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Nonsporing Anaerobic Bacteria in Foods

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NONSPORING ANAEROBIC BACTERIA IN FOODS

BY

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This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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INTRODUCTION

Anaerobic bacteria, with the exception of members of the spore forming genus Clostridium, are not detected in routine food examinations, and their occurrence in foods has seldom been reported in the literature. The purpose of this study is to determine first, the presence of anaerobes in processed foods and secondly, whether under certain conditions of food processing or storage, anaerobic multiplication could occur at rates high enough to produce spoilage or cause food borne illness.

Anaerobes are part of the normal microflora of the body of man and other animals. They are found on the skin, in the oral cavities, respiratory, intestinal and urogenital tracts. Anaerobes are also found in the soil, marine and fresh water sediments. Their requirements for anaerobiosis vary from organisms that are able to grow, though just barely, on the surface of solid media exposed to air to organisms that are unable to grow in the presence of .03 percent oxygen.

Most anaerobes are opportunistic pathogens. Upon obtaining access to tissue with an impaired blood supply or tissue that has been rendered necrotic, they can grow and cause sufficient tissue destruction to cause pathological conditions. All organs or tissues of the body may be involved in anaerobic infection. Genera frequently encoun-

tered in significant infections include Actinomyces, Arachnia, Bacteroides, Bifidobacterium, Clostridium, Eubacterium, Fusobacterium, Peptococcus, Peptostreptococcus, Propionibacterium, Treponema, and Veillonella (1)(11)(23).

The responsible pathogen has not been identified in approximately half of the foodborne disease outbreaks reported to the Center for Disease Control in the last five years. (5) Conventional food microbiology examinations detect the presence of aerobes, facultative anaerobes, and two spore forming obligate anaerobes, Clostridium perfringens and Clostridium botulinum. Whether food infections may be caused by other species of anaerobes following improper handling, processing or storage of foods that would provide suitable growth temperature, pH, medium requirements, O-R potential, and adequate incubation time for the multiplication of anaerobes in numbers sufficient to cause illness has yet to be determined.

LITERATURE REVIEW

Anaerobic microflora of foods

A bacteriological survey of 150 units of raw ground beef (13) yielded the presence of Clostridium perfringens in 9.3%, Peptostreptococcus and Clostridium cochlearium in 5.3%, Clostridium difficile in 4%, Bacteroides and Peptococcus in 2.7%, Clostridium bifermentans in 2%, and Clostridium butyricum, Clostridium capitovale, Clostridium chauvoei, Clostridium fallax, Clostridium septicum, Clostridium sordelli, Clostridium tetanomorphum, and Veillonella alcalescens in percentages ranging from 0.6 to 1.4. Isolates from the genera Bacteroides, Peptostreptococcus, and Peptococcus were not identified as to species.

Clostridium isolates have been recovered from meat during slaughter and processing in low numbers ranging from less than one spore per gram to two or three spores per gram of meat (28). The three most commonly occurring anaerobic spore formers in milk are C. sporogenes, C. perfringens, and C. butyricum. Clostridia identified as spoilage organisms of meats and sea foods include C. sporogenes, C. bifermentans, C. muscosum, C. parabifermentans, C. septicum, C. paraputrificum, and C. putrefaciens (8), (28).

Spoilage of cheese has been caused by C. sporogenes, C. lentoputrescens, C. pasteurianum, C. butyricum, C. tyrobutyricum.

Clostridium species that have been implicated in the spoilage of syrups, candies, canned fruits, tomato juice, olives and grape juice include C. butyricum, C. butylicum, C. acetobutylicum, C. beijerinckii, C. saccharobutyricum, C. multifermentans, C. pasteuranum, C. tyrobutyricum, C. sporogenes, C. bifermentans, and C. fallax. Thermophillic anaerobes responsible for spoilage of non-acid canned foods include C. thermosaccharolylicum, C. thermoaceticum, and Desulfomaculum nigrificans (8)(28).

The occurrence of C. botulinum on raw meats, fish, poultry, vegetables, milk, and cream and its subsequent toxin production following improper food processing has been thoroughly documented.

Other anaerobic or microacrophilic organisms found as part of the normal microflora of foods include the genera Propionibacterium, Lactobacillus, and Pediococcus, some species of which contribute to food spoilage and other species of which are used in food fermentations.

Food contamination

Any methods of food processing including slaughtering, slicing, mashing, grinding, milling, heating, freezing, and packaging may alter a food's ability to support microbial growth, expose a food to contamination, and alter the microbial flora. Cross contamination of foods can occur as contaminated and non-contaminated foods are processed with

the same equipment and personnel. Certain processing procedures may add to the load of contaminants.

Water scalding of poultry can result in contamination of edible parts of carcasses by contaminates from the respiratory system entering the circulatory system via the scald water (17). C. perfringens, normally not found in poultry hearts and livers, was isolated from 18% of the hearts and 36% of the livers of poultry which were water scalded in a commercial poultry processing plant. In 1971, Lillard isolated C. perfringens from 2.6% of commercially precooked broiler parts (6). C. botulinum was found in 9% of 1400 farmed trout examined in Great Britain, introduced in particulate matter suspended in the water supply of trout farms (4).

In an analysis of 150 raw ground beef samples (10) the overall mean count of coliforms was 4.9×10^3 per gram. The mean fecal streptococcus count was 1.8×10^3 per gram. The incidence of these organisms from fecal matter introduced into foods suggests that the contamination of foods with anaerobes which are present in fecal matter in counts of up to 10^{11} per gram may occur. Human fecal flora, investigated by Moore and Holdeman in 1974, yielded a mean count of 50.6×10^{10} organisms per gram (18). Two thirds of the isolates were from ten species of anaerobic bacteria. The most common isolates were Bacteroides vulgatus and Bacteroides distasonis. Anaerobic viable bacteria counts of 2.9×10^{11}

per gram dry weight were obtained in another study (1). In addition to the intestinal tract of man and other animals, anaerobes are also found in the mouth, conjunctival sac, the male urethra, the vagina, and the skin within the openings of the sebaceous glands.

Oxidation-reduction potential and oxygen exposure

A positive oxidation reduction potential does not appear to be the limiting factor in growth of certain anaerobic bacteria (27). The sensitivity of three strains of anaerobic intestinal bacteria freshly isolated from human feces to the differential effects of oxygen and adverse oxidation-reductional potential was measured. The presence of oxygen inhibited the multiplication of Clostridium perfringens, Bacteroides fragilis, and Peptococcus magnus at Eh-50 as well as Eh near 500 mV. In the absence of oxygen, no inhibition was observed at Eh + 325 mV. When grown in an aerated culture medium, 50% of P. magnus cells survived for one hour, 50% B. fragilis organisms were viable after three hours and 50% C. perfringens vegetative cells following five hours. (No C. perfringens spores were observed by staining.) No viable cells were detected after two hours for P. magnus, five hours for B. fragilis, and ten hours for C. perfringens.

Many food items could provide ideal conditions for anaerobic multiplication. After cooking, exposure to oxy-

gen may be limited only to the surface of the food. Also, the presence of facultative microorganisms may reduce the oxygen potential to the point where anaerobic multiplication can occur.

Temperature of foods

Studies of a mixed inoculum of two strains of Clostridium perfringens during heat processing of chicken parts, frozen storage, and subsequent flash frying showed a difference in heat resistance in spores between the two strains (14). One strain was completely eliminated, while the second showed a 10% spore survival rate.

Another study (6) showed that temperatures reached when cooking broiler parts was high enough to destroy surface contaminants but not high enough to kill C. perfringens, which had been disseminated through the circulatory system following scalding.

Repair of spores injured by ultra high temperatures (105°C for 8.5 minutes) occurred in meat broth and milk (2).

A recent study confirmed that microwave heating was less effective for microbial destruction than conventional heating when the same internal end point temperatures were used (7).

Large food items such as beef roasts or turkeys, and products prepared in large bulk containers, require a long time to cool even when placed under proper refrigeration.

Five hours are required to reduce the temperature of a four pound roast from 65° to 2°C, eight hours for a 6¾ lb. roast (28). After cooking, exposure to oxygen is limited to the surface only, leaving subsurface portions free of oxygen and with a reduced Eh. Cooking temperatures destroy vegetative cells of most microorganisms, including C. perfringens. Although cooking temperatures are insufficient for the destruction of C. perfringens spores they are sufficient to heat shock spores thereby stimulating their germination. After cooking, as the temperature declines the freshly germinated cells are able to multiply rapidly.

Those findings underscore the well-known fact that proper handling during preparation, distribution, and marketing is the effective way of preventing Clostridium perfringens food infections rather than cooking at a time and temperature combination sufficient to ensure the destruction of all spores.

Many foods are consumed in a rare state in which the heat used in preparation is not sufficient to kill the vegetative cells in the interior of the foods.

Freezing

The composition of foods in which bacteria are contained can increase or decrease the resistance of bacterial cells to freezing damage (24). Increased resistance is provided by viscous foods and by food components such as

proteins, simple and complex carbohydrates and triglycerides. Reduced resistance is found in the presence of certain ions, inorganic salts, acids, surface active components and certain enzymes.

The ability of C. perfringens to survive freezing is strain dependent (14). The presence of oxygen in the medium during freezing, storage, and thawing adversely affects viability. Cooling rates did not affect survival. The addition of glycerol increased survival significantly. Mean survival rate has been reported at 17% (14) and 12% (22) for C. perfringens. Cells present in a frozen product are probably in a state of sublethal cell injury, with enhanced sensitivity to selective agents. Death of microorganisms upon freezing is attributed to alteration of the cell membrane lipids during cooling, increased solute concentrations of intra- and extra-cellular liquid when frozen, and damage to cell permeability barriers during freezing and thawing. (22).

A review of the effects of freezing and storage on microorganisms in frozen foods suggest that the numbers of viable organisms markedly exceed those detected by conventional methods in food microbiology. Many organisms considered to be killed are only injured, and the viability can be determined by repair in a non-selective medium before conventional selective media are used to determine their presence.

Freeze injured organisms are more sensitive to selective media. Similar consideration should be given when evaluating the microbiological quality of semi-preserved products because conditions in such foods may produce sublethal injury to the microbial population present (22).

Susceptibility and resistance of anaerobes to antibiotics

The effect of antibiotics on the growth of anaerobes was studied in an effort to devise a selective medium that would inhibit the growth of facultative anaerobes and thus promote the isolation of obligate anaerobes from foods.

Bactericidal activity of five antimicrobial agents against 19 strains of Bacteroides fragilis were tested by Nastro and Finegold (20). These agents included rifampin, 7-chlorolincomycin, vancomycin, metronidazale and tetracycline. All 19 strains of Bacteroides fragilis were found to be resistant to vancomycin and eight were resistant to tetracycline. Forty clinical isolates of Bacteroides fragilis were tested against 24 antibiotics by Kislak (16). He reported that more than half of the strains tested were resistant to tetracycline, and that all were resistant to aminoglycosides, polymyxins and semi-synthetic penicillinase-resistant penicillins. Finegold and Sutter (12)(25)(26) found kanamycin the least active of five aminoglycosides when tested with 30 strains of Bacteroides fragilis and Bacteroides melaninogenicus and the most active against

Fusobacterium. The use of kanamycin and neomycin with or without vancomycin had been previously recommended for use in selective media for isolating gram negative and other anaerobes from mixed cultures (9). Kanamycin was preferable for isolating strains of Bacteroides and neomycin for isolation of Fusobacterium and Sphaerophorus. The susceptibility of anaerobic bacteria to carbenicillin, cefoxitin and related drugs was determined by Sutter and Finegold (25). Their findings showed that most of the strains tested were susceptible to penicillin G at clinically achievable blood levels with the exception of a majority of strains of B. fragilis strains. Ampicillin was found to be as active as penicillin G. Other semi-synthetic penicillins were found to be less active. Carbenicillin and cefoxitin were active against the majority of strains tested.

Combinations of antimicrobial agents against Bacteroides fragilis were tested by Busch, Sutter, and Finegold (3). A synergistic effect was noted with the combination of clindamycin and metromidazale. No antagonism was noted with any of the strains tested.

Rodriguez, Prieto, Sanchez, Gonzalez and Luengo (21) reported that Bacteroides strains were resistant to 500 ug/ml of phosphomycin, whereas Fusobacterium were sensitive of 62 ug/ml. Sensitivity testing was described as a rapid means of distinguishing Fusobacterium from Bacteroides spp.

Research objectives

Anaerobic non spore forming bacteria are not detected in routine food examinations, and their occurrences in foods has seldom been reported in the literature. The purpose of this study is to determine the occurrence of non-spore forming anaerobes, the identification of the isolates found, and the possible growth and multiplication in foods which might contribute to spoilage or food illness.

MATERIALS AND METHODS

Source of foods

Foods used in this study were purchased from commercial grocery stores. These foods included packaged refrigerated meats, fish, and meat spreads, and frozen precooked meats and fish.

Equipment and supplies

Culture methods included the use of pre-reduced media with an oxidiation-reduction potential of less than minus 150 millivolts. Culture transfers were made using a three place swing cannula, and glass Pasteur pipettes or stainless steel or platinum inoculating loops. Commerical CO_2 gas passed through copper heated to 300°C in a thermostatically controlled oven was used as a source of oxygen-free gas. The copper, when oxidized, was reduced by a flow of hydrogen gas.

The necks of stoppered culture tubes were flamed, the stopper removed with a hemostat, and a flame sterilized CO_2 cannula inserted. After inoculation of the tube, the stopper was placed over the tube containing the cannula long enough to purge the air from the neck of the tube before removing the cannula and stoppering the tube tightly.

Staining method

The Kopeloff Modification of the Gram stain was used

to determine gram reaction. (15)

Preparation of pre-reduced media

All media, staining reagents and solutions were prepared as described in the Anaerobic Laboratory Manual, 4th edition, Holdeman and Moore. (15).

Pre-reduced media was prepared by boiling, usually for 20 minutes, to drive off the oxygen and reduce the ingredients as determined by the resazurin Eh indicator, which changes from pink to colorless. Then the medium was cooled in an ice bath to room temperature with oxygen-free CO_2 bubbling through it. Reducing agent (cysteine hydrochloride) was added to lower the Eh further. The pH was adjusted to .2 pH unit above the desired preautoclaving pH with 8N NaOH or 6N HCl, and CO_2 bubbled through the medium until the pH was lowered to the desired value. Nitrogen was bubbled through the medium during dispensing and the tubes were flushed with nitrogen by using two cannulas soldered together, one dispensing the medium and the other delivering nitrogen gas. Rubber stoppers were inserted as the cannulas were withdrawn from the tubes.

Racked tubes were placed in presses to hold the stoppers securely in place during autoclaving at 121°C (15 pounds) for the desired time. Fast exhaust was used. Agar or gelatin, when used, was added to the tubes before the media was dispensed. All media contained resazurin,

cysteine hydrochloride, vitamin K and hemin. An exception is the acetyl methyl carbinol media which does not contain resazurin. Dilution blanks contained gelatin, salts solution, distilled water and resazurin. Carbohydrate concentrates were prepared by pre-reduced media preparation methods with resazurin and cysteine hydrochloride and stoppered under nitrogen gas.

Specific tests

Chopped meat (CM) was used as a culture maintenance medium and for determination of meat digestion, and portions were withdrawn and used for determination of motility and production of indol. Peptone yeast glucose (PYG) was used both for chromatographic analysis and for pH to determine acid production. Growth in PYG was compared to PYG-bile, PYG tween 80 and PYG-rumen fluid to determine inhibition or stimulation of growth. Biochemical reactions were determined by the addition of carbohydrate concentrates to peptone yeast (PY) basal medium. The concentrates were prepared as pre-reduced anaerobically sterilized solutions and were added dropwise to the PY medium at the time of inoculation. The final concentrations were as given in the summary table for media preparation in the Anaerobe Laboratory Manual, usually one percent or five tenths percent. Acid production from carbohydrates was determined by pH readings, and carbohydrates used included arabinose,

cellobiose, esculin, fructose, glucose, inositol, lactose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose, starch, xylose, dulcitol and galactose. Esculin hydrolysis was determined by adding ferric ammonium citrate. Starch hydrolysis was determined by adding Gram's iodine.

Acetyl methylcarbinol production was determined by using AMC media (PYG with no resazurin) to which alpha napthol and potassium hydroxide solutions were added. Ammonia production was determined by adding Nessler's solution to two drops of PYG culture. Gas production and resazurin reduction was noted by inoculation of PYG agar deeps which had been melted and cooled to 45°C. Gelatinase production was noted by inoculation of gelatin. Lactic and pyruvate utilization were determined by chromatographic comparison of lactate PY and pyruvate PY cultures to PY cultures for increased products. Spore production was determined when necessary by inoculation of a chopped meat agar slant, and by heating inoculated starch broth at 80°C for 10 minutes in a water bath followed by cooling to 37°C. Threonine conversion to propionate was determined by chromatographing ether extracts of threonine and PY cultures to ascertain the presence of more propionic acid in the threonine culture.

Gas chromatography

The gas chromatography used was the Dorman Anaerobic Bacteriology System (ANABAC) distributed by the Clinical Analysis Products Company (CAPCO), employing a dual thermal conductivity detection system with a linear chart recorder. The column used for the injection of volatile fatty acids and alcohols was packed with 15% Supelco-1220 H_3PO_4 on 100/120 chromosorb. The column used for non volatile fatty acids was packed with 10% Supelco-1000 1% H_3PO_4 on 100/120 chromosorb. Operating conditions were 8X attenuation (unless otherwise specifically noted), column temperature 150°, detector block temperature 150°C and helium carrier gas at 20 psi pressure, column 1 at 86 cc/min and column 2 at 70 cc/min, detector current 100 m.a. DC.

Volatile fatty acid and alcohol analysis was made by acidifying 1 ml samples of culture with H_2SO_4 to change the fermentation acids to the protonated form, (soluble in ether as well as water) adding ether, and adding NaCl for salting out the volatile fermentation acids (27).

The ether layer containing the short chain fatty acids was extracted and $MgSO_4$ added to remove dissolved water from the ether. An aliquot of 1^4 microliters of the ether extract was injected.

Methyl derivatives of pyruvic, lactic, fumaric and succinic acids were prepared by adding methanol and H_2SO_4 to PYG cultures, followed by heating at 55°C for 30 minutes

or holding overnight at room temperature, then adding water and chloroform and mixing. Fourteen microliters were then withdrawn from the chloroform layer containing the methylesters for injection.

Commercial standards (CAPCO company) of volatile fatty acids, non-volatile fatty acids, and alcohols were used. Injections were made using a Unimetrics Corporation teflon tipped glass syringe. Chromatographs from unknown cultures were compared to the chromatographs of standard solutions. Peak heights of fatty acids equal to or greater than standard solutions containing one milliequivalent per 100 ml aqueous solution were designated with capital letters. Peak heights less than standard solution (less than 1 meq per 100 ml.) were designated with small letters.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was done by the broth disc method (Anaerobe Laboratory Manual, Holdeman and Moore). (27). Prereduced BHI broth was inoculated with one drop of culture from a Pasteur pipet after antibiotic discs or antibiotic solutions were added. Tubes were incubated for 24 hours at 37°C. (Readings were also taken at 48 hours for slower growing organisms). Antibiotic tubes with 50% or more of the turbidity of the control culture were reported as resistant. Tubes with no turbidity or less than 50% of the control tube turbidity were reported sus-

ceptible. Tubes in which the turbidity was questionable were reported as indeterminate.

Cultural counts to determine anaerobes and facultative anaerobes

Food samples of approximately one gram were aseptically added while under a CO_2 cannula to 9.0 ml pre-reduced salts solution containing glass beads. The tubes were reweighed and the actual weights of the food samples were determined for later correction of cultural counts per gram. The contents of this tube, 10^{-1} dilution, was mixed using a vortex mixer, and serial dilutions were made under a CO_2 cannula by adding 1 ml to 9.0 ml of salts solution. Wide tipped pipets were used to measure the diluted food. These were prepared by cutting off the lower portion of a 10 ml pipette. CO_2 was drawn into the pipette routinely before any fluid was drawn into the pipette to prevent oxidation of the foods. Food dilutions were continued to a dilution of 10^{-5}. One ml of each dilution was added to molten and cooled pre-reduced brain heart infusion agar (BHIA). The tubes were inverted several times to mix the contents and then the agar rolled. The roll tubes were incubated at 37°C two days or at room temperature for 2-5 days. Roll tubes were marked with spiral lines and colonies counted at 40 x under an American Optical Stereo dissection microscope.

Anaerobe Isolation

Colonies picked from BHIA roll tubes were transferred to chopped meat glucose (CMG), peptone yeast glucose (PYG), and (aerobic) brain heart infusion broth. Organisms showing good growth in the BHI broth, 1+ to 4+, were discarded as facultative bacteria. Organisms showing no growth or doubtful growth in aerobic media and growth in pre-reduced media were identified. Isolates were checked repeatedly for aerotolerance with aerobic BHI media. Some facultative anaerobes did not grow aerobically upon primary isolation. Isolates growing only anaerobically were identified by gram staining, chromatographic analysis, and biochemical tests.

Cultural counts to determine growth of anaerobes in food

Anaerobic isolates obtained from foods were inoculated into the same food type from which they were originally isolated, and growth at 32°C was determined.

The foods were blended in a blender jar that had been flushed with nitrogen. Approximate one gram amounts of the blended food were then placed in preweighed nitrogen flushed tubes containing glass beads. The tubes were reweighed to determine the actual weight of the food added, and the foods were autoclaved at 121°C (15 lb) for 30 minutes. The food samples were inoculated with a PYG culture of the anaerobe that had been originally isolated from the food, and placed in a 32°C water bath. Counts were made

from the inoculum and from the inoculated foods at 0 hours, 6 hours, and 24 hours by adding one ml. of serial dilutions to molten and cooled BHIA. The tubes were mixed by inversion, rolled and incubated at 37°C for two days. Tubes containing 30-300 colonies were counted. The colony count was corrected according to the initial weight of the food sample, and counts from duplicate samples were averaged.

Cultural counts following freezing

Two of each food type of the weighed sterilized food samples were inoculated with anaerobes and then frozen and held at -18°C for two weeks. Cultural counts were then made to determine the reduction in numbers following freezing.

Growth curves of anaerobes

Growth curves of anaerobic isolates recovered from foods were determined at 37°C by inoculating side arm flasks containing 100 ml of pre-reduced anaerobically sterilized PYG broth with 2 ml of a 12 hour PYG culture. Absorbance readings were taken at timed intervals at 580 nm on a Bausch and Lomb Spectronic 20.

RESULTS AND DISCUSSION

Antibiotic susceptibility testing of bacteria

Antibiotic susceptibility testing of fourteen stock cultures of anaerobes and three facultative anaerobes was done using different concentrations and combinations of kanamycin, neomycin, colistin, polymyxin B, vancomycin, and streptomycin. Kanamycin and vancomycin were also tested in combination with bile. Kanamycin, table 1, inhibited the facultative anaerobes at a concentration of 352 µg/ml but also inhibited eight of the obligate anaerobes. Two species of Bacteroides and two of Clostridium were resistant. Neomycin at 12 µg/ml, table 2, did not inhibit the facultative tested. Colistin, table 2, inhibited just one of the facultative anaerobes at 4 µg/ml, as did polymyxin B at 120 units, table 3. Streptomycin, table 3 at 4 µg/ml did not inhibit the facultative anaerobes. The combinations of kanamycin at 88 µg with neomycin at 6 µg/ml, colistin at 2 µg/ml, polymyxin B at 60 units/ml and streptomycin at 2 µg/ml table 4, inhibited just one of the facultative anaerobes but also inhibited several obligate anaerobes. Bile, both alone and in combination with vancomycin and kanamycin inhibited many obligate anaerobes, table 5. Vancomycin at 7.5 µg/ml in combination with kanamycin at 352 µg/ml inhibited the facultative anaerobes as well as many of the obligate anaerobes. However, it was decided that this com-

bination might be useful in isolating some species of Bacteroides or Fusobacterium from food samples, and that a duplicate test of some of the samples would be made using this combination of antibiotics added to BHIA as a selective medium.

Table 1 Kanamycin Sensitivity

	Kanamycin 352 µg/ml	176 µg/ml	88 µg/ml
Bacteroides fragilis	R	R	R
Bacteroides thetaiotaomicron	R	R	R
Bacteroides ruminicola ss ruminicola	S	S	S
Clostridium perfringens type D	R	R	R
Clostridium clostridiiforme	R	R	R
Peptococcus magnus	S	S	R
Peptostreptococcus intermedius	S	R	R
Fusobacterium varium	I	R	R
Propionibacterium acnes	S	S	S
Eubacterium lentum	I	I	I
Leptotrichia buccalis	S	S	S
Peptococcus morbillorum	S	S	S
Bifidobacterium ericksonii	S	S	S
Actinomyces israelli	S	S	S
Streptococcus faecium	S	S	R
Streptococcus faecalis ss liquifaciens	S	R	R
Escherichia coli	S	S	I

R = Resistant
I = Indeterminate
S = Susceptible

Table 2 Neomycin and Colistin Sensitivity

	Neomycin		Colistin	
	6 µg/ml	12 µg/ml	2 µg/ml	4 µg/ml
Bacteroides fragilis	R	R	R	R
Bacteroides thetaiotaomicron	R	R	R	R
Bacteroides ruminicola ss ruminicola	I	I	I	I
Clostridium perfringens type D	R	R	R	R
Clostridium clostridiiforme	R	R	R	R
Peptococcus magnus	R	S	S	S
Peptostreptococcus intermedius	R	R	R	R
Fusobacterium varium	R	R	S	S
Propionibacterium acnes	I	I	R	R
Eubacterium lentum	I	I	I	I
Leptotrichia buccalis	R	R	R	R
Peptococcus morbillorum	I	I	I	I
Bifidobacterium ericksonii	I	I	S	S
Actinomyces israelli	I	I	I	I
Streptococcus faecium	R	R	R	R
Streptococcus faecalis ss liquifaciens	R	R	R	R
Escherichia coli	R	R	S	S

R = Resistant
I = Indeterminate
S = Susceptible

Table 3 Polymyxin B and Streptomycin Sensitivity

	Polymyxin B		Streptomycin	
	60 units/ml	12 units/ml	2 µg/ml	4 µg/ml
Bacteroides fragilis	R	R	R	R
Bacteroides thetaiotaomicron	R	R	R	R
Bacteroides ruminicola ss ruminicola	I	I	I	I
Clostridium perfringens type D	R	R	R	R
Clostridium clostridiiforme	R	R	R	R
Peptococcus magnus	S	S	S	S
Peptostreptococcus intermedius	R	R	R	R
Fusobacterium varium	S	S	R	R
Propionibacterium acnes	S	S	S	S
Eubacterium lentum	I	I	I	I
Leptotrichia buccalis	S	S	I	I
Peptococcus morbillorum	I	I	I	I
Bifidobacterium ericksonii	S	S	I	I
Actinomyces israelli	S	S	I	S
Streptococcus faecium	R	R	R	R
Streptococcus faecalis ss liquifaciens	R	R	R	R
Escherichia coli	S	S	R	R

R = Resistant
I = Indeterminate
S = Susceptible

Table 4 Antibiotic Combinations

	Kanamycin 88 µg/ml Neomycin 6 µg/ml	Kanamycin 88 µg/ml Colistin 2 µg/ml	Kanamycin 88 µg/ml Polymyxin G 60 units/ml	Kanamycin 88 µg/ml Streptomycin 2 µg/ml
Bacteroides fragilis	R	R	R	R
Bacteroides thetaiotaomicron	R	R	R	R
Bacteroides ruminicola ss ruminicola	S	S	S	S
Clostridium perfringens type D	R	R	R	R
Clostridium clostridiiforme	R	R	R	R
Peptococcus magnus	S	S	S	S
Peptostreptococcus intermedius	R	R	R	R
Fusobacterium varium	R	S	S	R
Propionibacterium acnes	S	S	S	S
Eubacterium lentum	I	I	I	I
Leptotrichia buccalis	S	I	R	R
Peptococcus morbillorum	S	S	I	I
Bifidobacterium ericksonii	S	S	S	S
Actinomyces israelli	S	S	S	S
Streptococcus faecium	R	R	R	R
Streptococcus faecalis ss liquifaciens	R	R	R	R
Escherichia coli	S	S	S	S

R = Resistant
I = Indeterminate
S = Susceptible

Table 5 Bile and Antibiotic Susceptibility

	Bile, 2%	Bile, 2% Vancomycin 7.5 µg/ml Kanamycin 352 µg/ml	Bile, 2% Vancomycin 7.5 µg/ml	Bile, 2% Kanamycin 352 µg/ml
Bacteroides fragilis	R	R	R	R
Bacteroides thetaiotaomicron	R	R	R	R
Bacteroides ruminicola ss ruminicola	S	S	S	S
Clostridium perfringens type D	R	S	S	S
Clostridium clostridiiforme	S	S	S	S
Peptococcus magnus	S	S	S	S
Peptostreptococcus intermedius	S	S	S	S
Fusobacterium varium	R	R	R	S
Propionibacterium acnes	S	S	S	S
Eubacterium lentum	R	S	S	S
Leptotrichia buccalis	S	S	S	S
Peptococcus morbillorum	S	S	S	S
Bifidobacterium ericksonii	S	S	S	S
Actinomyces israelli	S	S	S	S
Streptococcus faecium	R	S	I	S
Streptococcus faecalis ss liquifaciens	R	S	S	S
Escherichia coli	S	S	R	S

Table 6 Vancomycin and Kanamycin Susceptibility

	Vancomycin 7.5 ug/ml	Vancomycin 15 ug/ml	Vancomycin 7.5 ug/ml Kanamycin 352 ug/ml	Vancomycin 15 ug/ml Kanamycin 352 ug/ml
Bacteroides fragilis	R	R	R	R
Bacteroides thetaiotaomicron	R	R	R	R
Bacteroides ruminicola ss ruminicola	S	S	S	S
Clostridium perfringens type D	S	S	S	S
Clostridium clostridiiforme	S	S	S	S
Peptococcus magnus	S	S	S	S
Peptostreptococcus intermedius	S	S	S	S
Fusobacterium varium	R	R	R	R
Propionibacterium acnes	S	S	S	S
Eubacterium lentum	S	S	S	S
Leptotrichia buccalis	S	S	S	S
Peptococcus morbillorum	S	S	S	S
Bifidobacterium ericksonii	S	S	S	S
Actinomyces israelli	S	S	S	S
Streptococcus faecium	S	S	S	S
Streptococcus faecalis ss liquifaciens	S	S	S	S
Escherichia coli	R	R	S	S

Cultural counts from foods

The cultural counts obtained from the serial dilutions of foods plated in BHIA roll tubes consisted of both facultative anaerobes and obligate anaerobes. Transfers to aerobic BHI as well as prereduced chopped meat glucose (CMG) showed that most of the colonies were facultative anaerobes. From 20 to 40 colonies from each food type were picked from the BHIA roll tubes. Most grew aerobically as well as anaerobically and were discarded following two days incubation at 37°C. Some isolates that grew only anaerobically were discarded later as facultative anaerobes that would on original isolation grow only anaerobically.

The BHIA tubes containing vancomycin, 7.5 μg/ml, and kanamycin, 352 μg/ml, inhibited the growth of facultative anaerobes from one to four logarithms. The use of antibiotics was helpful in isolating Bacteroides species.

Anaerobic isolates from foods

Anaerobic isolates included species from the genera Bacteroides, Lactobacillus, Clostridium, Peptococcus and Peptostreptococcus. Anaerobes were isolated from six of the ten foods tested. Their characterization is given in the following tables.

Table 7 ROLL TUBE CULTURAL COUNTS PER GRAM OF FOOD

	BHIA x 10^2/gm	BHIA Vancomycin 7.5 µg/ml Kanamycin 352 µg/ml x 10^2/gm
Chicken Liver	3,000	6.3
Ground Beef #1	400	53
Ground Beef #2	140	18
Bologna	30,000	5.5
Ground Pork	1,400	.11
Ham Salad Spread #1	125	.81
Ham Salad Spread #2	19,100	
Oysters	610	
Fish Miniatures (frozen)	2	
Turkey Pot Pie (frozen)	130	

Table 8 ANAEROBE ISOLATES FROM FOODS

FOOD	ISOLATE
Chicken Liver	*Bacteroides vulgatus* *Lactobacillus plantarum*
Ground Beef #1	*Clostridium tertium*
Ground Beef #2	None
Bologna	*Bacteroides ruminicola* *ss brevis*
Ground Pork	*Lactobacillus* sp.
Ham Salad Spread #1	None
Ham Salad Spread #2	*Clostridium* sp.
Oysters	None
Fish Miniatures (frozen)	None
Turkey Pot Pie (frozen)	*Peptostreptococcus intermedius* *Peptococcus morbillorum*

Table 9 CHARACTERIZATION OF ISOLATE #1

Identity: Bacteroides vulgatus

Morphology and Gram stain: Gram negative bacillus

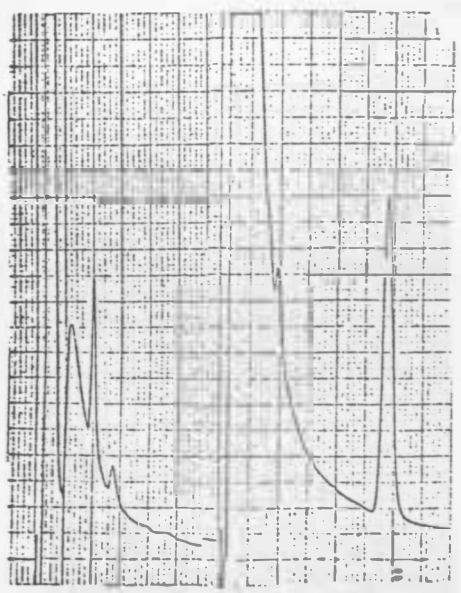
Food source: Chicken liver

GLC Products ASp

Acetic > 1 meq/100 mls

Succinic > 1 meq/100 mls

propionic < 1 meq/100 mls



Growth in PYG	++++	arabinose	A
PYG - Tween	++++	cellobiose	w
PYG - Bile S	++++	dulcitol	w
PYG - RF	+++	esculin	-
Indole	-	fructose	A
Motility	-	galactose	A
AMC	-	glucose	A
NH_3	+	inositol	w
Gas (agardeep)	+	lactose	A
Esculin hydrol	+	maltose	A
Starch hydrol	+	mannitol	w
Threo→prop	+	mannose	A
		melebiose	w
		raffinose	A
		rhamnose	A
		salicin	w
		sorbitol	w
		starch	A
		sucrose	A
		trehalose	w
		xylose	A

Table 10 CHARACTERIZATION OF ISOLATE #2

Identity: Lactobacillus plantarum

Morphology and Gram stain: Gram + bacillus

Food source: Chicken liver

GLC Products Las

Lactic > 1 meq/100 mls	Growth in PYG	++++	arabinose	A
acetic < 1 meq/100 mls	PYG - Tween	+	cellobiose	A
succinic < 1 meq/100 mls	PYG - Bile	+++	dulcitol	A
	PYG - RF	+++	esculin	A
	Indole	-	fructose	A
	Motility	-	galactose	A
	AMC	-	glucose	A
	NH_3	-	inositol	w
	Gas (agardeep)	-	lactose	A
	Esculin Hydrol	+	maltose	A
	Starch Hydrol	+	mannitol	A
	Gelatin	-	mannose	A
	Arg.	-	melebiose	A
			raffinose	A
			rhamnose	A
			salicin	A
			sorbitol	w
			starch	A
			sucrose	A
			trehalose	A
			xylose	A

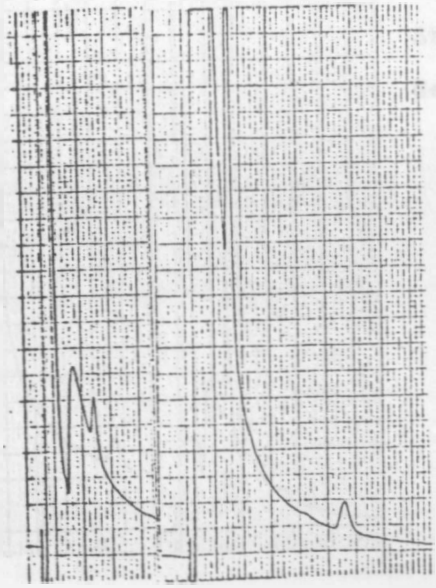


Table 11 CHARACTERIZATION OF ISOLATE #3

Identity: Clostridium tertium

Morphology and Gram stain: Gram + bacillus

Food source: Ground beef

GLC Products Alpbs

Acetic > 1 meq/100 mls	Growth in PYG	++++	arabinose -
lactic > 1 meq/100 mls	PYG - Tween		cellobiose -
propionic < 1 meq/100 mls	PYG - Bile	++++	dulcitol w
butyric < 1 meq/100 mls	PYG - RF		esculin w
succinic < 1 meq/100 mls	Indole	-	fructose A
butanol 1 meq/100 mls	Motility	-	galactose w
	AMC	-	glucose A
	NH_3	-	inositol -
	Gas (agardeep)	+	lactose A
	Esculin Hydrol	+	maltose A
	Starch Hydrol	-	mannitol W
	Gelatin	-	mannose A
			melebiose A
			raffinose -
			rhamnose -
			salicin -
			sorbitol -
			starch -
			sucrose A
			trehalose -
			xylose w

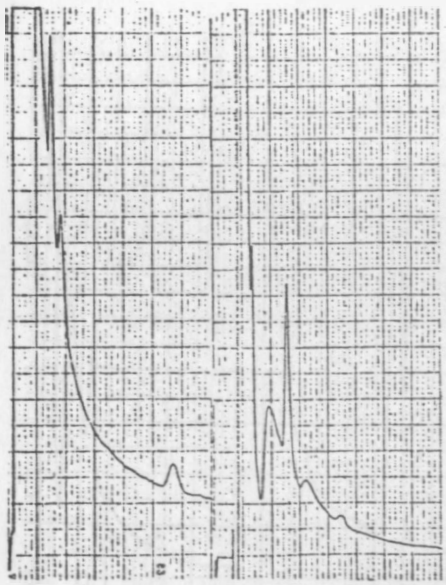


Table 12 CHARACTERIZATION OF ISOLATE #4

Identity: Bacteroides ruminicola ss brevis

Morphology and Gram stain: Gram negative bacillus

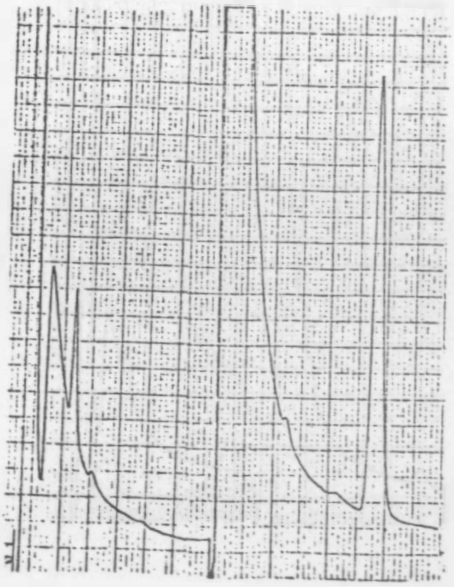
Food source: Ring balogna

GLC Products ASp

Acetic > 1 meq/100 mls

Succinic > 1 meq/100 mls

propionic < 1 meq/100 mls



Growth in PYG	++++	arabinose	A
PYG - Tween		cellobiose	w
PYG - Bile	-	dulcitol	w
PYG - RF		esculin	w
Indole	-	fructose	A
Motility	-	galactose	w
AMC	-	glucose	A
NH_3	+	inositol	w
Gas (agardeep)	+	lactose	A
Esculin Hydrol	+	maltose	A
Starch Hydrol	+	mannitol	-
		mannose	A
		melebiose	w
		raffinose	w
		rhamnose	A
		salicin	w
		sorbitol	w
		starch	A
		sucrose	A
		trehalose	w
		xylose	A

Table 13 CHARACTERIZATION OF ISOLATE #5

Identity: Lactobacillus spp.

Morphology and Gram stain: Gram + bacillus

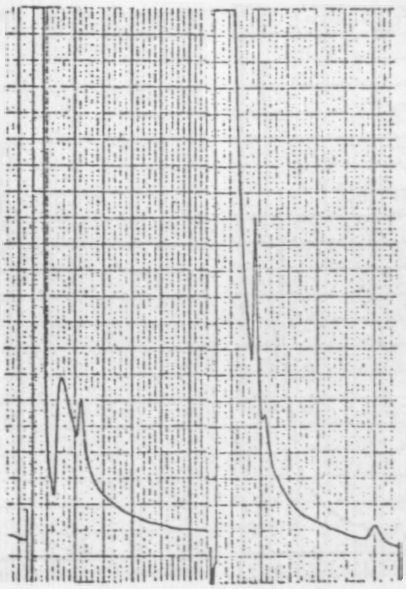
Food source: Ground pork

GLC Products Las

Lactic > 1 meq/100 mls

acetic < 1 meq/100 mls

succinic < 1 meq/100 mls



Growth in PYG	++++	arabinose	A
PYG - Tween	++	cellobiose	-
PYG - Bile	-	dulcitol	-
PYG - RF	+	esculin	-
Indole	-	fructose	A
Motility	-	galactose	A
AMC		glucose	A
NH_3	-	inositol	-
Gas (agardeep)	-	lactose	-
Esculin Hydrol	+	maltose	A
Starch Hydrol	-	mannitol	-
Gelatin	-	mannose	A
Argenine	-	melebiose	A
		raffinose	A
		rhamnose	w
		salicin	A
		sorbitol	-
		starch	-
		sucrose	A
		trehalose	A
		xylose	w

Table 14 CHARACTERISTICS OF ISOLATE #6

Identity: Clostridium sp.

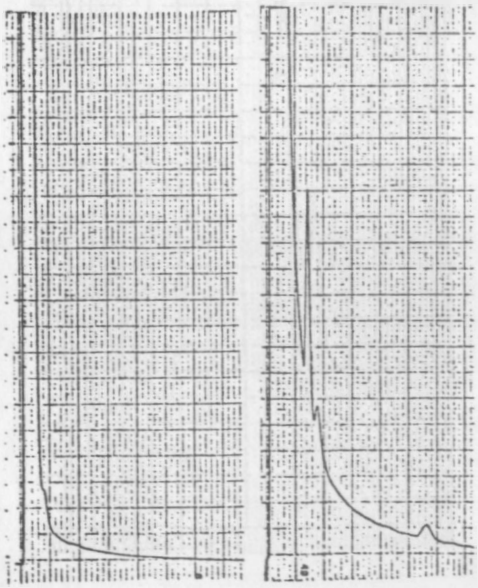
Morphology and Gram stain: Gram + bacillus

Food source: Ham salad spread

GLC Products Ls

Lactic > 1 meq/100 mls

succinic < 1 meq/100 mls



Growth in PYG	++++	arabinose	w
PYG - Tween	++	cellobiose	w
PYG - Bile	-	dulcitol	w
PYG - RF	++++	esculin	-
Indole	-	fructose	A
Motility	-	galactose	w
AMC	-	glucose	A
NH_3	+	inositol	-
Gas (agardeep)	+	lactose	w
Esculin Hydrol	+	maltose	A
Starch Hydrol	-	mannitol	w
Gelatin	-	mannose	A
Argenine		melebiose	w
		raffinose	A
		rhamnose	w
		salicin	w
		sorbitol	-
		starch	-
		sucrose	A
		trehalose	A
		xylose	A

Table 15 CHARACTERIZATION OF ISOLATE #7

Identity: Peptostreptococcus intermedius

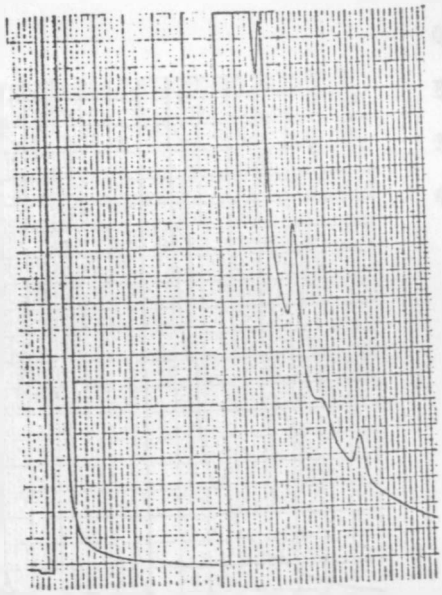
Morphology and Gram stain: Gram + cocci

Food source: Frozen turkey pie

GLC Products Ls

Lactic > 1 meq/100 mls

succinic < 1 meq/100 mls



Growth in PYG	++++	arabinose	w
PYG - Tween	++++	cellobiose	A
PYG - Bile	-	dulcitol	w
PYG - RF	++++	esculin	w
Indole	-	fructose	A
Mortility	-	galactose	A
AMC	-	glucose	A
NH_3	-	inositol	A
Gas (agardeep)	-	lactose	A
Esculin Hydrol	+	maltose	A
Starch Hydrol	-	mannitol	w
Gelatin	-	mannose	w
		melebiose	A
		raffinose	A
		rhamnose	w
		salicin	A
		sorbitol	w
		starch	w
		sucrose	A
		trehalose	A
		xylose	w

Table 16 CHARACTERIZATION OF ISOLATE #8

Identity: Peptococcus morbillorum

Morphology and Gram stain: Gram + cocci

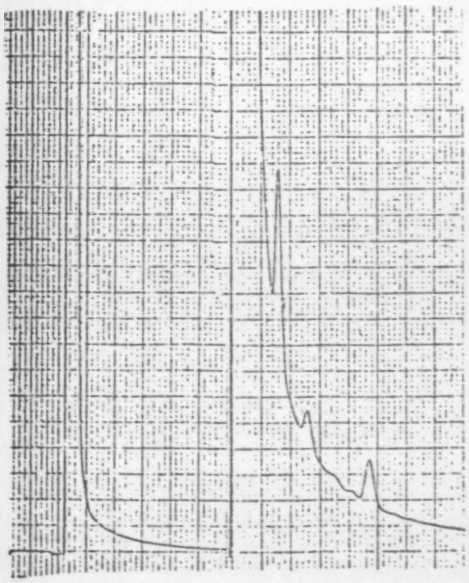
Food source: Frozen turkey pie

GLC Products Ls

Lactic > 1 meq/100 mls

succinic < 1 meq/100 mls

Growth in PYG	++++	arabinose	w
PYG - Tween	++	cellobiose	-
PYG - Bile	-	dulcitol	-
PYG - RF	++++	esculin	-
Indole	-	fructose	w
Mortility	-	galactose	-
AMC	-	glucose	A
NH_3	-	inositol	-
Gas (agardeep)	+	lactose	w
Esculin Hydrol	-	maltose	A
Starch Hydrol	-	mannitol	w
Gelatin	-	mannose	A
		melebiose	w
		raffinose	w
		rhamnose	w
		salicin	-
		sorbitol	-
		starch	-
		sucrose	A
		trehalose	w
		xylose	-



Growth curves of the anaerobic isolates

Growth curves of four of the anaerobes isolated from foods showed a lag phase lasting from four to nine hours when inoculated into PYG media and incubated at 37°C. Absorbance readings were taken at 30 minute intervals on a Bausch and Lomb Spectronic 20. Growth curves of Peptococcus morbillorum, Peptostreptococcus intermedius, Bacteroides vulgatus and Bacteroides ruminicola ss brevis are plotted on Figures 1 through 4, and a composite of the four growth curves is given in Figure 5.

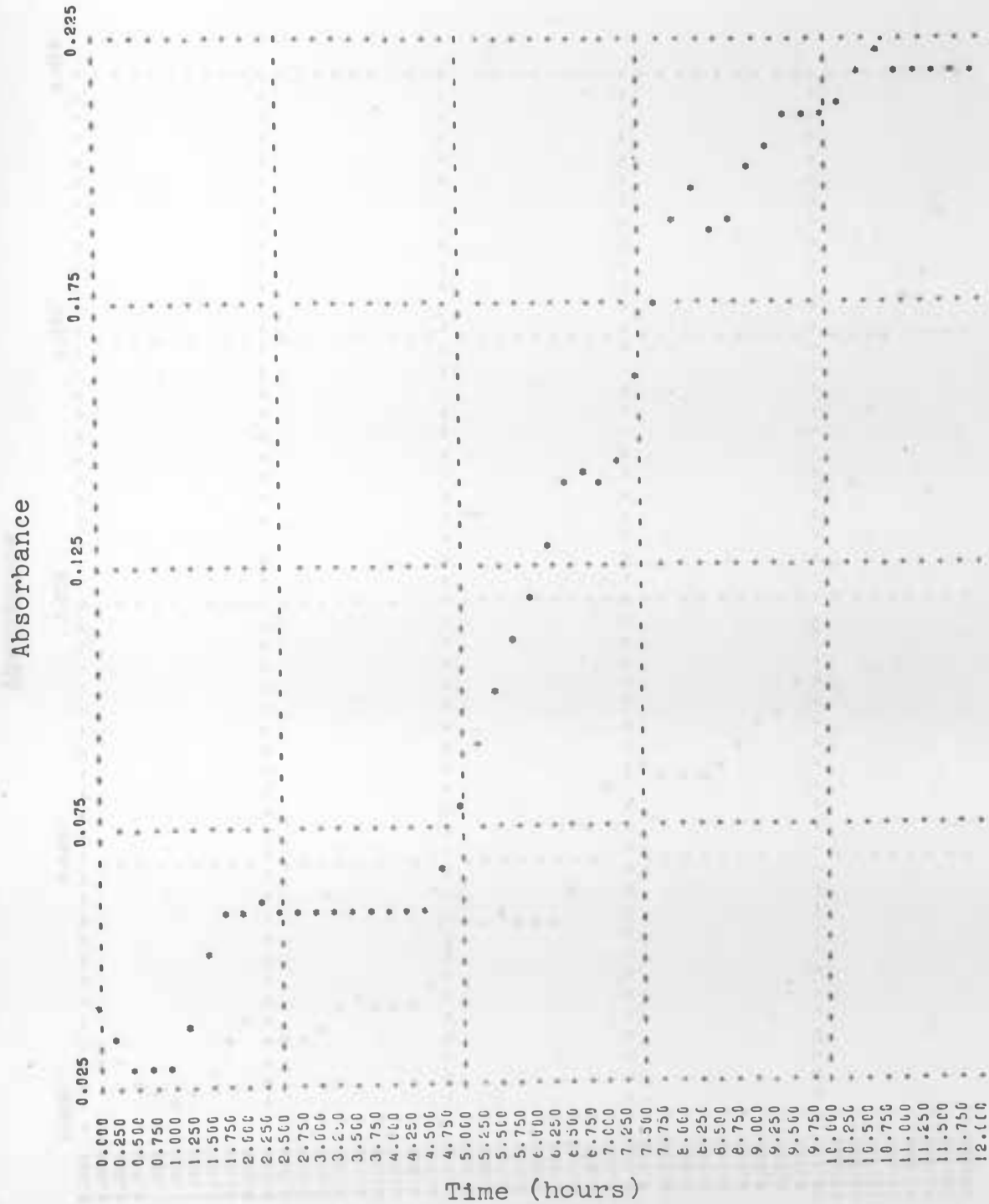


Figure 1 Growth Curve of Peptococcus morbillorum

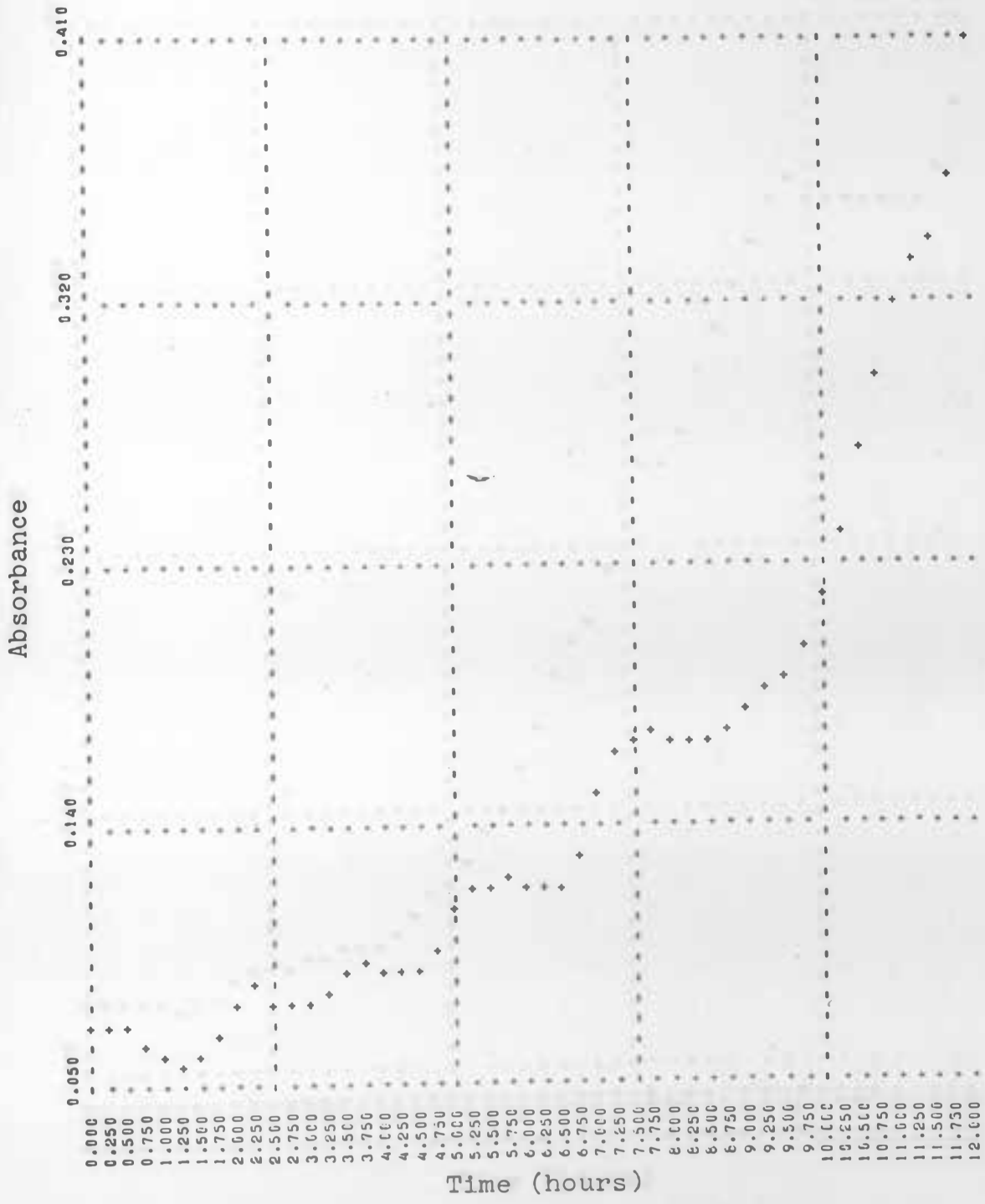


Figure 2 Growth Curve of Peptostreptococcus intermedius

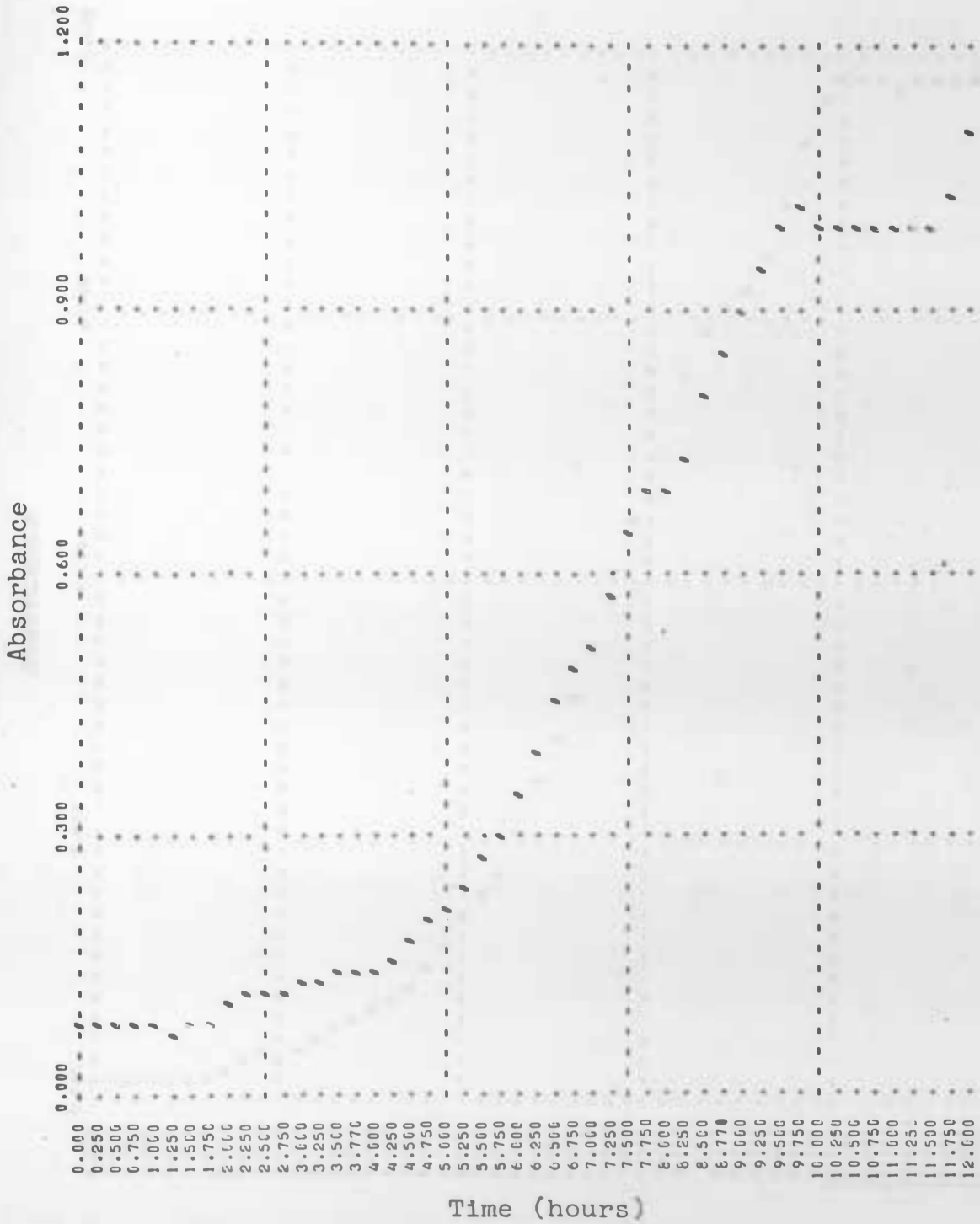


Figure 3 Growth Curve of Bacteroides vulgatus

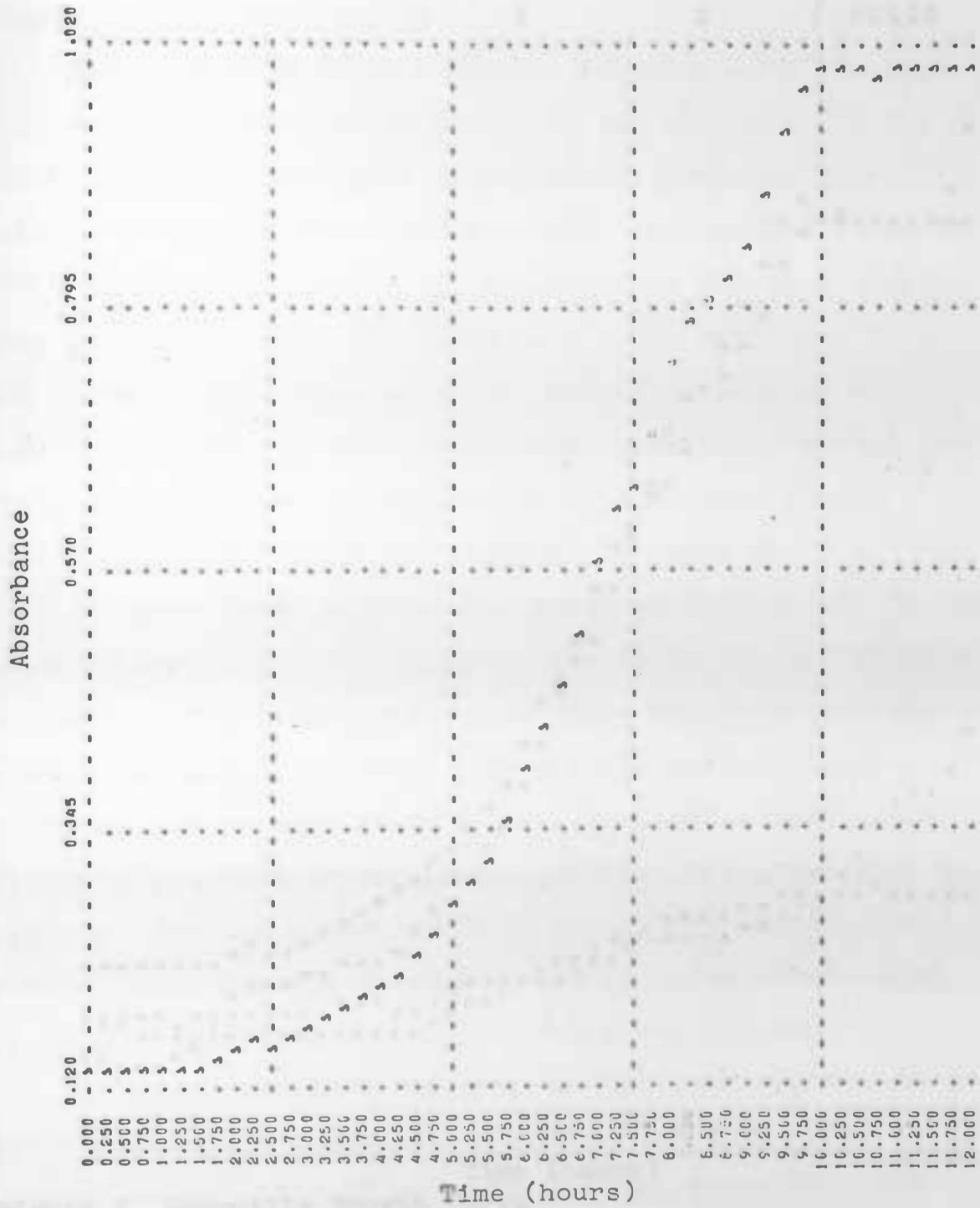


Figure 4 Growth Curve of Bacteroides ruminicola ss brevis

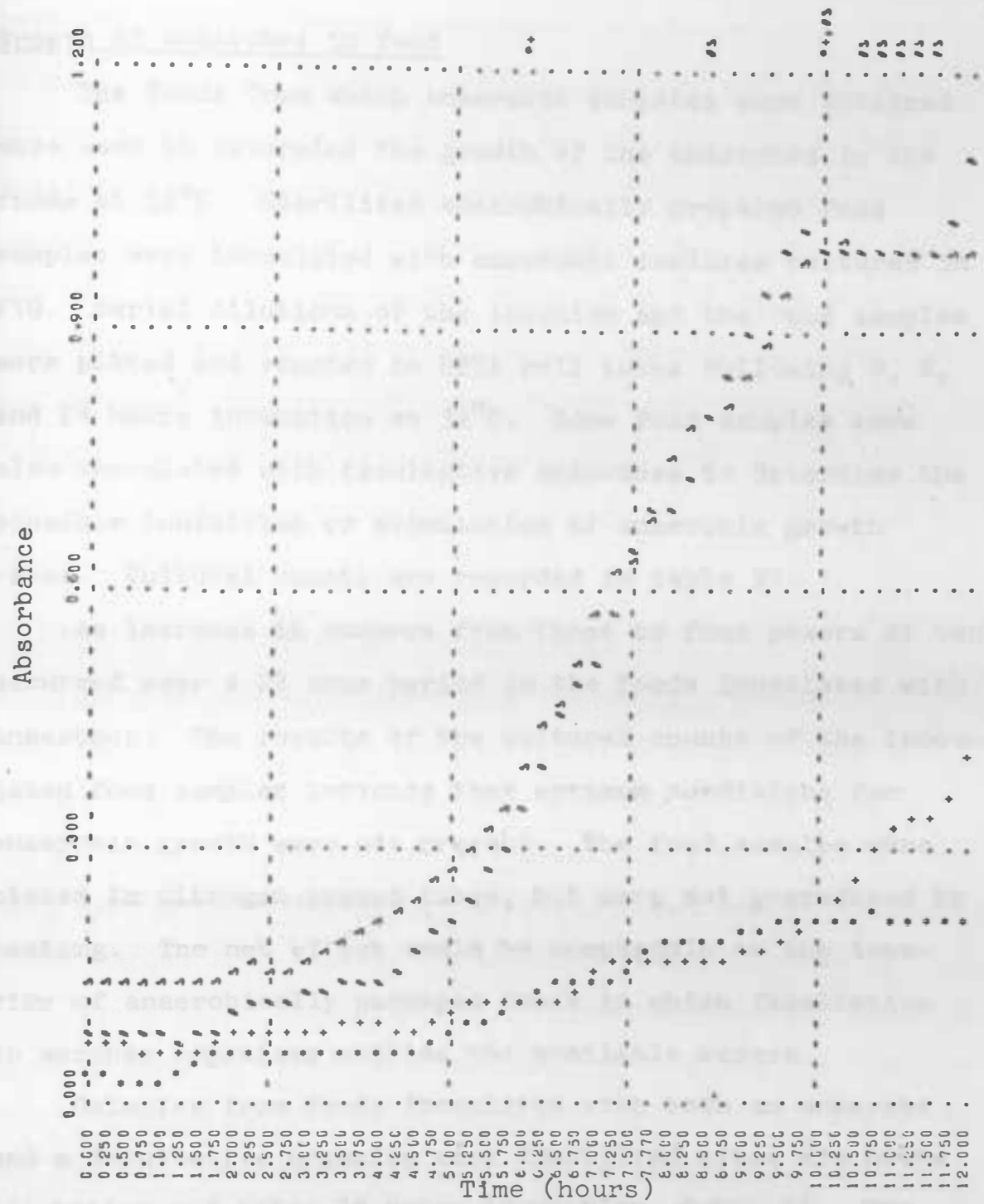


Figure 5 Composite Growth Curve

Peptococcus morbillorum = *
Peptostreptococcus intermedius = +
Bacteroides vulgatus = #
Bacteroides ruminicola ss brevis = &

Growth of Anaerobes in food

The foods from which anaerobic isolates were obtained were used to determine the growth of the anaerobes in the foods at 32°C. Sterilized anaerobically prepared food samples were inoculated with anaerobic isolates cultured in PYG. Serial dilutions of the inoculum and the food samples were plated and counted in BHIA roll tubes following 0, 6, and 24 hours incubation at 32°C. Some food samples were also inoculated with facultative anaerobes to determine the possible inhibition or stimulation of anaerobic growth rates. Cultural counts are recorded in table 17.

An increase in numbers from three to four powers of ten occurred over a 24 hour period in the foods inoculated with anaerobes. The results of the cultural counts of the inoculated food samples indicate that optimum conditions for anaerobic growth were not present. The food samples were placed in nitrogen gassed tubes, but were not prereduced by heating. The net effect could be comparable to the interior of anaerobically packaged foods in which facultative or aerobic organisms utilize the available oxygen.

Colonies from foods inoculated with both an anaerobe and a facultative organism were identified after six hours incubation and after 24 hours incubation, table 18. The colonies were picked, stained, and identified by morphology. Streptococcus faecalis outgrew the anaerobe in the bologna inoculated food sample, and Bacteroides ruminicola was not

detected in the colonies examined at six hours or at 24 hours incubation. Bacteroides vulgatus was not overgrown by the Streptococcus faecalis in the chicken liver samples. Ten percent of the colonies examined after 24 hours incubation were gram negative rods. Bacteroides vulgatus would not have been detected in the final dilution tube from the food sample of the logarithmic growth rate if the organism had not been roughly equivalent to that of Streptococcus faecalis. The turkey pie samples inoculated with Peptococcus morbillorum and Escherichia coli also showed equivalent growth rates.

Survival of anaerobes in frozen foods

Survival rates of anaerobes following freezing was determined by inoculating the sterile food samples in the nitrogen gassed tubes, mixing, and freezing for one week at $-18^{\circ}C$. Cultural counts of serial dilutions of the food samples were made to determine the viable cell count. Survival rates varied from four to fifty-eight percent, table 19.

Survival of bacterial cells following freezing depends upon the rate of freezing, the rate of thawing, the temperature of storage, the media in which the cells are suspended, and the inherent resistance of the individual species or strain. Proteins, simple and complex carbohydrates, and triglycerides present in foods provide increased resistance of bacterial cells to freezing.

Table 17 CULTURAL COUNTS OF INOCULATED FOOD

Food plus Organisms	Inoculum	0 hrs.	6 hrs.	24 hrs.
	x 10^4/ml	x 10^4/gm	x 10^4/gm	x 10^4/gm
Chicken liver				
Bacteroides vulgatus	248	86	285	46,100
plus *Streptococcus faecalis*	200		95,100	236,000,000
Bologna				
Bacteroides ruminicola ss *brevis*	17.6	.361	3.81	3,030
plus *Streptococcus faecalis*	200		511	123,000,000
Turkey pie				
Peptococcus morbillorum	40	20	10,810	70,000
plus *Escherichia coli*	220		51,100	181,000,000

Table 18 IDENTIFICATION OF ORGANISMS FROM FOODS INOCULATED WITH MIXED CULTURES OF BACTERIA

Food and Organisms	Percentage of Colonies 6 hours	24 hours
Chicken liver		
Bacteroides vulgatus	48	10
Streptococcus faecalis	52	90
Bologna		
Bacteroides ruminicola ss brevis	--	--
Streptococcus faecalis	100	100
Turkey pie		
Peptococcus morbillorum	42.9	51.5
Escherichia coli	57.1	48.5

Table 19 COLONY COUNTS AFTER FREEZING

Food and Organism	Inoculum x 10^4/ml	Immediate Recovery x 10^4/gm	After Freezing x 10^4/gm	Percent Survival
Chicken liver				
Bacteroides vulgatus	248	86	3.6	4.2
Bologna				
Bacteroides ruminicola ss brevis	17.6	.36	.21	58.3
Turkey pie				
Peptococcus morbillorum	40	20	2.9	14.5

CONCLUSIONS

1. Anaerobic nonsporing bacteria are sometimes present in foods in numbers at least as high as 10^3 per gram.

2. Obligately anaerobic bacteria can be isolated from commercial foods with increased frequency through the use of selective media such as the vancomycin - kanamycin selective media.

3. Multiplication of anaerobes in foods can occur to high levels, (10^7/g), under certain conditions including an above normal food storage temperature, anaerobic packaging of the food, or the possible presence of associated aerobic or facultatively anaerobic bacteria. These may lower the available oxygen present to a level where growth of anaerobic bacteria can occur.

4. The possibility exists that nonsporing anaerobic bacteria, when present in foods in high numbers, could be responsible for foodborne disease.

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