when it was not added. No doubt many anaerobes were inhibited or were suppressed by the presence of aureomycin and probably its use in the enrichment or isolation procedure is of doubtful value.

It was noticed in some of the deep-agar shake cultures that, at dilutions of 10^4-10^5, the numbers of colonies present prevented the picking of isolated colonies. Because isolations could be made from the higher dilutions, it was thought that a larger percentage would be found at the lower dilutions. To test this possibility, 35 x 200 mm. test tubes were selected and the dilutions placed in approximately 100 ml. of T.S.G. medium. This procedure enabled one to pick isolated colonies; however, difficulty was experienced in

getting the plugs from the tubes in a condition suitable for picking. Although isolates were found in the milk sample by plating, no anaerobes were found in the colonies picked from the tubes. This procedure held no advantage over the deep-agar plates previously described.

Aerobic and Anaerobic Platings of Samples

On several occasions in the latter phases of the study, several samples of cream, cheese and milk were plated in duplicate. The same milk sample was plated on two separate days following enrichment, accounting for the two counts for each sample. One series was placed under anaerobic conditions, the other was incubated aerobically. Table 5 shows the results of some of these platings. When the cream samples were plated in duplicate, plates incubated anaerobically had higher bacterial counts than those incubated aerobically. In two cases (samples 22 and 36) anaerobes were not isolated even though counts were higher on plates incubated anaerobically. When counts are higher on anaerobic incubation and anaerobes are not isolated, it may indicate either that the procedure used was not conducive to their isolation, or that facultative bacteria were stimulated when incubated under anaerobic conditions. The majority of the cheese and milk samples had anaerobic plate counts which were

Table 5. Plate counts of raw milk, cream and cheddar cheese from plates incubated aerobically and anaerobically for 7 days at 35-37° C.

Source	Source no.	Counts per ml. from plates incubated: Aerobically	Anaerobically
Cream	Vat 2[a]	251×10^6	48×10^7
Cream	Vat 3[a]	265×10^6	41×10^7
Cream	22	44×10^8	57×10^8
Cream	52[a]	194×10^6	79×10^8
Cream	36	177×10^6	240×10^6
Cream	33[a]	222×10^6	456×10^6
Cheese	I[a]	168×10^6	164×10^6
Cheese	II[a]	68×10^6	73×10^6
Cheese	III[a]	144×10^6	145×10^6
Cheese	IV[a]	224×10^6	235×10^6
Cheese	V	236×10^6	236×10^6
Cheese	VI	175×10^6	306×10^6
Cheese	VII	32×10^6	37×10^6
Milk	20	113×10^2	117×10^2
Milk	20[a]	30×10^8	37×10^8
Milk	19[a]	127×10^7	140×10^7
Milk	19[a]	49×10^6	88×10^6

[a]Samples from which anaerobes were isolated.

comparable to the aerobic counts; however, cheese sample V did have a higher anaerobic count.

Characteristics of the Anaerobic Isolates

The source, medium used, method of isolation and final group classification for all of the anaerobes isolated in this study are shown in Table 6. Of the 239 non-sporulating anaerobes, 135 were isolated from raw milk, 83 from raw cream, 15 from Cheddar cheese and six from rumen liquid. For these isolations, 19 samples of milk were obtained, 11 of which were patron samples, while eight were vat or tank samples. The first of these samples did not produce any isolates, as the agar content of the medium was not sufficient for picking the colonies formed. Thus, this sample was not considered in figuring averages. Aliquots of four patron samples (7, 8, 9 and 11) were frozen and held for approximately 30 days at less than 0° C. They were thawed at 32° C. and plated separately. The anaerobes obtained from these platings were recorded in Table 6 as sample 13. In all other discussion the isolates from these platings were considered with the other isolates from the original patron samples. Of the 2,611 colonies picked from milk samples, 5.25 per cent were found to be non-sporulating anaerobes.

Table 6. Source, medium used, method of isolation and final group classification for non-sporulating anaerobic bacteria isolated between July 1, 1951 and January 10, 1953.

Cultures no.	Source	Medium	Method	Final group classification
[1,2,9-12,26]7[a,b]	Milk, P-3[c]	Eugonagar-L[d]	Tube	none
3-8,17,19	Milk, P-2	Eugonagar	Tube	I
13, [14]7	Rumen, 2	Eugonagar	Tube	I
[15]7	Milk, P-3	Eugonagar	Tube	none
16	Milk, P-3	Eugonagar-L	Tube	I
18	Rumen, 2	Eugonagar	Plate	I
20	Rumen, 2	Eugonagar-L	Plate	I
21	Milk, P-2	Wilkowske	Tube	I
22,24	Milk, P-4	T.S.G.V-8[e]	Tube	I
23	Milk, P-4	T.S.G.V-8	Plate	I
25	Milk, P-4	T.S.G.P.[f]	Tube	I
27	Milk, P-4	T.S.G.P.	Plate	I
28,29	Milk, P-4	T.S.G.P.R.[e]	Tube	I

[a]Numbers in brackets were lost after isolation and preliminary characterization.

[b]The culture numbers are listed in the order in which they were isolated between July 1, 1951 and January 10, 1953.

[c]P indicates patron sample, followed by patron number.

[d]L indicates that sodium lactate replaced glucose in the medium.

[e]V-8 or R, shows that 10 per cent V-8 Juice or Rumen liquid, respectively, had been added to T.S.G.P. medium.

[f]T.S.G.P. Trypticase-soy-glucose plain agar

Table 6 (continued)

Cultures no.	Source	Medium	Method	Final group classification
30, [31]	Rumen, 3	T.S.G.P.R.	Tube	I
32,33	Milk, P-5	T.S.G.P.A.[g]	Tube	I
34,35	Milk, P-7	T.S.G.P.	Plate	I
36-38	Milk, V-10[h]	T.S.G.[i]	Tube	I
39-43	Milk, F-13[j]	T.S.G.	Plate	I
44	Cream, P-68[c]	T.S.G.	Plate	I
45-48,64-76	Cream, V-4[h]	T.S.G.	Plate	I,1;II,13; III,3
49,50	Milk, P-12	T.S.G.	Plate	II
51-60	Cream, P-11	T.S.G.	Plate	I,1;II,9
61	Cream, P-57	T.S.G.	Plate	I
62,(17)[k]	Milk, V-10	T.S.G. (T.S.G.A.)	Plate	I
63	Cheese, 1	T.S.G.	Plate	I
77-107	Cream, P-5	T.S.G.	Plate	II
[108,109]	Cream, P-6	T.S.G.	Plate	II

[c] P indicates patron sample, followed by patron number.

[g] A. shows that 0.00020 mg./ml. aureomycin was added to T.S.G.P. medium.

[h] V indicates vat sample, followed by vat number

[i] T.S.G. Trypticase-soy-glucose agar

[j] F indicates patron samples frozen, followed by sample number

[k] Numbers in parenthesis are of number additional anaerobic cultures isolated but not numbered.

Table 6 (continued)

Cultures no.	Source	Medium	Method	Final group classification
[110]	Cream, P-27	T.S.G.	Plate	II
111	Cream, P-57[a]	T.S.G.	Plate	II
[112]	Cream, P-50	T.S.G.	Plate	II
113-131	Milk, V-15	T.S.G. (NBS)[1]	Plate	I,1;II,18
132-142, (4)	Milk, V-15	T.S.G.	Plate	II
143-146	Milk, P-12	T.S.G.	Plate	I
147-151,154-159	Milk, V-18	T.S.G.	Plate	I
152,153	Milk, V-17	T.S.G.	Plate	I
160-184	Milk, V-19	T.S.G.	Plate	I,23;II,1 IV,1
185-188	Milk, V-20	T.S.G.	Plate	I
189	Cream, P-33	T.S.G.	Plate	I
190,191	Cream, V-3	T.S.G.	Plate	I;II
192,193	Cream, P-14	T.S.G.	Plate	I
194,195	Cream, P-55	T.S.G.	Plate	I
196	Cream, P-46a	T.S.G.	Plate	I
197,198	Cream, P-26	T.S.G.	Plate	I
199,200	Cream, P-52	T.S.G.	Plate	I
201-203	Cream, P-30	T.S.G.	Plate	I

[a]Numbers in brackets were lost after isolation and preliminary characterization.

[1]NBS indicates 1 ml. of a 1 per cent Nile blue sulfate solution was added to 100 ml. T.S.G. medium.

Table 6 (continued)

Cultures no.	Source	Medium	Method	Final group classification
204,205	Cream, P-33	T.S.G.	Plate	I
206	Cream, V-2	T.S.G.	Plate	I
207	Cheese, I	T.S.G.	Plate	I
208-212	Cheese, II	T.S.G.	Plate	I
213,214,216	Cheese, III	T.S.G.	Plate	I
215,217	Cheese, IV	T.S.G.	Plate	I
218	Cheese, I	T.S.G.	Plate	I
219,220	Cheese, VI	T.S.G.	Plate	I

Of the 33 samples of raw cream, 28 were patron samples and five were vat samples. A total of 847 colonies was picked from the plates and 9.8 per cent were found to be non-sporulating anaerobes. From the 13 samples of cheese and three of rumen liquid, 5.6 per cent and 1.26 per cent non-sporulating anaerobes were isolated, respectively. Totals of 268 and 476 colonies were picked from cheese and rumen liquid, respectively. From all the dairy products examined, 4,202 colonies were picked, of which 5.74 per cent of the total were non-sporulating anaerobes. Only two sporulating anaerobes were found in this study.

Anaerobes were not isolated from four milk samples, 15 cream samples, six cheese samples and one rumen-liquid sample. Anaerobes were isolated from 78 per cent of the milk samples, 55 per cent of the cream samples, 67 per cent of the rumen liquid samples and 62 per cent of the cheese samples. Of the 67 samples examined, 63 per cent contained non-sporulating anaerobes.

The large majority of the cultures were isolated on T.S.G. medium and by means of platings. Of the 239 isolates, 187 were isolated from T.S.G. and the remaining isolates were found on the other media used. The plating procedure resulted in 202 isolates, while 37 isolates resulted when using deep-agar shake tubes. Isolations were made from the various products during January, May, June, and July (142),

and with a rather large number also being isolated during November and December (69). The breakdown by months of the 2,611 colonies picked from milk is as follows:

Month	Total	Anaerobes
January	478	2
May	598	27
June	266	5
July	624	59
October	145	2
November	453	36
December	47	4
	2,611	135

Of the 847 colonies picked from cream, 602 colonies were picked from June samples, with 65 anaerobic isolates, and from 245 colonies picked from December cream samples, 18 anaerobes were isolated. From four samples of pasteurized Cheddar cheese 6 or 7 months old, 11 anaerobes were isolated, while from six samples of raw milk Cheddar 40 days old, only three anaerobes were isolated.

In most cases, all the anaerobes isolated from a sample were finally placed in one group (Table 6). Relatively few anaerobes were isolated from each of these samples. The anaerobes isolated from each of four out of 13 vat samples and one out of 39 patron samples were placed in two or three groups. A large number of anaerobes was isolated from each of these samples. The majority of the anaerobes isolated from each of these five samples was placed in one group. Since a vat contains milk or cream from different sources, it

would seem logical to find a more varied anaerobic flora in a vat sample than in a patron sample.

The isolated anaerobic cultures as listed in Table 6 were divided into four groups on the basis of morphology, biochemical tests and fermentation studies. Group I consisted of 115 isolates from 39 different sources, including milk, cream, cheese and rumen liquid. Group II consisted of 88 isolates from nine different sources of milk and cream. Group III consisted of three isolates from one sample of cream and Group IV consisted of one isolate from milk. Before any of the group characteristics shown in Table 7 were determined, each culture was purified by three or four successive single colony isolations.

Morphologically the organisms of all four groups consisted of small, straight, gram-positive, non-motile, short rods, which occurred singly and in pairs. Cells from all groups but Group IV formed short chains. Electron micrographs of Groups I, II and III cells are shown in Figure 1. Photomicrographs of cells of the four groups are shown in Figure 2. Groups I and III cells measured 0.5-0.7 μ in width and 0.7-1.3 μ in length. Group II cells were 0.4-0.7 μ in width and 0.8-1.6 μ in length, while those of Group IV measured 0.4-0.6 μ in width and 0.7-1.0 μ in length. The sub-surface colonies of the four groups formed in T.S.G. medium usually were lens-shaped, while surface colonies were

Table 7. Characteristics of four groups of non-sporulating anaerobes isolated from milk, cream, cheddar cheese and rumen liquid.

Characteristics	Group I	Group II	Group III	Group IV
Morphology:				
Gram reaction	+	+	+	+
Cell size	0.5-0.7μ x 0.7-1.3μ	0.4-0.6μ x 0.8-1.6μ	0.5-0.7μ x 0.7-1.3μ	0.4-0.6μ x 0.7-1.0μ
Colony, surface	circular	circular	punctiform	punctiform
Colony, sub-surface	lens-winged	lens	lens	lens
Colony size	2-5 mm.	1-3 mm.	0.5-1 mm.	0.5-1 mm.
Growth in semi-solid	clusters	strings	strings	strings
Motility	non-motile	non-motile	non-motile	non-motile
Acidity in milk	0.32-0.34%	1.5-2.0 %	0.32-0.33%	0.27-0.29%
pH in milk	5.5-5.9[a]	3.8-4.3[a]	5.7-5.8[a]	6.1-6.2[a]
Coagulate milk	115/0	88/0	0/3	0/1
Reduce litmus	115/0	88/0	0/3	0/1
Growth:				
5°C.	0/18	0/13	0/3	0/1
10°C.	38/77	86/2	3/0	0/1
21°C.	102/13	86/2	3/0	1/0
30°C.	115/0	88/0	3/0	1/0
37°C.	115/0	88/0	3/0	1/0
45°C.	14/101	0/88	1/2	1/0
Thermal Resistance:				
60°C. for 10 minutes	7/108	56/32	3/0	0/1
60°C. for 20 minutes	2/113	29/59	3/0	0/1
60°C. for 30 minutes	0/115	16/72	3/0	0/1
80°C. for 10 minutes	0/115	0/88	0/3	0/1
Acids Produced:				
Acetic	74/0	88/0	3/0	1/0
Propionic	74/0	0/88	3/0	0/1
Lactic	0/115	88/0	0/3	0/1
Utilize lactate	115/0	0/88	3/0	1/0

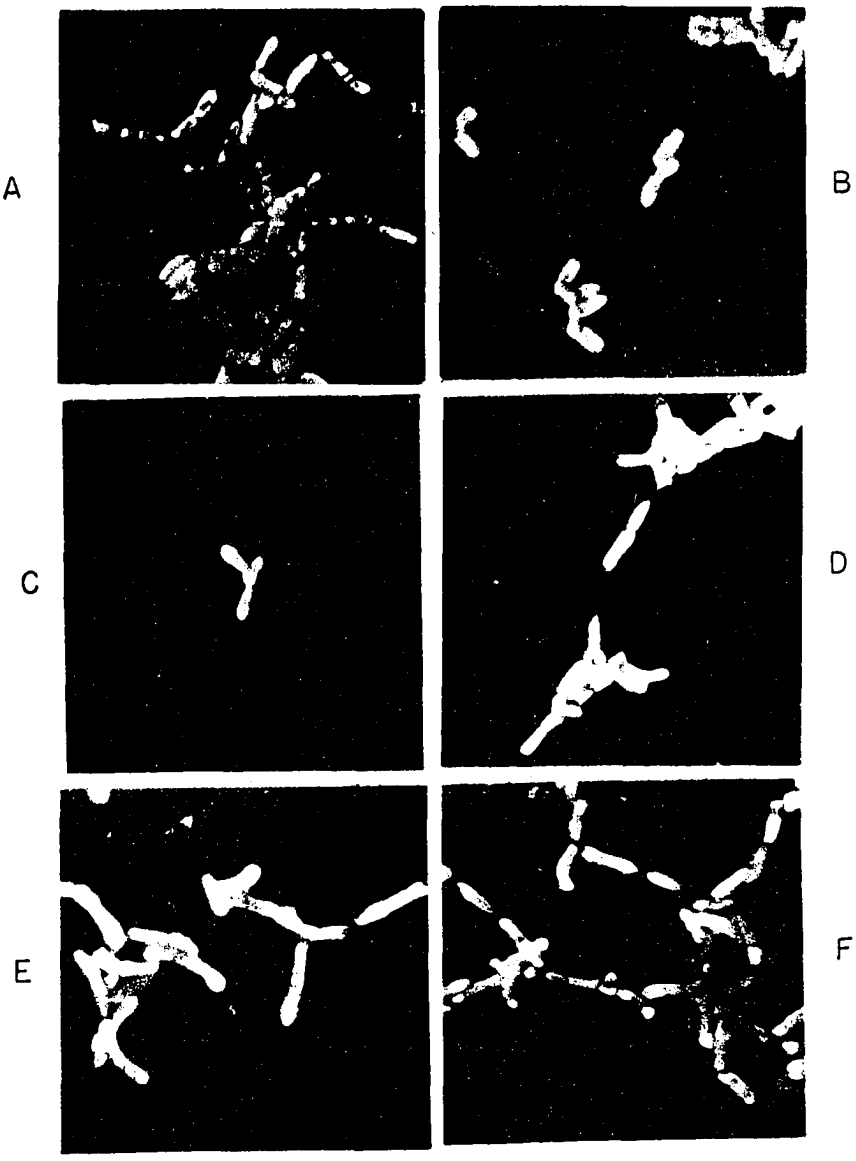
80°C. for 10 minutes	0/115	0/88	0/3	0/1
Acids Produced:				
Acetic	74/0	88/0	3/0	1/0
Propionic	74/0	0/88	3/0	0/1
Lactic	0/115	88/0	0/3	0/1
Utilize lactate	115/0	0/88	3/0	1/0
Biochemical tests:				
Catalase	115/0	0/88	3/0	0/1
Hydrogen sulfide	0/115	0/88	0/3	1/0
Ammonia	0/115	0/88	0/3	0/1
Reduce nitrate	103/12	3/85	3/0	1/0
Indole	114/1	0/88	0/3	0/1
Skatole	0/115	0/88	0/3	0/1
Proteolytic	113/2	0/88	0/3	0/1
Lipolytic	30/85	0/88	0/3	0/1
Hemolytic (Ox-blood)	6/65	0/72	0/3	0/1
Fermentation:				
Arabinose	0/70	53/7[b]	3/0	0/1
Cellobiose	0/70	51/10	0/3	0/1
Fructose	68/2[b]	60/0[b]	3/0	0/1
Galactose	70/0	51/9[b]	3/0	0/1
Glucose	70/0	60/0	3/0	0/1
Inulin	0/70	15/45[b]	0/3	0/1
Lactose	0/70	52/8[b]	0/3	0/1
Maltose	1/69	52/8[b]	3/0	0/1
Mannose	70/0	37/23[b]	3/0	0/1
Raffinose	0/70	46/14[b]	1/2	0/1
Sucrose	1/69	55/5[b]	0/3	0/1
Xylose	0/70	6/54	0/3	0/1
Mannitol	60/10	7/53	3/0	0/1
Sorbitol	62/8	7/53	2/1	0/1
Salicin	0/70	48/12[b]	3/0	0/1

a -First number indicates number of cultures positive, and second number indicates number of cultures negative, i.e. 38/77 shows 38 cultures positive, 77 negative.

b -The negative cultures have been lost; thus, further checking could not be completed.

Figure 1. Electron micrographs of organisms of Groups I, II, and III (4,200x)

A,B - 62, 18, Group I
C - 64, Group III
D.E.F - 137, 103, 120, Group II


A
B
C
D
E
F

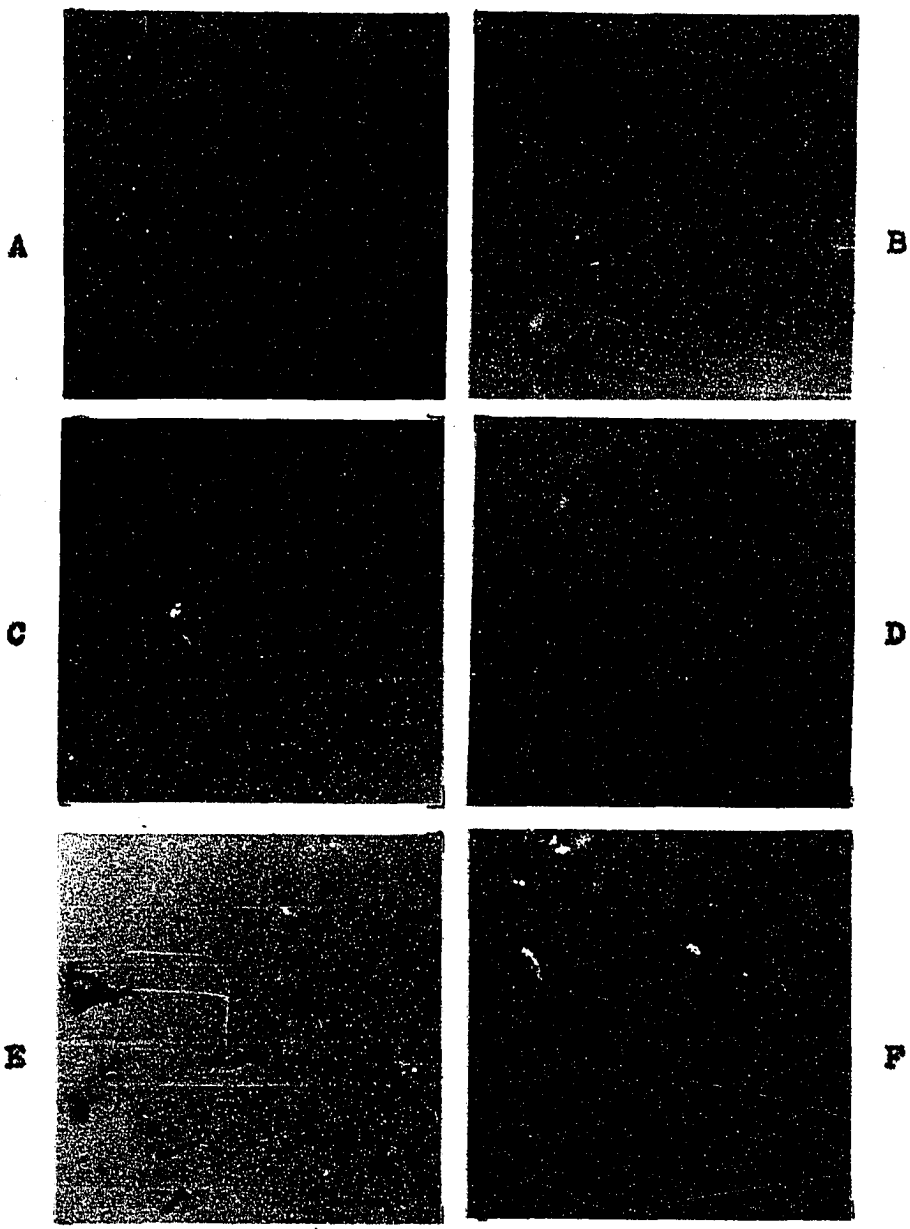


Figure 2. Photomicrographs of organisms of Groups I, II, III and IV (1,700x).
A - 184 Group IV
B - 74 Group III
CD - 30 Group I
EF - 103,120 Group II

punctiform or circular, translucent, greyish-white, convex, glistening and smooth and had an entire margin. When Group I cultures were incubated 7-10 days, they tended to form large, winged, brownish-yellow sub-surface colonies. Sometimes a cloudy concentric ring was formed around these winged colonies.

The electron micrographs in Figure 1 illustrates clearly the morphology of cells of Groups I, II and III. The pairs and short chains common to Group I are shown in Figures 1A and 1B. The side-by-side arrangement of the cells was typical of most Group I cultures. Some of the cells shown in Figure 1A have a shrunken appearance in the middle, perhaps the result of the preparation procedure or technic. This shrunken appearance has been observed with other electron micrographs. Cultures of Group III had a club shaped and solid appearance as shown in Figure 1C. The cells of Group II (Figures 1D, 1E and 1F) were somewhat larger with thicker spots or bodies at the ends of the cells. In some instances, two of these spots were seen in the same cell. The spots are not shown as clearly in Figure 1D as in 1E and 1F, because the focus was on the cell shapes in Figure 1D.

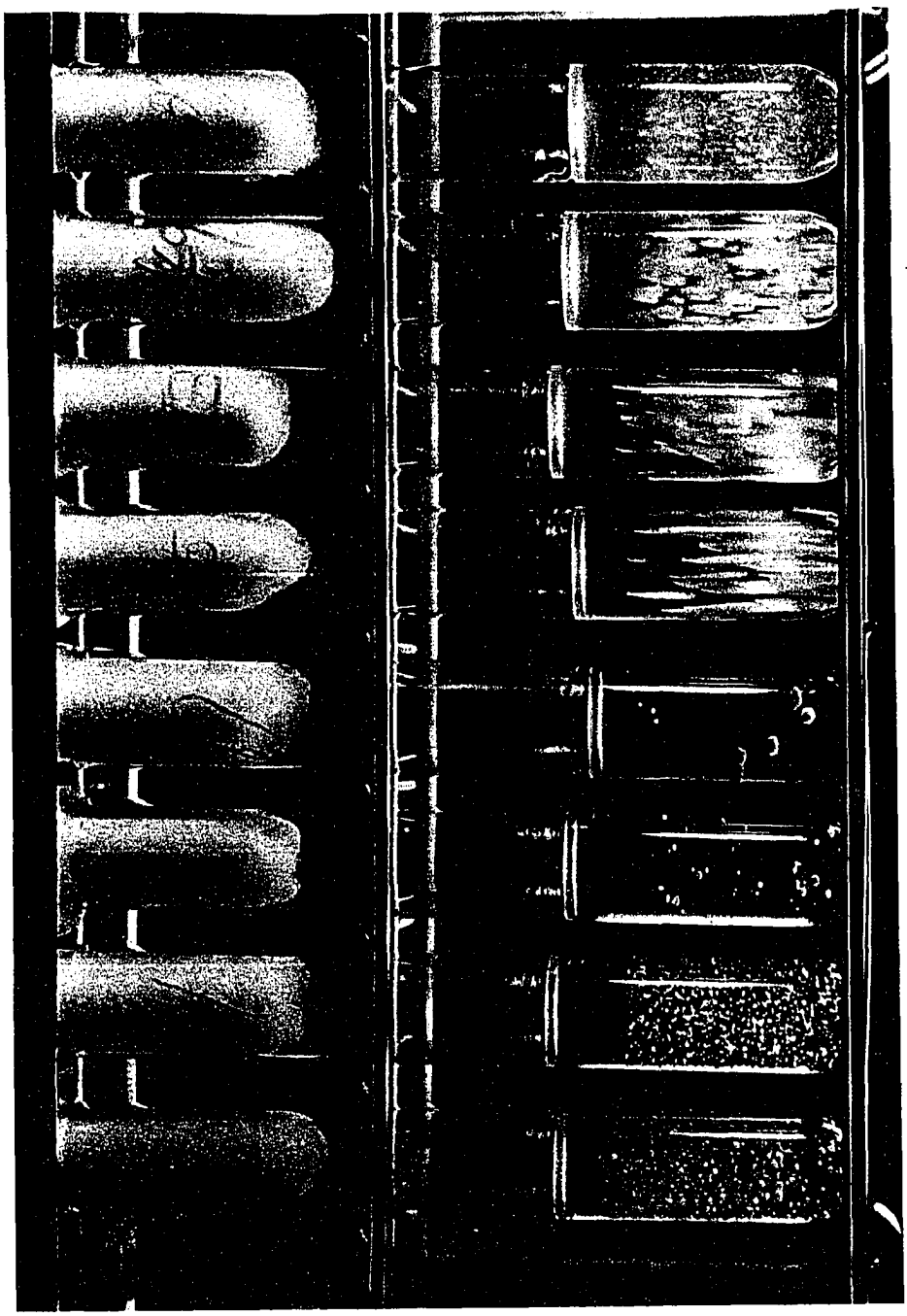
The photomicrographs of Figure 2 show cells of Groups I, II, III and IV. Cells of Group IV (Figure 2A) appear as stretched cocci, when grown in the T.S.G. broth, but usually appear as short rods in the semi-solid T.S.G. medium. The

small size and the pair arrangement is typical of culture 184. Culture 74 of Group III, as shown in Figure 2B, illustrates the side-by-side arrangement of the cells and the clumps commonly observed. Figures 2C and 2D show a typical culture of Group I with the pair and short chain formations. The larger size cells of two cultures of Group II are shown in Figures 2E and 2F. The thicker spots or bodies can be observed, but not as well as in the electron micrographs in Figure 1.

The growth characteristics of two groups in semi-solid medium are shown in Figure 3. Cultures of Group I produced "clusters" and are shown in the four tubes on the left, while cultures of Group II produced "strings" and are shown in the four tubes on the right. Cultures of Groups III and IV, although not shown in Figure 3, also produced "strings". The type of growth has been found to be characteristic of the group at all times.

Organisms of Group II coagulated milk and reduced litmus, while those of Group IV did not. Some cultures of Group I failed to coagulate milk in test tube quantities, but all cultures coagulated and reduced litmus milk in larger quantities. This phenomenon probably was due to the inability of some cultures to initiate growth. Cultures of Group I, being proteolytic, digested the protein of milk soon after coagulation took place. Group III organisms did not coagulate

Figure 3. Growth characteristics of non-sporulating anaerobes in semi-solid T.S.G. medium. The "clusters" produced by Group I are shown in the four tubes on the left, and the "strings" produced by Group II are shown in the four tubes on the right.



milk but reduced litmus milk very slowly only at the bottom of the test tubes and then only when a heavy inoculum was used and the period of incubation was extended to 18-21 days. Organisms of Groups I, III and IV produced very little titratable acidity in milk, while members of Group II produced an average of 1.7 per cent with a range of 1.5 to 1.9 per cent. The pH's of the milk samples after incubation for 7 and 14 days correlate with the titratable acidities. The cultures of Group I did not produce enough acid to give an acid coagulum; therefore, they must produce a coagulating enzyme.

All the cultures of all the groups grew at 30 and 37° C., but all cultures tested failed to grow at 5° C. The Group IV culture grew at 45° C., as did some cultures of Groups I and III. The majority of the cultures of all the groups grew at 21° C. Group II and III cultures also grew well at 10° C., as did a small fraction of the Group I cultures, but the latter did so only after a long period of incubation. Those cultures that failed to grow at the other temperatures after 30 days did grow when incubated at 35-37° C., indicating a satisfactory culture medium and inoculum.

Most of the cultures of Groups I and IV did not survive 60° C. for 10 minutes. A relatively few Group I cultures survived 10 and 20 minutes at 60° C. Only a small proportion of the cells in any one culture usually survived these

treatments. Group III cultures survived 60° C. for 30 minutes. The majority of Group II cultures survived 60° C. for 10 minutes but only a few cultures survived 20 and 30 minutes. A large number of cells survived the 10 minute treatment, while only a relatively few survived 20 and 30 minutes. The majority of those surviving the two higher temperatures were isolated from two milk samples. No cultures survived 80° C. for 10 minutes, indicating that spores were not formed under the conditions employed in this study.

Only six cultures of Group I hemolyzed bovine blood; two of these were beta hemolytic and four alpha. Eggerth (1953) used human blood in conducting his studies of hemolysis. A preliminary study was made to determine if any differences would be encountered between bovine and human blood. A definite beta hemolysis was observed on the human blood with two cultures, one of these being beta hemolytic on bovine blood and the other gamma. A further limited study was conducted to compare rabbit, bovine, human and sheep blood. The results of this study are shown in Table 8. The cultures hemolyzed rabbit and human blood more than they did bovine or sheep blood. Probably in the main study in which only bovine blood was used, many cultures were called non-hemolytic because the slight action was difficult to interpret.

In limited pathogenicity studies, one strain from each of the four groups was injected subcutaneously into mice.

Table 8. Beta hemolysis by selected Group I anaerobes on four types of blood.

Culture no.	Degree of hemolysis on blood from			
	Rabbit	Human	Bovine	Sheep
13	+++	++	+	-
24	+++	++	+	-
30	+++	++	+	-
52	+++	++	+	-
156	+++	++	+	-
167	+++	++	±	-
197	+++	++	+	-
209	+++	++	+	-
214	+++	++	+	-

None of the cultures proved to be pathogenic.

Group I cultures usually fermented fructose, galactose, glucose, mannose, mannitol and sorbitol. A few cultures failed to ferment mannitol, sorbitol or both of these compounds. Only one culture (149) fermented maltose and sucrose. Group III cultures were somewhat similar to those of Group I and fermented arabinose, fructose, galactose, glucose, maltose, mannose, mannitol, sorbitol and salicin. One culture (64) fermented raffinose but failed to ferment sorbitol. The culture of Group IV failed to ferment any of the carbohydrate compounds tested. The majority of the Group II cultures fermented arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, raffinose, sucrose and salicin. A few cultures fermented inulin, mannitol, sorbitol and xylose. Some Group II cultures and two Group I cultures were not available for retesting; thus, further fermentation studies could not be made to check their failure to ferment arabinose, fructose, galactose, lactose, maltose, raffinose, sucrose and salicin. All eight cultures of Group II that failed to ferment lactose produced enough acid in milk to reduce the pH to 3.8-3.9. Thus, it probably should be assumed that these cultures do ferment lactose. It was observed that some cultures failed to initiate growth in small quantities of the fermentation medium, due to a low inoculum. It might be assumed that these cultures which are no longer available

could ferment the sugars and glucoside as did the majority of cultures in their respective groups. Eighteen of the 23 cultures that failed to ferment mannose were lost. The remaining five were retested and the earlier findings were substantiated, as were findings for five of ten cultures that did not ferment cellobiose. Those available Group II cultures that fermented certain sugars or alcohols, whereas the majority did not, were retested and earlier findings were confirmed for fermentation of inulin (7 cultures), xylose (4 cultures), mannitol (4 cultures) and sorbitol (4 cultures). The remainder of the cultures in each case were not available for retesting. Those few cultures of Group II which did not ferment some of the sugars were isolated from two cream sources. No indication of a definite fermentation pattern could be observed with these cultures. The three cultures of Group II which reduced nitrates also fermented mannitol, sorbitol, xylose, inulin and mannose.

Since cultures of Group I were proteolytic, nine representative cultures were selected and inoculated into skim milk to determine the acid-soluble nitrogen after incubation for 7 days at 35-37° C. Besides the control, one culture of Group II was included. The results are shown in Table 9. All cultures of Group I increased the acid-soluble nitrogen more than did the control and the one culture of the non-proteolytic Group II. Cultures 22 and 44 were the most active,

Table 9. Nitrogen soluble in trichloroacetic acid produced by anaerobes inoculated into sterile skim milk.

Culture	Source of culture	Sol. N in 5 g. milk (as ml. 0.1 N NaOH)
Group I		
5	Milk	4.75
6	Milk	4.90
19	Milk	5.10
22	Milk	5.52
33	Cream	4.50
44	Cream	5.65
61	Cream	3.30
71	Cream	4.55
63	Cheese	4.40
Group II		
51	Cream	2.47
Control, milk sample		2.50

while 61 was the least. The source of the isolate apparently did not influence the results.

Total Developed Acid, Volatile Acid and Lactic Acid Produced by Anaerobes

It has been shown that cultures of Group II produced lactic and acetic acids (Table 7). In order to determine how much lactic acid, volatile acid and total developed acid these cultures produced in skim milk, one per cent inoculum was used and the milk was incubated for 9 days at 35-37° C. The total developed and volatile acids were determined on a 50 g. sample and converted to mmols. per 100 g. milk. The distillation apparatus used for the volatile acids was the same as that described by Ramsey and Patterson (1945). The distillation was carried out as rapidly as possible, collecting the 200 ml. of distillate in approximately 45 minutes. Oleic acid was used as an antifoaming agent. Lactic acid was determined by the method of Barker and Summerson (1941). The results of this experiment are shown in Table 10. Results with only two representative cultures of Group I are shown, since the other cultures tested gave the same results. The other 12 cultures for which data are given belong to Group II.

The total developed acid ranged from 9.9 to 23 mmols. for Group II cultures, and was 1.7 for Group I cultures. Of the total developed acid, cultures of Group II produced

Table 10. Total developed acidity, volatile acidity and lactic acid produced in skim milk by cultures of Group I and II.

Culture no.	Acid found (mmols./100 ml. milk) Total developed acid	Lactic acid	Volatile acid	Volatile acid x 1.75[a]	% accountable
Group I					
20	1.70	--	1.06	1.85	109
32	1.72	--	1.10	1.92	111
Group II					
47	22.02	10.22	5.92	10.36	93
53	10.74	4.44	2.90	5.08	88
78	20.02	9.33	5.76	10.08	97
80	17.00	8.00	4.88	8.54	97
87	20.84	9.77	6.00	10.50	97
91	23.18	10.66	6.62	11.59	96
103	21.24	9.99	5.42	9.49	92
115	15.70	8.22	4.82	8.44	106
120	9.92	4.66	2.44	4.27	90
125	11.44	5.33	3.24	5.67	96
130	21.40	9.99	6.00	10.50	96
135	12.60	6.00	3.74	6.55	99

[a]Correction factor for incomplete recovery of volatile acids.

approximately 46 per cent inactive lactic acid and approximately 30 per cent volatile acid. This accounted for 69-83 per cent of the total acid when the uncorrected data were used. It has been shown that cultures of Group II produced only acetic acid. Ramsey and Patterson (1945) showed that with the apparatus used, only 57±2 per cent of the acetic acid was distilled over. Therefore, the amount of volatile acid was corrected by multiplying by a factor of 1.75 to account for the acetic acid that was not distilled over. If this is accurate, then from 88-106 per cent of the total developed acid are accounted for, with an average of 95 per cent. Because Group I cultures produced propionic acid in addition to the acetic acid, the 1.75 factor probably is not accurate for them, but was included for comparison purposes.

Characteristics of *Bacteroides pseudoramosus* and *Lactobacillus bifidus*

Because Groups I and II were similar to *B. pseudoramosus* and *L. bifidus*, respectively, cultures of these organisms were obtained from the American Type Culture Collection as strains *B. pseudoramosus* No. 8489, and *L. bifidus* No. 4962. The morphological, biochemical and fermentation studies shown in Table 7 were conducted on these cultures.

Under the conditions of the tests, the cells of *B. pseudoramosus* were gram-positive, non-motile, straight, short

rods which occur singly, in pairs or short chains; the cells are approximately 0.5 μ in width and 1.0 μ in length. The culture forms lens-shaped and winged sub-surface colonies and forms circular, translucent, greyish-white, raised to convex, glistening, smooth surface colonies with entire margins. Culture 8489 grew as "clusters" in semi-solid T.S.G. medium. Culture 8489 reduced litmus slowly and coagulated milk at a pH of 5.7, produced a titratable acidity of 0.33 per cent and produced bitter and rancid flavors in milk. Culture 8489 grew well at 30, 35-37 and 45° C. and after a long incubation period grew at 21° C. but not at 10° C. Culture 8489 failed to survive 60° C. for 10 minutes. Propionic and acetic acids were produced and lactates utilized. This culture was catalase-positive, reduced nitrates, produced indole, was proteolytic and slightly lipolytic, was non-pathogenic and hemolyzed human, rabbit, bovine and sheep blood. Culture 8489 did not produce hydrogen sulfide, ammonia or skatole. Culture 8489 fermented fructose, galactose, glucose, mannose, mannitol and sorbitol.

The cells of L. bifidus were gram-positive, non-motile, straight, long rods approximately 0.5 μ in width and 3-5 μ in length, occurring singly, in pairs or long chains. Lens-shaped sub-surface colonies and punctiform, translucent, cream-colored, slightly raised, glistening, smooth and entire-margin surface colonies were characteristic. This

culture grew as "strings" in semi-solid T.S.G. medium, and tended to be microaerophilic. Culture 4962 reduced litmus milk when a heavy inoculum was used, coagulated milk at a pH of 4.6 and produced a titratable acidity of 0.80 per cent. Culture 4962 grew well at 30, 35-37 and 45° C., but not at 10 or 21° C. It survived a temperature of 60° C. for 30 minutes. Culture 4962 was catalase-negative, did not reduce nitrates, did not produce indole or skatole, was non-proteolytic, non-pathogenic, non-hemolytic, produced lactic acid and fermented fructose, galactose, glucose, lactose, maltose and mannose, otherwise being rather inactive biochemically.

Characteristics of Seven Cultures of the Genus Propionibacterium

Seven cultures of the genus Propionibacterium which were obtained from the collection of the Bacteriology Department, Iowa State College, included: P. freudenreichii ATCC 6207, P. shermanii, P. thoenii ATCC 4872, P. zeae ATCC 4964, P. peterssonii ATCC 4870, P. arabinosum ATCC 4965, and P. pentosaceum ATCC 4875. Some of the morphological, biochemical and fermentation tests outlined in Table 7 were determined on these cultures to enlarge the description in Bergey's Manual (Breed et al., 1948) in order that characteristics not already known could be compared with those of Group I cultures.

Morphologically the cells in the cultures of the genus Propionibacterium appeared to be the same as Group I cultures, except for cultures of P. arabinosum and P. pentosaceum, which gave the appearance of involution forms. The fermentation pattern appeared to be very much the same for each culture as shown in Bergey's Manual (Breed et al., 1948). All the cultures utilized lactates, did not produce indole, skatole, ammonia or hydrogen sulfide, were not proteolytic, lipolytic or hemolytic on bovine blood and grew rapidly at 35-37, 30 and 21° C. Contrary to the description in Bergey's Manual, P. shermanii and P. freudenreichii both reduced nitrates under the conditions of the tests; however, none of the other cultures were able to do so. All cultures produced a yellow or cream-colored growth in agar stabs, except P. thoenii which produced a reddish-brown agar stab. P. pentosaceum, P. arabinosum and P. thoenii were catalase-negative or weakly positive. P. shermanii, P. freudenreichii and P. thoenii grew as "strings" in semi-solid T.S.G. medium, while the other cultures grew as modified "clusters". P. shermanii and P. freudenreichii grew well at 45 and 10° C., while P. peterssonii and P. pentosaceum grew at 45° C. but not at 10° C. P. freudenreichii and P. arabinosum failed to coagulate milk; however, the other five cultures coagulated milk, giving a pH of 4.52-4.90. Two cultures survived 60° C. for 30 minutes, one for 20 minutes and two for 10 minutes,

while two cultures did not survive 60° C. for 10 minutes.

Ability of Anaerobes to Grow in Partially Defined Media

In order to determine how nutritionally fastidious these anaerobes were, three media were chosen to study the ability of these anaerobes to grow in a partially defined medium. These media were a modification of Niven's medium (Collins et al., 1950), Delwiche's medium (Delwiche, 1949), and the tryptophan assay medium used by Potter and Nelson (1952). Previous experiments indicated that tryptophan might be needed for the growth of the anaerobes. Because Collins's and Potter's media did not contain tryptophan, it was added at the rate of 0.2 g. per liter to another series of each medium. Delwiche's medium contained tryptophan; therefore, reticulogen[1] was added at the rate of 0.2 ml. per liter to another series of this medium. All media were made as broths. Eleven cultures representative of all four groups were selected for this study. The cells were centrifuged in a high-speed centrifuge and washed four or five times with Potter's medium. The cells were resuspended in the medium and inoculations were made by adding a drop of the culture from a pipette into 6 ml. of each of the three media. The tubes were placed immediately in a desiccator under anaerobic

[1] A commercial liver extract prepared by Eli Lilly and Co., Indianapolis, Ind.

conditions and incubated at 35-37° C. until growth appeared or until a week had elapsed. Most cultures showed growth in 24 hours. No more than four transfers were made. The results are shown in Table 11.

Cultures of Groups I and II and B. pseudoramosus, Culture 8489 grew in both Potter's and Collins's media only when tryptophan was added, and in Delwiche's medium only when reticulogen was added. Groups III and IV grew in both series of all media. Seven cultures of the genus Propionibacterium grew well in Delwiche's medium; however, growth was faster in the medium with the added reticulogen. Groups III and IV grew as a concentrated sediment in the bottom of the test tubes, while Groups I and II grew throughout the media. L. bifidus, Culture 4962 did not grow well in any of the above media.

Effect of Adding Anaerobes to Milk

Milk which had been steamed on three consecutive days was dispensed aseptically in 100 ml. and 1000 ml. quantities, inoculated with 1 per cent inoculum and incubated at 35-37° C. After 3 days incubation the 1000 ml. milk samples had coagulated and developed flavors that could be detected. Group I cultures produced bitter and rancid flavors, while Group II cultures produced a clean acid and a "green culture"

Table 11. Suitability of three partially defined media for growth of anaerobes.

Medium	Number of serial transfers through which the cultures survived (4 transfers maximum)										
	Group I				Group II				Group III		Group IV
	4[a]	18	117	212	53	103	115	130	64	74	184
Potter's	0	0	0	1	2	1	0	0	4	4	4
+ Trypto-phane	4	4	4	4	4	4	4	4	4	4	4
Collins's	0	0	0	2	2	0	0	0	4	4	4
+ Trypto-phane	4	4	4	4	4	4	4	4	4	4	4
Delwiche's	1	0	0	1	0	1	0	0	4	4	4
+ Reticu-logen	4	4	4	4	4	4	4	4	4	4	4

[a]Strain number

flavor. After 9 days the cultures in the 100 ml. samples produced the same flavor defects. Group IV had a putrid, hydrogen sulfide odor and flavor. Group III cultures were rather inactive and did not produce enough flavor to detect.

Effect of Adding Anaerobes in the Manufacture of Cheddar Cheese

Cultures of Groups I, II and III were added in the manufacture of Cheddar cheese in order to determine their effect on proteolysis and flavor development. Four cultures of Group I and one each of Groups II and III were used. The four cultures of Group I represented milk, rumen liquid and cheese sources. The cheese was judged by experienced judges at the end of 1, 3 and 6 months. The flavor scores and criticisms of the cheese at the judging periods are shown in Table 12.

The experimental cheese after curing for one month were either curdy, acid or flat and had a slight fermented flavor; however, the flavor defects were more pronounced in the cheese held for 2 weeks at 15.5° C. (60° F.). None of the cheese were given flavor scores above the controls and only four of the 12 were given scores equal to those of the controls. The remainder of the cheese were scored 0.5-1.0 point lower than the controls which did not contain the test organisms. A few experimental cheese were criticized for

Table 12. Flavor scores and criticisms of Cheddar cheese made from milk inoculated with anaerobes from Groups I, II and III

Vat, group and culture number	Score and criticism after curing at: 10° C.	Score and criticism after curing at: 10, 15.5, 10° C.
	One month curing	
I, Control	39, flat	38.5, acid
II, I-42	38.5, sharp	38.5, curdy
III, I-63	38.5, acid	37.5, whey taint[a]
IV, II-103	38.5, sl. fermented[a]	38.5, sl. fermented
V, Control	39, flat	39, flat
VI, I-18	39, flat	38.5, sl. fermented[a]
VII, I-62	38.5, acid	38, acid
VIII, III-64	39, flat	38, whey taint
	Three months curing	
I, Control	39, acid	39.5, acid
II, I-42	38, acid, bitter[a]	38.5, acid
III, I-63	38, acid, bitter	38.5, acid
IV, II-103	39, sl. bitter	39.5, flat
V, Control	39.5, more flavor	39.5
VI, I-18	39, whey taint	39
VII, I-62	38, bitter, musty[a]	38.5, acid
VIII, III-64	38.5, sl. fermented[a]	38, sl. fermented
	Six months curing	
I, Control	39.5, cheddar, flavor[a]	39, acid, sl. bitter
II, I-42	38, acid[a]	38.5, sl. acid
III, I-63	39, sl. acid	37.5, acid, mealy[a]
IV, II-103	39.5,	38, acid, sl. fermented
V, Control	40	38, acid, bitter[a]
VI, I-18	39	38.5, acid
VII, I-62	39,	38.5, acid
VIII, III-64	38, acid, bitter	38.5, acid, bitter[a]

[a]Indicates cheese was criticized for having a weak body. Other cheese was not criticized for body defects.

having a weak body. After curing for 3 months, the cheese held at 10° C. (50° F.) were scored somewhat lower than the cheese held for 2 weeks at 15.5° C. An acid, bitter, or slightly fermented flavor began to be detected by the judges in the cheese which contained cultures of Groups I and III. Only cheese from Vat IV, containing the culture of Group II, were given scores equal to the control. The experimental cheese were not criticized for the fermented flavor after curing for 6 months, as was the case after curing for 1 and 3 months. The flavor defects most often found by the judges were acid or slightly bitter. The cheese cured at 10° C. generally were scored higher than the cheese cured for 2 weeks at 15.5° C. The cheese from Vats VI, VII and VIII scored 0.5 point higher than the control when cured for 2 weeks at 15.5° C., but Vats VI and VII scored lower than the duplicate cheese cured at 10° C. In no instance did the experimental cheese give indication of having a flavor which was considered more characteristic than the controls; however, none of the anaerobic cultures added to the milk in the manufacturing process caused any abnormal fermentations in the cheese.

DISCUSSION

Strains of Group I agreed very closely in characteristics to Bacteroides pseudoramosus No. 8489 received from the American Type Culture Collection. This organism had been isolated from the human intestinal tract and described by Eggerth (1935). Due to limited descriptions published by other workers previous to Eggerth's, comparison with their organisms is difficult to make. However, presumably Group I cultures also are related closely to Bacillus pseudoramosus described by Distaso (1912), Bacteroides pseudoramosus described by Castellani and Chalmers (1919), and Ramibacterium pseudoramosum described by Prévot (1938). Morphological and fermentation tests of Group I cultures and Culture 8489 agree well. Culture 8489 grew at 45° C., but not at 10° C., whereas the majority of Group I cultures did not grow at either temperature. Under the conditions of study, Culture 8489 survived 60° C. for 10 minutes, while cultures of Group I generally did not survive this treatment. The biochemical features of Culture 8489 were the same as those of Group I, except that Culture 8489 was only slightly lipolytic and only 30 of 115 cultures of Group I were lipolytic. However, both Group I cultures and Culture 8489 produced rancid and bitter flavor defects in milk. Cultures of Group

I tended to form winged sub-surface colonies more than Culture 8489; however, the differences in the source of isolation, the relatively longer period of maintenance, and medium of maintenance may account for this. Cultures of Group I also resemble a number of anaerobes isolated from dairy products by Wilkowske (1948) who obtained seven cultures from rumen liquid, seven cultures from cream and 12 cultures from milk. Because of similar characteristics, such as production of propionic and acetic acids, utilization of lactates, proteolysis, production of indole, reduction of nitrates, catalase activity, growth temperature range and failure to survive 60° C. for 10 minutes, they were considered identical or very closely related. Wilkowske's cultures usually fermented only galactose and glucose and not mannitol. Mannose, fructose or sorbitol were not tested; thus, these can not be compared as his cultures were not available for retesting or for checking other qualities. Some of Wilkowske's cultures from milk and rumen liquid were lipolytic and many were proteolytic. Cultures from cream did not produce indole and results of tests for nitrate reduction and proteolysis were questionable; thus, they could be intermediates between Groups I and III. Because of the tendency for the anaerobes to grow slowly or not at all when small inocula were used, these characteristics are difficult to interpret and Wilkowske's cultures could be considered associated with Group I cultures. Group I cultures differ

from the few characteristics described by Distaso (1912) for Bacillus pseudoramosus and Castellani and Chalmers (1919) for Bacteroides pseudoramosus by not fermenting lactose and sucrose and by generally growing at 21° C. Some of the description given by Castellani and Chalmers may have been taken in part from the work of Distaso. King and Rettger (1942), working with the gram-positive non-sporulating anaerobes from the human intestinal tract, isolated two cultures that were catalase-positive and produced large quantities of volatile acids, chiefly propionic. These cultures were not available from the authors but may be similar to Group I cultures.

Bacteroides pseudoramosus has not been given a place in the sixth edition of Bergey's Manual (Breed et al., 1948); however, appendix III includes Prévot's (1938) classification of the genus Ramibacterium under the genus Lactobacillus. Therefore Group I cultures, which have been shown to resemble Bacteroides pseudoramosus, have no established taxonomic position. Eggerth (1935) concluded that the gram-positive species differed sufficiently from the gram-negative species of Bacteroides to justify classifying them either in the genus Lactobacillus or in a separate genus. After studying 10 of Eggerth's 12 cultures, Pederson (1945) found that some cultures definitely belonged to the genus Lactobacillus and some to the genus Butyribacterium. His studies, contrary to

the findings reported here, showed that B. pseudoramosus produced considerable dextro rotatory lactic acid and little volatile acids. He assumed that acetic was the only volatile acid formed. He regarded B. pseudoramosus as a form intermediate between the genus Lactobacillus and the proteolytic and parasitic types of non-sporulating anaerobes.

After reading the opinions of these workers, it is evident that cultures of Group I, similar to Culture 8489 and B. pseudoramosus, do not belong in the gram-negative genus Bacteroides, nor should they be placed in the genus Lactobacillus. They should not be placed in the latter genus because they do not produce lactic acid, actually utilizing lactate, do not form an acid coagulum in milk, are proteolytic, are catalase-positive and are morphologically distinct. Group I cultures have some characteristics similar to those of the genus Propionibacterium, and may be considered as being closely related to this genus. These characteristics include production of propionic and acetic acid, utilization of lactate, catalase activity, similar morphology, growth temperature range and lack of thermal resistance. They could be placed in this genus without changing the generic description given in the sixth edition of Bergey's Manual (Breed et al., 1948). However, none of the 11 species now listed in the genus Propionibacterium show any appreciable similarity to the members of Group I. These conclusions

were strengthened by the comparative tests on the seven species of the genus Propionibacterium checked in this study. If cultures of Group I were placed in the genus Propionibacterium, the genus would have to be expanded to include cultures which reduce nitrates, produce indole, are proteolytic, lipolytic and hemolytic, produce a small amount of titratable acidity in milk, coagulate milk without acid, do not grow well at 21° C., do not survive 60° C. for 10 minutes and do not grow in Delwiche's medium unless modified by adding reticulogen. The other alternative would be to place Group I cultures in a separate genus in the family Lactobacteriaceae, one closely related to the genus Propionibacterium. It appears that the two groups have enough basic differences to warrant the latter action. The characteristics that could be used to separate the cultures of Group I from the genus Propionibacterium would be: proteolysis, lipolysis, hemolysis, reduction of nitrates, production of indole, production of a low titratable acidity in milk, more anaerobic character, slower growth at 21° C. and more fastidious nutritional requirements. Cultures of Group I do show a homogeneity even though the 115 cultures had different sources and had been isolated on several occasions from different samples. This homogeneity seems to justify the establishment of a separate genus. The genus name Ramibacterium as used by Prévot (1938) is suggested here.

Thus, the name most suitable for the cultures of Group I would be Ramibacterium pseudoramosum (Prévot). The taxonomic position for this genus should be close to the genus Propionibacterium in the family Lactobacteriaceae and not under the genus Lactobacillus.

The characteristics of Group II cultures would seemingly place it in close association with the genus Lactobacillus. These organisms produce acetic acid and inactive lactic acid, are catalase-negative, non-proteolytic, do not reduce nitrates, do not produce indole or skatole, and produce 1.5-1.9 per cent titratable acidity in skim milk. However, the cells appear to be somewhat smaller than those commonly associated with the genus Lactobacillus. Cultures of Group II were stimulated by the addition of Tween 80 to the medium. This compound has been shown to be a growth stimulant for members of the genus Lactobacillus (Williams and Fieger, 1946; Hutchings and Boggiano, 1947). Group II cultures do resemble in some respects the known strains of L. bifidus, L. acidophilus and L. bifidus types I and II (Weiss and Rettger, 1938). These organisms probably are more closely related to types I and II L. bifidus than to the other species mentioned. Group II organisms differ from L. bifidus in that the cells are somewhat smaller, with no branching or club-shape forms; more acid is produced in milk; growth occurs at 21 and 10° C.; the cultures do not become

microaerophilic; and arabinose, cellobiose, maltose, mannose and sucrose are fermented, but not mannitol, sorbitol and xylose. Group II cultures resembled L. bifidus type II, but did not ferment xylose and produced inactive lactic acid. Unlike either types I and II, Group II cultures produce approximately 50 per cent of the total acid as volatile acid, chiefly acetic and some formic and approximately 45 per cent as inactive lactic acid. The L. bifidus Culture 4962 obtained from the American Type Culture Collection was not related closely to cultures of Group II. Cultures of L. bifidus types I and II were not available from Rettger. The ability of Group II cultures to ferment a large variety of sugars is similar to that of some of the bacteria isolated from the rumen. However, nowhere in the literature could reference be found to organisms apparently closely related to these of Group II. Sijpesteijn (1948, 1951) gave an incomplete description of Ruminococcus flavefaciens, whose cells resemble those of Group II. R. flavefaciens cells were smaller and produced a yellow pigment; the cultures produced butyric acid and small amounts of lactic acid, contrary to Group II cultures. In some respects Group II cultures resemble the A-1 type of Lewis and Rettger (1940), except that Group II cultures coagulate milk regularly, do not grow well at 45° C. and grow at 10° C. Most cultures of Group II survived 60° C. for 10

minutes, while only a few cultures survived 60° C. for 30 minutes. The fermentation pattern of Group II cultures did not fit closely to the A-1 type. Some strains isolated from rumen liquid and milk by Wilkowske (1948) have many similarities to members of Group II, and probably could be considered to be closely related. However, these cultures of Wilkowske's were not available for further testing. If the cultures of Group II are placed into the present scheme of the genus Lactobacillus, probably the taxonomic position would be in the group with L. bifidus, where they would be separated from L. helveticus and L. acidophilus by being "anaerobic in freshly isolated cultures ". They then could be separated from L. bifidus by morphology, acid production and on fermentation characteristics. It therefore is proposed that the cultures of Group II be named Lactobacillus anaerobius.

Group III cultures resembled Group I cultures except that they did not coagulate milk or reduce litmus, survived 60° C. for 30 minutes, did not produce indole, were not proteolytic, lipolytic or hemolytic and fermented arabinose, maltose and salicin, did not form winged sub-surface colonies and grew as "strings" in semi-solid T.S.G. medium. They resembled Group I cultures in the acidity developed in milk, growth temperature range, production of propionic and acetic acids, utilization of lactates, catalase activity and reduction of nitrates. Therefore, this group could be placed as a second

species under the genus Ramibacterium (Prévot). This species might be designated as R. lacticum.

The one culture of Group IV (No. 184) did not resemble any of the other three groups of this study, although it had some characteristics in common with Groups I and II (Table 7). Culture 184 is closely related to Bacteroides siccus isolated and described by Eggerth and Gagnon (1933) and reclassified by Prevot (1938) into the genus Spherophorus. Culture 184 differs from B. siccus in that it is gram-positive, reduces nitrates and does not produce acid from fructose; however, they are similar in morphology, in their failure to produce indole, to coagulate milk, to ferment arabinose, cellobiose, glucose, lactose, mannitol, sorbitol, salicin and xylose and are non-proteolytic and non-pathogenic. A culture of B. siccus was not available for comparison purposes. Neither genus, Bacteroides or Spherophorus, would provide a satisfactory position for Culture 184 since the two genera are restricted to gram-negative organisms. Further isolations should be made to determine whether additional organisms having the same characteristics as Groups III and IV may be found and to determine the probable limits of variation within these types.

Although several media could have been used for the isolation of the non-sporulating anaerobes, the T.S.G. medium proved satisfactory. No doubt a medium might be

developed which would encourage the growth of anaerobic cultures that have not been found in this study. This possibility should be explored further, as the diversity of types encountered suggests that other non-sporulating anaerobic bacteria might be found in dairy products.

Vaspar, 3 per cent agar, and other material that will give absolute anaerobiosis does not need to be used for routine maintenance of anaerobic cultures. If the medium contains a reducing agent in sufficient quantities, anaerobic growth will be initiated without the above sealing materials. Probably the best medium for routine maintenance of the anaerobes is the semi-solid type medium used in this study. In many instances, particularly in Groups II and IV, cultures would grow faster in the semi-solid medium than in the solid medium. When the semi-solid medium is used, any aerobic contamination or a change of the anaerobic tendency toward being microaerophilic can be observed. Throughout this study, the "aerobic zone", the portion of the medium above the growth and under the meniscus, varied with each culture as the temperature of incubation, the amount of reducing agent in the medium and the amount of inoculum or number of cells present changed. When observed just as growth was evident, the aerobic zone was the widest, but as growth continued, the zone narrowed. This phenomenon probably was due to the culture adjusting the conditions in

this aerobic zone, so that growth was extended. At no time could the aerobic zone width be used as a means of separating cultures for sub-grouping.

V-8 Juice and rumen liquid had been added to media by other workers to stimulate the growth of organisms, and their use for the dairy anaerobes has been shown to be effective. The V-8 Juice was favored because it was readily available and probably would be less subject to change than the rumen liquid. Rumen liquid might be used in future studies, as its effect could be due to a different factor which was not contained in the V-8 Juice. The slight stimulatory effect noted with the cell-free extract of S. lactis on the dairy anaerobes may be similar to the effect noted on rumen anaerobes by Clostridium sporogenes (Sijpesteijn, 1951).

The dairy anaerobes generally were stimulated by the addition of Tween 80 to the medium as has been shown to be the case for members of the genus Lactobacillus (Williams and Fieger, 1946). A stimulatory effect was noted with sodium lactate, but not with sodium acetate, propionate or butyrate. Cultures of Groups I, III and IV, which utilize lactate were stimulated by sodium lactate, while members of Group II which do not utilize lactate, probably were not stimulated. Combinations of pyruvic acid, lactate and acetate added to Tween 80 did not increase the stimulation provided by Tween 80 alone. Pyruvic acid did not stimulate the growth of dairy

anaerobes when added to the medium, as was the case with the intestinal anaerobes (West et al., 1942). The intestinal anaerobes studied by West et al. were the A-1, A-2 and A-3 types as described by Lewis and Rettger (1940) and these have been shown to differ from the anaerobes isolated in this study.

The kind of reducing agent used in the medium appears to be of minor importance with the anaerobes; however, the amount needed by the anaerobes depends on the particular reducing agent used. As has been shown by Vennesland (1939), slow growth was encountered if too much reducing agent was added. The amount of cysteine hydrochloride might be increased up to 0.5 per cent in the T.S.G. medium; further studies need to be carried out to determine the ideal Eh for the anaerobes to initiate growth and maintenance of the cultures. The results indicate that a medium reaction of pH 6.4-6.8 is more favorable than the higher pH's used by those working with rumen and intestinal anaerobes. The dairy anaerobes have been isolated from products with a slightly acid reaction and thus their optimum pH might be different than that for those anaerobes isolated from the intestinal tract. Sijpesteijn (1948) has shown that the rumen anaerobes also grew best at the slightly acid pH. The optimum growth temperature seems to be 35-37° C. for the dairy anaerobes; however, either a higher or a lower temperature may be a

means of encouraging growth of other anaerobes which would not grow at the 37° C. incubation temperature.

Before a quantitative isolation procedure for obligate anaerobes is worked out, the development of the large population of facultative anaerobes usually found in dairy products must be selectively prevented or distinctive characteristics of the obligate anaerobes must be found that will be apparent during the course of the isolation procedure. Several procedures were tried during the course of the study, but none entirely solved the problems. If a procedure could be found, then milk, cream and other dairy products could be plated out directly and the total number of obligate anaerobes in the product could be determined. Of the three chemical agents tested, only sodium azide was effective in eliminating the facultative types before acting on the anaerobes. However, the difference between effective and inhibitory concentration (2×10^{-3} and 4×10^{-3}) was not great enough to warrant further testing. Other bacteriostatic agents might prove to be more effective. Two of the four antibiotics tested, aureomycin and terramycin, were quite successful in inhibiting the facultative types at 0.00050 mg. per ml. of medium, but not the obligate anaerobes until 0.00100 mg. levels. It was believed, in a few instances, that some anaerobes were not isolated when the antibiotics were used; however, they probably offer some possibilities

for use in developing a procedure to eliminate the facultative types. Antibiotics such as diplococcin and other new antibiotics produced by the lactic streptococci as described by Hirsch and Gunsted (1951) definitely should be tested. A cyanide-sensitive system which could be used on the facultative anaerobes for an isolation procedure for the obligate anaerobes was not found. The possible use of Nile blue sulfate or other oxidation-reduction indicators listed by Hewitt (1950) as a means to select anaerobic colonies should be studied further.

Anaerobes were found in 63 per cent of the total 67 samples, as compared to human stools where anaerobes were found in approximately 90 per cent of the samples. (Eggerth and Gagnon, 1933, and Weiss and Rettger, 1937) Of the above samples, the lowest percentage of cultures isolated and found to be anaerobes was from the cream samples, while the highest was from the milk samples. Non-sporulating anaerobes apparently are present in the majority of dairy products, but they seem to constitute only a relatively small proportion of the total bacterial population. Approximately 6 per cent of the total 4,202 colonies picked were anaerobes. The remainder were either facultative or failed to initiate growth after picking. This figure is quite low compared with 21 and 40 per cent from rat and human feces, respectively, as found by Lewis and Rettger (1940). The low percentage of isolates

from colonies picked can be attributed in part to the fact that in the early phases of this study all colonies were picked, while in later phases, the appearance of anaerobic colonies aided selection of anaerobes. Therefore, experience possibly would increase efficiency. Some procedure should be developed to improve the efficiency of isolating dairy anaerobes.

After giving the deep-agar shake tubes a fair trial, the plate method was considered the best procedure for the isolation of anaerobes if the plates were poured in a deep-agar layer, and a top layer poured after the first layer had hardened. Probably a more effective method would be to find a deeper petri plate with a smaller diameter. In this way, the advantage of the shake cultures, and the relative ease of picking from the plates would be available. It would also provide a means to pour a thin bottom and top layer, and to have sufficient agar to prevent drying when incubated over a longer period of time. The period of incubation could be extended from 7-10 days to 14-20 days. Further experiments should be conducted to determine if other types of anaerobes could be isolated by using this method.

There seems to be no advantage in duplicate platings of the samples, with both anaerobic and aerobic incubations. In these experiments only a few of the milk samples showed higher anaerobic counts, while Wilkowske (1948) found only eight of

52 samples to have higher anaerobic counts. Perhaps duplicate platings may be useful to determine the efficiency of a new medium, or the addition of some chemical agent or antibiotic.

Eggerth (1935) indicated that he frequently found B. pseudoramosus to be present in the laboratory air; therefore the source of the organisms in the milk supply might be dust, hay or feed. Group I cultures were also found in the rumen liquid; thus, this might be also a source, as no doubt these organisms could be found in the mouth of the bovine and possibly in the fecal material, as it has been found in the human intestinal and respiratory tracts. However, these sources were not tested in this study. A study should be made to determine the possible sources of the anaerobes in dairy products.

These organisms probably could not be found in pasteurized products unless they entered as post-pasteurization contamination. After entering the product, the chances are that the product would be consumed before the anaerobes appeared in the numbers required to cause the flavor defects. Anaerobes were found quite easily in pasteurized milk Cheddar cheese 6 or 7 months old; however, no other pasteurized dairy products were examined. The incidence in pasteurized milk products presumably would be very small; however, this assumption should be verified.

Only one concentration of anaerobic starters was added to the cheese made in this study and other amounts and strains might give different results. The one culture of Group II seemed to give a desirable flavor, whereas cultures of Group I and III produced a flavor which is undesirable, although not objectionable. A combination of Group I and II cultures might be more useful in future studies.

SUMMARY AND CONCLUSIONS

1. The important physiological characteristics of the gram-positive, non-motile, non-sporulating anaerobic rods and their occurrence in rumen liquid, cream, milk and cheese have been studied.

2. Although the non-sporulating anaerobes accounted for a small percentage of the total flora of the samples, they were isolated from approximately 63 per cent of the samples.

3. Of 4,202 colonies picked from 59 raw products and 8 pasteurized products, 239 were found to be non-sporulating anaerobes. The remainder of the picked colonies were either facultative anaerobes or failed to initiate growth. Morphological, biochemical and fermentation studies were conducted on 207 isolated anaerobes and these organisms were divided into four groups. Group I consisted of 115 isolates from milk, cream, cheese, and rumen liquid. Group II consisted of 88 isolates from milk and cream. Group III consisted of three isolates from cream, and Group IV of one isolate from milk.

4. Cultures of Group I were related closely to Bacteroides pseudoramosus, as described by Eggerth (1935). They differ only in a few minor characteristics. B. pseudoramosus, ATCC 8489, and cultures of Group I failed to produce

hydrogen sulfide and lactic acid as had been reported in the literature. Because the genus Bacteroides has been reserved for only gram-negative strains, the taxonomic position of the isolated dairy anaerobes is not defined. On the basis of the studies presented, it has been proposed that cultures of Group I be placed in the genus Ramibacterium (Prévot), which would be included in the family Lactobacteriaceae and placed close to the genus Propionibacterium. The three cultures of Group III were considered under this genus and designated R. lacticum.

5. Cultures of Group II closely resembled members of the genus Lactobacillus in many ways; however, they did not closely resemble any one of the known species, but had some characteristics in common with L. bifidus types I and II of Weiss and Rettger (1938). Group II cultures differ from these types by being able to produce more acid in milk, growing at 21 and 10° C., not becoming microaerophilic, not forming branching or club-shaped forms, and fermenting arabinose, cellobiose, maltose, mannose and sucrose, but not mannitol, sorbitol and xylose. They produce approximately 50 per cent of the total acid as volatile acid, chiefly acetic and some formic acid and approximately 45 per cent as inactive lactic acid. Because it was believed that cultures of this group should be placed in the genus Lactobacillus, a new

species name is proposed, L. anaerobius.

6. The T.S.G. medium used throughout the study seemed to be favorable in the isolation of non-sporulating anaerobes from dairy products. The use of Tween 80 and V-8 Juice gave added stimulus to the dairy anaerobes. The medium was adjusted to pH 6.3-6.5 by the phosphate buffer and cysteine hydrochloride was the reducing agent used.

7. Two antibiotics, aureomycin and terramycin, at a concentration of 0.00050 mg. per ml. were found to be effective in inhibiting some facultative anaerobes, while allowing the obligate anaerobes to grow. This addition proved successful in some anaerobic isolations. The use of three bacteriostatic agents was not too effective in inhibiting facultative anaerobes.

8. The best procedure found in isolation of the non-sporulating anaerobes from dairy products was to plate the samples after enrichment at room temperature using deep-agar plates with an overlay. Plates were incubated under anaerobic conditions for periods of 7-10 days.

9. Group I cultures caused a bitter and rancid flavor in milk, when incubated 3-9 days at 37° C. Group II cultures caused an acid flavor which was not objectionable. Group III cultures did not produce any flavor in milk, while the Group IV culture produced a hydrogen sulfide odor and flavor, which was very objectionable and seemed to be detected earlier than

the other flavors. The chances that any of these abnormal flavors would develop in pasteurized products would be very slight, because the anaerobes have a low thermal resistance. If the milk was held for long periods of time at a favorable temperature, and if these organisms entered after pasteurization, the defect probably would appear. Probably other organisms would outgrow the slower growing anaerobes.

10. The possible use of Group I and II cultures in the breakdown of the body of pasteurized milk Cheddar cheese should be investigated further, although they do produce a slight fermented or bitter flavor. One culture of Group II did not give any objectionable flavor in the cheese and this group might be considered for use in Cheddar cheese.

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