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THE USE OF CHROMATOGRAPHIC TECHNIQUES IN THE IDENTIFICATION OF BACTEROIDES SPECIES

A THESIS

Submitted in partial fulfilment of the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY

by

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JULY 1983

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To my daughter, Luma, who was two years old at the beginning of this project and had to spend long hours away from me at that very young age when she needed me most. I am most grateful for her love and affection and wish her forgiveness for the long four years taken to finish this project.

DEDICATION

To Maher

Luma

and

The memory of a brave fighter and a loving brother Issam Sartawi (1934-1983)

ORIGINALITY

All the work presented in this thesis has been carried out by the author except where acknowledged and has not previously been presented for a degree at this university or any other institution.

ABBREVIATIONS

NCTC	National collection of type culture
NCIB	National collection of industrial bacteria
ATCC	American type culture collection
VPI	Virginia polytechnic institute
GC	Gas chromatography
GLC	Gas liquid chromatography
GSC	Gas solid chromatography
SCFA	Short chain fatty acids
A	Acetic acid
P	Propionic acid
IB	Isobutyric acid
B	Butyric acid
IV	Isovaleric acid
V	Valeric acid
L	Lactic acid
S	Succinic acid
O	Oxalic acid
M	Malonic acid
PYG	Peptone yeast glucose
CMG	Cooked meat glucose
CM	Cooked meat
VFAs	Volatile fatty acids
n-VFAs	Non-volatile fatty acids
FFAP	Free fatty acid phase
h	Hour
m	Meter
min	Minute
MV	Millivolt

MS	Milliseconds
µl	Microliter
µg	Microgram
W/V	Weight by volume
SD	Standard of deviation
ss.	Subspecies
DEGA	Diethylene glycol adipate
i.d.	Internal diameter
PGLC	Pyrolysis gas liquid chromatography
GS.MS	Chromatography mass-spectrometry
WCOT	Wall coated open tubular
SCOT	Support coated open tubular
S	Similarity
CVA	Canonical varaite analysis
S.C.	Subcutaneous
i.m.	Intramuscular
FAB	Fastidious anaerobe broth
RPM	Revolution per minute
OD	Optical density
MK-9	Menaquinone with nine isoprene unit
MK-10	" " ten " "
C_{14}	Tetradecanoic fatty acid
C_{15}	Pentadecanoic fatty acid
C_{16}	Hexadecanoic fatty acid
>	Greater than
<	Less than

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ABSTRACT

Identification of various Bacteroides species studied in this thesis employed a range of morphological biochemical and some chemotaxonomic techniques such as short chain fatty acid analysis by gas chromatography. Also pyrolysis gas chromatography of whole cells was used, with special reference to the ability of this technique in identifying bacteroides organisms involved in an infection process.

Biochemical techniques showed the use of API 20A, the miniaturised system of identification, to be superior to the conventional methods for its more consistant and rapid results. Differentiation of different species was not possible on the basis of biochemical tests alone.

Gas liquid chromatography (GLC) of fermentation end products of metabolism showed a varied profile ranging from a complex pattern consisting of A,P, iB, IV, L and S (B.asaccharolyticus and B.melaninogenicus ss. macacae) to a simple pattern consisting of A,P,L and S produced by the closely related B.fragilis group. A third profile was produced by B.ruminicola ss. brevis and B.melaninogenicus ss. melaninogenicus and consisted of A,L and S.

Although the use of GLC has greatly improved the taxonomy of Bacteroides species, its quantitative aspects have not been fully explored. A strictly standardized quantitative GLC analysis employed in this thesis has pointed out the presence of quantitative

differences in the production of these acids which varied greatly from one species to another. It also showed the closely related B.fragilis group to be differentiated from each other on the basis of the ratio of fatty acids produced. In addition the ratio of S:A at 12 h of incubation gave valuable results in differentiating various species. The use of two different media, cooked meat glucose (CMG) and peptone yeast glucose (PYG) showed fermentation products to be effected by the media used and the length of incubation time. CMG medium showed a higher rate of fatty acids production when compared to PYG.

The pathogenic capabilities of selected bacteroides species was also tested using pure cultures (B.fragilis, B.melaninogenicus ss. macacae and B.asaccharolyticus) and mixed cultures (E.coli, St.faecalis and Cl. sporogenes and B.asaccharolyticus). Pure species were shown to be capable of producing localized abscesses at the site of injection at an inoculum level of 10^8 cells.ml^{-1} (0.5 ml) and without the help of additives. The size of the abscesses have been shown to be inoculum dependent with higher number of cells giving bigger abscesses. Severe infection with B.asaccharolyticus is shown to be accomplished either through a high level inoculum of pure culture (0.5 ml of 10^{10} cells.ml^{-1}) or the use of small level inoculum in the presence of certain helper organisms (0.5 ml of 10^5 cells.ml^{-1}). Fatal infections coincided with the use of a high level inoculum (1 ml of 10^{10} cells.ml^{-1}) and the presence of B.fragilis and B.asaccharolyticus within the same animal.

The application of pyrolysis gas liquid chromatography (PGLC) in conjunction with canonical variate analysis shows clearly the ability of this technique to discriminate Bacteroides at subspecies level and to detect the heterogenity existing within strains of the same species (B.fragilis and B.asaccharolyticus). It was also succcessful in differentiating the closely related non-pigmented species with the exception of B.fragilis (NCTC 8560) and B.distasonis. These PGLC findings on the relationships within the Bacteroides species studied correlate extremely well with the present taxonomic positions of these species with the exception of B.melaninogenicus ss. melaninogenicus (VPI 9085). PGLC was also applied for direct analysis of pus samples from pure induced Bacteroides infections. The results show that this technique provides a rapid and sensitive method for identification and characterization of these species. In addition it is possible to use this technique for the identification of unknown infecting Bacteroides species. This can be accomplished by applying canonical variate analysis to the pyrolysis data, thus, identifying the unknown by allocation into a pre-established canonical variate plots.

CHAPTER 1

INTRODUCTION

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1.1 Clinical Importance of Bacteroides

Bacteroides are Gram-negative, non-sporing, anaerobic bacilli belonging to the family Bacteriodaceae (Pribram, 1933). They are mostly pleomorphic although there are some species with characteristic and uniform morphology, and most species are non-motile. These organisms have been isolated from intestinal tracts of man and animals (Barnes & Goldberg, 1962 and Mitsuoka, et. al., 1974). They are the predominant flora of the gastrointestinal tract and inhabit body cavities lined by mucous membranes, such as the oropharynx and urogenital tract (Levison, 1973).

Bacteroides species are the commonest cause of anaerobic infections in man (Finegold, et. al., 1972; Finegold & Rosenblatt, 1973). Such infections are usually endogenous in origin where these species may invade the adjacent tissues that are the centre of some pathological change. General predisposing factors to infection including; debility, diabetes, malignant disease, anaemia, the trauma of abdominal surgery, especially bowel and gynaecological surgery, and the physiological process of childbirth. These organisms cause a variety of infections which are not commonly initiated in the vicinity of their normal habitats, where characteristically deep seated, foul smelling abscesses are produced (Willis, et. al., 1974). While some 30 species of Bacteroides are listed in Bergy's Manual (1974), only two are

clinically important, these are B.fragilis and B.melaninogenicus.

B.fragilis is a common commensal of the large intestine in man and animals (Donaldson, 1954). Of 290 strains isolated from faeces of healthy subjects, Holdeman & Moore (1970) found 0.7% to be B.fragilis, 47% B.vulgatus, 17% B.thetaiotaomicron, 9% B.distasonis and 3% B.ovatus. However, in clinical infection B.fragilis is the most frequently isolated anaerobe, 70%, (Tabaqchali, 1979). Its role in bacteremia and septicaemia is well documented (Gleb, et. al., 1970; Felner, et. al., 1971; Wilson, et. al.,1972; Danielsson, et. al., 1974a; Lambe, et. al., 1975). It has been implicated in endocarditis (Masri, et.al., 1972; Felner, 1974), anaerobic empyema (Sullivan, et. al.,1973), abdominal wound infections following surgical or other mechanical trauma (Thadepalli,et.al., 1973), septic abortion (Rotherman, et. al., 1969)and joint infections (Ziment, et. al., 1969).

The B.melaninogenicus group of organisms comprises pigment producing Bacteroides when grown on media containing blood. They are common inhabitants of the mouth (Socransky, et. al., 1963),(1) lower gastro-intestinal tract (Drasar, et. al., 1969) and gingival crevices of man (Gibbons, et.al., 1963;(7); Socransky, et.al., 1963). They have been isolated in association with other bacteria from serious types of clinical infections, e.g. tooth abscess (Burdon, 1928; Kerébel & Sedallian, 1972)(11) soft tissue infections (Nobles, 1973), liver abscess (Sabbaj, et.al., 1972), lung abscess (Burdon, 1928 and 1932; Cohen, 1932;

Shevky, et.al., 1934), an infected surgical wound (Oliver & Wherry, 1921), appendicitis and peritonitis (Altemeier, 1938). However, these organisms are well known for their role in periodontal disease (Weiss, 1937; Macdonald, et.al., 1960; Macdonald & Gibbons, 1962; Socransky, 1970), (11). and one report has indicated that these organisms comprise up to 66% of total cultivable flora in advanced periodontitis (Slots & Hausmann, 1979).

1.2 History of Anaerobic Classification

The pathogenic potential of the non-sporing anaerobic bacteria and in particular Bacteroides was recognized as early as 1897 by Veillon & Zuber. However, tetanus with its striking clinical features and its impressive mortality had captured the attention of Hippocrates as early as 460B.C. Acute necrotizing ulcerative gingivitis (Vincent's gingivitis) was also known in the fourth century B.C. when Xenophon described Greek soldiers suffering from foul-smelling breath and sore mouths.

Antonie Van Leeuwenhoek (1680) was the first to discover anaerobiosis. The process was re-discovered by Pasteur in 1861. He assigned the terms aèrobes and anaèrobies to microorganisms which live with or without oxygen respectively and described butyric acid fermentation due to an anaerobic spore-forming bacillus (now known to be Clostridium butyricum).

In 1889 Kitasato first grew anaerobes (including Clostridium tetani) on solid media and obtained them in pure cultures.

In 1893, Veillon reported recovery in pure culture of an obligately anaerobic micrococcus from a foetid suppurative bartholinitis. Four years later, in a brief but classic paper, Veillon & Zuber (1897) reported the association of Gram-negative, non-sporing anaerobic bacteria (Bacteroides) in a variety of gangrenous suppurative lesions and assigned them the name Bacillus fusiformis.

Tissier (1908) reported that the predominant microorganisms of the human colon were anaerobes and five "types" were described.

Tunnicliff (1907, 1919 and 1923) published several papers on the occurrence of fusiform bacteria (fusiform at this time referred to members of Bacteroides group) from several sources including the gums and tonsils of healthy individuals and from cases of gingivitis.

Krumwiede & Pratt in 1913 isolated 15 strains of fusiform bacteria from different sources and classified them into two groups, one which fermented glucose and the other glucose and sucrose.

In 1919 Castellani & Chalmers referred to Bacteroides, as those organisms which are non-spore forming rods, but did not state any reaction to Gram stain. Gram-positive organisms were later excluded from the genus by Weiss & Rettger (1937).

Oliver & Wherry in 1921, isolated black pigmented bacteria from the mouth, faeces, urine and and infected wounds and assigned them the name Bacterium melaninogenicum. Knorr in 1922 isolated four strains of fusiform bacteria from carious teeth and these isolates were assigned to three groups by morphological criteria. He considered all strains belonged to the same genus and suggested the generic name Fusobacterium to replace the generic name of Bacillus used earlier.

The first detailed systematic analysis was published by Eggerth & Gagnon (1933) in which 18 species were described. Following the earlier suggestion of Castellani & Chambers (1919), the new generic name Bacteroides was adopted by Eggerth & Gagnon (1933) and speciation was based on differential morphology and fermentation of 20 carbohydrates. The species described were designated as B.fragilis (Syn.: B.convexus), B.vulgatus, B.distasonis, B.ovatus and B.thetaiotaomicron. These organisms were later regarded as being similar to B.fragilis and were confirmed as subspecies of B.fragilis (Moore & Holdeman, 1973) with the exception of B.convexus which was shown by Werner (1969) to be identical with B.fragilis and was termed B.fragilis ss. fragilis by Moore & Holdeman (1973). These authors also regarded B.thetaiotaomicron (Distaso, 1912) as subspecies of B.fragilis and labelled it B.fragilis ss. thetaiotaomicron.

The work of Prévot which extended over three decades and which is presented in his manual (1966) represented the first attempt to employ more reliable taxonomic criteria such as acid end product analysis, quantitation of purines and pyrimidines, measurement of oxidation reduction potential and the use of a variety of experimental animals for pathogenicity tests. The genus Bacteroides, Prévot referred to as Ristella and several new species were described but these were based mainly on morphological characteristics.

In 1957, the editors of Bergy's Manual divided the family Bacteroidaceae, on the basis of morphological differences, following an earlier suggestion of Weiss & Rettget (1937) and based specifically upon the work of Prévot. Genera described were: Bacteroides, Dialister, Sphaerophorus, Fusobacterium and Streptobacillus. Thirty species were included in the genus Bacteroides, most species fermented glucose and rarely lactose and sucrose. Three species were considered to be proven pathogens, B.fragilis, B.serpens and B.melaninogenicum.

Bryant et.al., (1958) successfully isolated a new species from the rumen of cows and assigned them the name B.ruminicola. They divided the species into two subspecies, B.ruminicola ss. ruminicola and B.ruminicola ss. brevis, on the basis of morphology and growth requirements.

In 1964 another new species was isolated from the human mouth by Loesche et.al., (1964). They

isolated bacteroides-like organisms and divided them into two subspecies, B.oralis ss. oralis and B.oralis ss. elongatus. The latter strain was found by Holdeman & Moore (1972) to resemble Fusiformis nucleatus var ochraceus which was first described by Prévot et.al., (1956). Holdeman & Moore (1972) re-classified the two organisms isolated, as B.oralis and B.ochraceus.

Finegold et.al., (1967) preferred a division into groups rather than species. On the basis of morphology, physiological tests and antibiotic susceptibility patterns, five groups were described; three of these groups were B.fragilis, B.oralis and B.melaninogenicus.

Several workers believed that the latter system of classification used too few tests for such a heterogeneous collection of strains (Spiers, 1971) and a numerical method was suggested. Barnes & Goldberg (1968) compared 72 strains of Gram-negative, non-sporing anaerobes by numerical phenetic analysis (Sneath 1957).

Based on their DNA homology studies Cato & Johnson (1976) restored the species rank to B.fragilis, B.ovatus, B.vulgatus, B.distasonis and B.thetaiotaomicron. This was later accepted and used by Holdeman, Cato & Moore in their Anaerobic Manual (1977).

Bacterium melaninogenicum was the name originally given by Oliver & Wherry (1921) to the organisms they isolated. These organisms have been variously

classified as Haemophilus melaninogenicus (Bergy's Manual, 1939), Ristella melaninogenicus (Prévot, 1938), Fusiformis nigrescens (Schwabacher, et.al., 1947). Editors of Bergy's Manual (1939) accepted the term Bacteroides melaninogenicus which was proposed by Roy & Kelly (1939).

Sawyer, et.al., (1962) divided B.melaninogenicus into three groups of the basis of biochemical characteristics. These groups were later recognised as subspecies by Cato, et.al., (1970) and confirmed as B.melaninogenicus ss. melaninogenicus, B.melaninogenicus ss. intermedius and B.melaninogenicus ss. asaccharolyticus by Moore & Holdeman (1973) on the basis of carbohydrate fermentation and acid end product analysis. Williams, et.al., (1975) and Shah, et.al., (1976) in a later study recommended the elevation of B.melaninogenicus ss. asaccharolyticus to species rank. This was accepted by the International Taxonomic Subcommittee for Gram-negative Anaerobic Rods (Finegold & Barnes, 1977) and B.asaccharolyticus was amended to a species level.

Further investigation by Shah & Collins (1980) on the species of B.asaccharolyticus revealed the presence of two subgroups within the species based on lipid analysis and DNA/DNA reassociation studies, (7), (9).

A new subspecies, B.melaninogenicus ss. macacae, was isolated by Slots & Genco (1980). They based their classification on; biochemical,

immunological properties and acid end product analysis. The strain was isolated from experimentally induced periodontitis in a monkey.

1.3 Review of Methods used for Bacteroides Identification

Much confusion has existed in the classification of Gram-negative, non-sporing anaerobic bacilli. A number of clearly different organisms have been given the same name and similar organisms have been given different names. Werner (1969) first described a method for demonstrating that B.convexus was a similar organism to B.fragilis.

A wide variety of approaches have been adopted in order to identify these organisms. Biochemical tests of anaerobic bacteria were found to be of taxonomic value and they were used in conjunction with other tests as basis for Bacteroides current system of classification (Holdeman, et.al., 1977); Dowell & Hawkins, 1976. Most workers have used these tests to aid their identification of Bacteroides species, Bryant, et.al., (1958); Loesche & Gibbons (1965); Starr, et.al., (1973); Mitsuoka, et.al., (1974); Holbrook & Duerden (1974); Schreckenberger & Blazevic (1974 & 1976); Williams, et.al., (1975); Cato & Johnson (1976); Shah, et.al., (1976); Smibert & Holdeman (1976); Holbrook, et.al., (1977); Holland, et.al., (1977); Finegold & Barnes (1977); Shayegani, et.al., (1978); Slots & Genco (1980); Occhionero, et.al., (1982) and Chindler & Idlbek (1982).*(10),(11) Chromatographic analysis of fermentation

end products was noted as an easy and rapid method for identification of Bacteriodaceae (Cato, et.al., 1970; Moore, 1970 and Holdeman & Moore, 1974),(8)(9). This technique will be discussed in detail in a later section.

Identification of these organisms at a less differentiated level has been based on tolerance tests using:

(i) Antibiotic resistance (Barnes & Goldberg, 1962; Finegold, et.al., 1967; Ingham, et.al., 1970; Sutter & Finegold, 1971; Werner, 1972; Mitsuok, et.al., 1974; Peach, 1975; Jones & Fuchs, 1976; Draper & Barry, 1977; Holbrook, et.al., 1977; Borriello, et.al., 1979 and Essers, 1982).

(ii) The effect of dyes on growth (Baird-Parker, 1957; Barnes & Goldberg, 1962; Suzuki, et.al., 1966, Holbrook, et.al., 1977).

(iii) Sensitivity of bile salts (Shimada, et.al., 1970; Draper & Barry, 1977; Holbrook, et.al., 1977).

Ultraviolet red-fluorescence has been noted in B.melaninogenicus (Myers, et.al., 1969; Shah, et.al., 1979) and may be useful for early identification of these organisms as the observation of fluorescence can be made before black pigment is produced in the colony.

The ratio of DNA base composition indicates fundamental similarities or differences between bacteria. This property has been studied by Sebald (1962); Sebald & Veron (1963); Johnson (1973); Williams, et.al., (1975); Shah, et.al.(1976).

Cell wall analysis of B.melaninogenicus has been carried out by several workers, Hofstad & Kristoffersen (1971), Kasper (1976) and Shah, et.al., (1976 and 1980),(12).

A large number of characteristics have been studied for numerical taxonomy studies of the Bacteroides by Barnes & Goldberg (1968) and Sundqvist (1976),(3).

Other methods found useful for identification of Bacteroides species, have included:

(i) The separation of protein by gel electrophoresis (Storm, et.al., 1976; Hofstad, 1976; Swindlhurst, et.al., 1977; Poxton & Brown, 1979; Mansheim & Coleman, 1980),(4),(5),(10),(11).

(ii) Serological grouping of Bacteroides species (Werner, 1969; de La Cruz & Cuadra, 1969; Sharpe, 1971; Reinhold, 1971; Beerens, et.al., 1971; Lambe & Moroz, 1976; Lambe & Jerris, 1976; Hofstad & Fredriksen, 1979; Meisel-Mikolajczyk & Grzelak-Puczyska, 1981.

(iii) Direct fluorescent antibody staining (Griffin, 1970; Garcia, et.al., 1971; Lambe, 1974; Stauffer, et.al., 1975;

Mansheim, et.al., 1979; Grezelak-Puczynsk and Mikolajczyk, 1981.

(iv) The production of bacteriocin-like substances by some species of B.fragilis (Podhaisky & Reinhold, 1970; Collee, et.al., 1974; Watt, et.al., 1974).

1.4 Chromatography and Bacteroides

Gas chromatography (GC) developed largely since 1951, has become the preferred method for rapidly and accurately analyzing many volatile substances. In essence, the volatile material is injected into a column containing a liquid absorbant supported on an inert solid. The basis for the separation of the component of the volatile material is the difference in the partition coefficients of the component as they are carried through the column by an inert gas such as helium or nitrogen. Two types of detectors are generally in use, thermal conductivity and hydrogen flame ionization detectors. The latter are widely used for fatty acid analysis, they have extreme sensitivity, a wide linear response, and are insensitive to water.

Gas liquid chromatography (GLC) analysis of fermentation end products is of immense value to the study of obligately anaerobic bacteria. The most intensively investigated end products in this context are the volatile fatty acids of the series formic to heptanoic and certain other short chain

carboxylic acids (notably lactic and succinic). These end products are generated by anaerobes as a result of fermentation of carbohydrates and amino acids, and are excreted in low concentrations into the growth environment. GC has the potential to analyse minute quantities of closely related chemical substances and is eminently suited for rapid separation, sensitive detection and accurate quantification of these anaerobic bacterial metabolites.

The application of this technique with respect to anaerobes was initiated and extensively explored by workers at the Anaerobe Laboratory of Virginia Polytechnic Institute (Eiken, 1958; Moore, 1970; Cato & Salmon, 1976; Finegold & Barnes, 1977). Their studies of a wide range of anaerobic bacteria showed that qualitative and quantitative differences in end products of metabolism were associated with different genera and species, and that these characteristic were stable, and, therefore, of taxonomic value. The fundamental importance of this concept had great impact on the development of a more rational classification of anaerobic bacteria, particularly the non-sporing anaerobes.

Although acid production in bacteria, especially in anaerobes, has long been known to be a constant property of genera or species, it was the introduction of GC into bacteriology (Moore, et.al., 1966) that revolutionized the analysis of many products which formerly required labour and time consuming methods. Whereas many morphological

and biochemical characters of anaerobes are found to be unreliable for both classification and identification, the acid end products of metabolism can generally be considered as characters of prime taxonomic importance. The fatty acid formed as an end product of fermentation, is therefore of particular importance in the current system of classification (Holdeman, et.al., 1977).

Classification of members of the Bacteroidaceae on the basis of their fatty acid end products was first attempted by Guillaume, et.al., (1956) by means of distillation and paper chromatography and later improved and used by, Charles & Barrett (1963); Slifkin & Hercher (1974). GLC was developed by Moore, et.al., (1966); Werner (1969) and Cato et.al., (1970).

Cato, et.al., (1970) and Moore (1970) noted the use of acid end product analysis in the identification of Bacteriodaceae. The results of analysis were used in conjunction with the result of more conventional biochemical test to identify Gram-negative, non-sporing anaerobes. Their system of identification has been followed by several authors, Loesche & Gibbons (1965); Barnes & Goldberg (1968); Werner, et.al., (1971); Werner (1974); Harding, et.al., (1975); William, et.al., (1975) and Shah, et.al., (1976),(8),(9).

Sasaki & Takazoe (1974) studied 116 strains of B.melaninogenicus and classified them using GLC into three types based on the pattern of their

volatile fatty acids. They also found a correlation between acids production and carbohydrates fermentation pattern.

Mayhew, et.al., (1975) studied the effect of time and growth media on fatty acids production by B.fragilis using GLC. They assayed the fatty acids chromatographically in 5 different media: sterile ascitic fluid; chopped meat glucose; ascitic fluid, chopped meat glucose (1:1); peptone yeast and peptone yeast glucose medium. The strains studied were four strains of B.fragilis and one strain of B.vulgatus isolated from clinical specimens. Their results showed acetic and succinic to be the prominent end products encountered and they were readily detected within 24 hours. Propionic, isobutyric, butyric, isovaleric and lactic were usually recorded in more limited quantities. They also found out that maximum rates of bacterial multiplication, glucose catabolism and acids production coincided with the first 24 hours in carbohydrate supplemented media. Extended incubation (672h) was found to favour substantial increase in succinate production, in three of five media. Their findings suggest that media composition and incubation time are an important determinant on the production of fatty acids by B.fragilis and B.vulgatus.

Wüst, (1977) applied GLC for presumptive diagnosis of bacteraemia, with the results suggesting that quantitative GLC is a useful and rapid

method for detection of obligately anaerobic bacteria in blood cultures.

Another techniques in gas chromatography that was popular with most authors in this field due to its reliability and speed was gas solid chromatography (GSC). A technique that was used to a lesser extent was head-space chromatography which was used for the analysis of the acidic end products and alcohols resulted from metabolic fermentation.

Shah, et.al., (1976) carried out a quantitative analysis of acid end products on 45 strains of black pigmented bacteroides using GSC. The analysis was carried out in conjunction with other tests such as; biochemical tests; cell wall analysis; DNA base composition analysis; electrophoresis and antibiotic susceptibility tests. Their results showed 22 strains of B.melaninogenious ss. intermedius produced isobutyric, isovaleric and succinic acids. Eight strains of B.melaninogenicus ss. melaninogenicus produced acetic and succinic acids, whereas B.melaninogenicus ss. asaccharolyticus produced a mixture of acetic, propionic, isobutyric, butyric, isovaleric and lactic acids. Their results showed the three subspecies to have a different fatty acid profile which is useful for their identification. Based on the results of various tests, they recommended B.melaninogenicus ss. asaccharolyticus to be given a species rank.

Characterization of anaerobic bacteria using quantitative GSC was carried out by Bohannon, et.al.,

(1978). They studied Clostridium novyi; Cl.septicum; Cl.sporogenes; B.vulgatus and Propionibacterium acnes in two different growth media, cooked meat (CM) and peptone yeast glucose (PyG) media. Their results showed that cultures can be characterized by distinctive molar proportions of acid fermentation products in the growth medium.

Deacon, et.al., (1978) used GSC for identification of Bacteroidaceae, but described it as GLC. They recommended the technique for routine hospital work due to its accuracy and speed. They analysed 180 isolates from the family Bacteriodaceae and evaluated the effect of different media on the production of fatty acids. They studied B.fragilis; B.thetaiotaomicron; B.vulgatus; B.ovatus; B.distasonis; B.oralis; B.ruminicola; many heterogenous strains of B.melaninogenicus and many strains belonging to different genera of the family Bacteriodaceae. The five media used were different strengths of BM medium. The results showed that all strains were identified to generic level by simple microscopy, colonial observation and GSC analysis, but further tests were required to allow species or subspecies identification for most strains.

Lindner & Marcelis (1978) studied the use of GSC for identification of Bacteroides species and the effect of different growth conditions on the level of fatty acids produced. 56 freshly isolated strains of bacteroides from stool and

lesions were found using quantitive GSC to belong to the species, B.fragilis; B.vulgatus; B.distasonis; B.thetaiotaomicron and B.capillosus. They also found that the presence of haemin affected the type and quantity of acid produced. Head-space gas chromatography was used by Larsson, et.al., (1978) to study the identification of anaerobic bacteria. The end products detected using the above technique were; alcohol and volatile fatty acids. They successfully differentiated B.fragilis; Cl.perfringens and Propionibacterium acnes using the above technique. In addition two stationary phases, FFAP and PorPak Q, were also compared for their suitability for fatty acid and alcohol separation with the result that PorPak Q was found to be more suitable.

As mentioned earlier, GC is applicable only to the analysis of volatile compounds. Non-volatile substances must be chemically modified or thermally degraded to lower boiling components. Pyrolysis-GC, on the other hand, offers a method for thermally degrading a complex molecules to yield more volatile substances with a small molecular weight.

1.5 Pyrolysis Gas Chromatography

Pyrolysis is the transformation of complex materials into a series of lower molecular weight substances through the agency of heat alone. It

is carried out in a chemically inert environment and, in practice, at temperatures between 200° and 1200°C. Reaction in the presence of oxygen (combustion) tends to yield uniform volatile fragments, e.g. CO_2, NH_3, H_2O, but in pyrolysis no net oxidation can occur, thus molecules are cleaved at their weak point to form smaller, more reduced fragments which are more characteristic of the original material (Drucker, 1976). Pyrolysis for the purpose of determining the chemical composition of polymers has been in use since 1860. Pyrolysis was first allied to spectrometry by Zemany (1952) who showed that complex materials decompose in reproducible fashion provided the pyrolysis parameters are carefully standardized. The adaption of a pyrolyser to gas chromatography was first reported by Davison, et.al., (1954), who pyrolysed chemical polymers at 650°C and produced characteristic patterns.

The impetus for the application of pyrolysis to microbiological problems came from the United States Space Exploration Programme which developed and built a miniaturized automated system to test for the presence of extraterrestrial life (Wilson et.al., 1961). The original system was intended for the surveyor series of Lunar landing craft, but it was never used. However, a pyrolysis gas chromatography mass spectrometry experiment was included on the Viking series of Mars Landers to

search for organic constituents in the Martian soil. The interest in using pyrolysis techniques to identify microorganisms stemmed directly from this work by the National Aeronautic and Space Administration (N.A.S.A.).

Application for pyrolysis techniques other than in microbiology have been extensively reviewed by Levy (1966) and May, et.al., (1977). In addition, the full range of biomedical uses for pyrolysis have been reviewed by Irwin & Slack (1978).

(A) Technical Aspects of Pyrolysis

(1) Pyrolysers

A major factor in the use of pyrolysis is the design of the instrument, which ideally should have a small "dead volume" and should allow pyrolysis to be carried out as close to the column inlet or ion source as possible. There are two broad categories of pyrolysers:

(a) Continuous mode units: This type usually consist of a cylindrical oven held at constant temperature into which a miniaturized sample boat is introduced. Lack of control of the temperature/time characteristic of the sample have made these devices

obsolete for precise analytical work and they have no future in pyrolysis of microorganisms.

(b) Pulse mode units: This type comprise three basic types

(i) Filament pyrolysers - usually resistive heating of platinum ribbons or coil, see Levy, et.al., (1972).

(ii) Curie-point pyrolysers - inductive heating of ferro-magnetic wires, see Giacobbo & Simon (1964); Bühler & Simon (1970).

(iii) Laser heating system have been suggested by Folmer & Azarrago (1969).

In Curie-point pyrolysis the solid sample is applied to a ferromagnetic wire (typically 100mm x 0.5mm) which is then placed in the work coil of a high frequency oscillator. Eddy currents are induced causing the surface temperature to rise. At the Curie-point, the wire becomes para magnetic and can no longer induct power, which promotes a drop in temperature causing the wire to become ferromagnetic and recommence heating. Thus at the

Curie-point the wire is acting as a thermostatic switch, the temperature being determined by its chemical composition (Dyson & Littlewood, 1968). A pure iron wire has a Curie-point of 770°C and a pure nickel wire, 358°C. Common alloys of the two have Curie-points between these two extremes but clearly the choice of pyrolysis temperature is more limited than with the resistive heating methods.

Actual pyrolysis temperatures used in studies on microorganisms have varied from 500°C (Oyama, 1963) to 1200°C (Cone & Lechowich, 1970) with the majority of authors using temperatures in the narrow range 770°C - 900°C. There is general agreement that varying pyrolysis temperature above 500°C and below 1000°C does not noticably affect the pyrolysate, given standardisation of the temperature rise time.

(2) Chromatographic Methodology

A wide variety of instruments and chromatographic conditions have been employed but some aspects of the methods have become relatively standardized. Nevertheless, there is still a long way to go before a unified methodology can be recommended for microbiological application. If this were to happen, the primary area of standardization would be the analytical column and the parameter for controlling the temperature of

the gas chromatograph oven during the analysis cycle.

Most separations in pyrolysis gas liquid chromatography PGLC have been performed with packed columns which consist of tubing packed with a granular, porous support material impregnated with a liquid stationary phase. The porous structure of the support results in a fine dispersion of the liquid as a thin film which provides a large interfacial area between the stationary phase and the gaseous moving phase, and a short diffusion path for the solute molecules in the liquids.

Packed columns used for PGLC have been constructed either from glass (Vincent & Kulik, 1970) or copper (Reiner, 1965) and have varied in length from 1.8m (Cone & Lechowich, 1970) to 6m (Reiner, 1967). The latter figure reflects the capacity of most chromatograph ovens. Bore sizes for packed columns have varied between 0.76mm (Reiner, 1967) and 4mm (Vincent & Kulik, 1970). Since PGLC involves temperature programming of the gas chromatograph ovens, packed column are used in pairs so that the second may act as a "blank" to compensate for the increase in detector current resulting from bleed of the stationary phase at elevated temperatures.

Another type of column used for PGLC is the capillary column of which there are two types.

Wall coated open tubular (WCOT) colums are long small bore tubes with the inner wall coated directly with the liquid phase. In support coated open tubular (SCOT) columns the inner wall is coated with a porous support layer which is impregnated with the liquid phase. Generally capillary columns have greater separating efficiencies than packed column because of their higher number of theoretical "plates" (Mitruka, 1975). Capillary columns used for PGLC have been constructed from either stainless steel (Meuzelaar & In't Veld, 1972) or glass (Needleman & Stuchberg, 1977). PGLC employing short packed column is regarded as "low resolution" and those using capillary as "high resolution". This terminology is linked with the ability of a column system to separate the individual chemical constituent of a pyrolysate and resolve them as individual peaks on a pyrogram.

As there is no single parameter which can describe the retention behaviour of a liquid phase the term polarity is used to classify them. Generally the greater the polarity of a liquid phase, the greater the retention of a polar solute relative to that of a non-polar solute with a similar boiling point. Simmonds (1970) in a pyrolysis gas chromatography-mass spectrometry study found as many non-polar as polar products in bacterial pyrolysate. Needleman & Stuchberg (1977) eluted similar numbers of components from,

a non-polar Apiezon L, 4m packed column as they did from a polar Carbowax 20M column of exactly similar dimensions. Most PGLC studies have been Carbowax 20M which is both polar and tolerant of temperature up to 200°C, but there is no clear theoretical justification for this. The need to operate at temperatures in excess of 200°C to separate high boiling-point products of the pyrolysis of microorganisms has created a requirement for a polar stationary phase with better thermostability. Some authors e.g. Reiner & Kubica (1969) have used terephthalic acid terminated variety of Carbowax 20M which has a higher thermostability, probably as high as 250°C. French, et.al., (1981) examined the suitability of eight solid stationary phases for PGLC of microorganisms. Five different bacterial genera were well discriminated and some differentiation was achieved between different isolates of Streptococcus mutans but similarity between pyrograms was unrelated to orthodox taxonomic grouping. The best discrimination was achieved with Chromsorb 104 followed by Chromosorb 101 and Tenax -GC.

Pyrolysate of whole cells consists of components of widely varying boiling-points. Thus temperature programmed gas chromatography is employed in which the temperature of the column is increased gradually during the analysis.

The advantage of this is to allow the solutes to move through the column at a temperature which is optimal for their separation when the initial temperature and the heating rate are appropriate. Temperature programming has been used by most authors and apart from the early studies of Garner & Chi Yuan (1964) there has only been one report on isothermal p.g.l.c. in conjunction with flame ionization detector in which French, et.al., (1981) pyrolysed different microorganisms isothermally at 100°C. However, Myers & Smith (1972) have reported the application of a rubidium chloride nitrogen detector to pyrolysis products of biological samples, using an isothermal analysis at 160°C.

Temperature programming has usually been from 60 - 80°C to between 160°C and 200°C at varying rates depending on the type of column system in use. Capillary columns are used with rates as low as 5°C/min (Meuzelaar, et.al., (1975a) whilst packed column use 10 or 12°C/min (Cone & Lechowich, 1970; Reiner, 1965). A similar distinction can be drawn for carrier gas flows which are usually in the ranges 15 - 40 ml/min for packed columns (Reiner, et.al., (1972) and 2 - 6 ml/min for capillary columns (Meuzelaar et.al., (1975b).

(3) Sample Preparation

Methods of sample preparation prior to PGLC are dependent on the nature of the material under analysis and the type of pyrolyser but are all designed to limit interference from extraneous organic material. For pyrolysis of microorganisms this means avoiding contamination from medium components because the pyrograms of media closely resemble the pyrograms of bacteria especially when using low resolution columns (Fontanges, et.al., 1967). High resolution columns, on the other hand, easily distinguished a mycoplasma culture medium from the mycoplasmas themselves (Quinn, 1976).

The general approach of most workers have been to prepare samples as free from medium components as possible using a scheme such as that of Wickman (1977).

In Curie-point pyrolysis the sample is applied to a 0.5 mm diameter ferromagnetic conductor. Meuzelaar & in't Veld (1972) prepared suspensions in carbon disulphide and coated clean wires by drop wise addition. Carbon disulphide is particularly useful in this respect as it evaporates away easily after coating and is not detected by the flame ionisation detector of the gas chromatograph.

An alternative method was developed by Oxborrow, et.al., (1976) who prepared bacterial samples for PGLC by growing cells on membrane filters placed on the top of an ordinary culture plate. They found that cells could be harvested from the filters and provide reproducible pyrograms without any further preparation.

Fungi represent a special problem for pyrolysis as there are a number of possible ways of collecting samples which had been discussed by many authors such as, Vincent & Kulik (1970); Sekhon & Carmichael (1972); Kulik & Vincent (1973); Stretton, et.al., (1976) and Taylor (1976).

(4) Importance of Secondary Reactions

In pyrolysis the primary rupture of bonds is frequently followed by a complex series of competitive reactions between the radical and the fragment formed (Levy, 1967). The course of these secondary reaction is largely dependent on the experimental conditions and the design characteristics of the pyrolysis unit (Pyrolyser). The design of the pyrolysers which offer minimum dependence of the thermal fragment pattern upon secondary reactions is a major objective because it should lead to a maximum

correlation between the molecular structure of the parent material and the qualitative-quantitative characteristic of the pyrolysis products (pyrolysate).

The two most important factors affecting the extent of secondary reactions and hence the nature of the pyrolysate are the quantity of sample to be pyrolysed and the temperature/time characteristic of the pyrolysis regime (Levy, 1967). It has been shown repeatedly that the quantity of pyrolysis products formed from secondary reactions is directly related to the quantity of material pyrolysed (Jones& Moyle, 1961; Simon & Giacobbo, 1965). For many years, it was thought that the final equilibrium temperature of pyrolysis was a critical factor in determining pyrolysate composition, but Farré-Rius & Guiochon (1968) showed that in a practical situation polymer decomposition was controlled by the heating rate of the sample. At equilibrium temperature the half decomposition time of many chemical polymers is several orders of magnitude smaller than the time taken to reach that temperature. Meuzelaar & in't Veld (1972) suggested that since the bulk material of bacteria is polymeric, the statement may be expected to hold true for microbiological specimens as well. The direct effect of the

temperature/time profile on the yield of pyrolysis products was shown by Ericsson, et.al., (1977) using Bacteroides fragilis. Ericsson found that long temperature rise times allowed high boiling point compounds to escape from the pyrolyser before true pyrolysis. So high pyrolysis temperature with short temperature rise are desirable.

(5) Reproducibility and Repeatability

Reproducibility may be defined as a comparison of the performance of an analytical system at a given time or over a short period of time, whereas repeatability is the comparison over long periods where factors, not apparent in day-to-day reproducibility, may become extremely important.

Reproducibility can be of two types: (i) basic reproducibility of successive analysis, and (ii) inter-laboratory reproducibility. If basic reproducibility was a problem, very little work would have been done with PGLC, Meuzelaar, et.al., (1975b) evaluated five successive pyrograms obtained during unattended operation of the automated PGLC system. Thirty well defined peaks were measured on each pyrogram and the peak height values proved reproducible

to within ± 5%. Very often no attempt has been made to quantify reproducibility and pyrograms have been compared by visual assessment (Oxborrow, et.al., (1977a, b). Nevertheless, there is little doubt that the kind of figures quoted by Meuzelaar, et.al., (1975b) are generally applicable.

Repeatability can be adversely affected by two factors (i) changes in the analytical conditions, and (ii) deviations from a standardized procedure for preparation of samples. Analytical parameters duch as carrier gas flow rate or temperature programming regimes must be standardised (Sibley, et.al., 1973; Sweeley, et.al., 1974).

In practice, most of the changes in the analytical system are a result of slow deterioration of the chromatography column. Quinn (1974) noticed changes in the resolution of pyrolysate compounds after 200 analyses using a 4% Carbowax 20M capillary column. Needleman & Stuchbery (1977) experienced similar problems with a Carbowax 20M SCOt column. They noticed that the first few coils of the column became grossly discoloured (almost black), whilst the remainder had turned to light brown. This resulted in leading and tailing of larger peaks of the pyrogram as well as a decrease in the resolution of some smaller components. Both Quinn (1974) and Needleman and Stuchbery (1977) attributed this degradation to condensation of high boiling-point tar residues on the first section of the column, which interferes with the

initial adsorption of the pyrolysate.

Mitchell & Needleman (1978) suggested a more automated approach to solving this problem using a technique known as back-flushing, where the direction of carrier flow is reversed after the more volatile sample components are vented off to the atmosphere and do not reach the analytical column. Stack, et.al., (1977) were able to complete over 1500 analyses using a 15% Carbowax 20M 3M packed column. This was attributed to regular monthly servicing during which all column connections were cleaned and the first 10 cm of column repacked with fresh material.

Column degradation can also be due to loss of stationary phase caused by continual high temperature operation. Carbowax 20M has been the preferred phase of PGLC studies of biological materials. Although it is temperature stable up to 225°C, it bleeds from the column at temperature above 140°C. Since temperature programming nearly always goes beyond this point, gradual diminution of the amount of phase on the column is inevitable.

The problem of column ageing is exacerbated by the difficulty in replacing an analytical column with one exactly similar in performance. Sekhon & Carmicheal (1973) found that analyses of a dermatophyte run on a fresh analytical column did not group with the original analyses when added to the library of data. On the other hand, Haddadin,

et.al., (1973) found that programs remained essentially similar when a column was replaced with a new one of identical specifications. Stack, et.al., (1977) calculated mean values for 15 peaks from five cultures which were pyrolysed into two different Carbowax 20M packed columns. Similarity(S) values were calculated between each individual set and a set representing the mean for all five cultures. These five S values for each column were found to match to within 5%, the two mean S values matching to within 3%. It thus seems possible to prepare columns with similar separation properties, but this is usually a result of experience, rather than any hard scientific knowledge.

Any departures from a standardised microbiological methodology have a marked effect on reproducibility. As a general rule changing the growth medium alters the pyrogram (Garner & Chi Yuan, 1964; Oyama & Carle, 1977; Cone & Lechowich, 1970; Meuzelaar, et.al., 1975a; Gutteridge & Norris 1980). Reiner & Ewing (1968) examined pyrograms of E.coli strains grown on blood agar base or peptone yeast base and found that although there were differences between the fingerprints, a number of characteristic peaks remained unaltered. Oxborrow, et.al., (1977b) grew six strains of Bacillus species on three different media and found several differences between the pyrograms,

particularly those peaks most characteristic for individual species. They also tested the effect of growing samples on six different manufacturer's batches of trypticase soy agar and found that there was no noticeable effect on the reproducibility of the fingerprint.

The effect of growth time on pyrogramme reproducibility has also been investigated with respect to Bacillus species by Oxborrow, et.al., (1977a) They noticed a "marker" peak for the physiological process of sporulation as well as a number of other changes in the pyrograms as the culture age. They concluded that this aspect of PGLC needs to be standardised as far as possible, an approach adopted by most workers. In contrast, Reiner, et.al., (1969) found no significant differences between the pyrograms of 2 and 6 weeks old cultures of mycobacteria. The most recent report on effect of different growth conditions on PGLC of the three different species was published by Gutteridge & Norris (1980), in which they used high resolution PGLC to discriminate E.coli, Pseudomonas putida and Staphylococcus aureus grown under a variety of conditions. They found that changing the culture media drastically altered the quantitative aspects of pyrograms of all three organisms, but the effects of culture time and incubation temperature were less severe.

Lambert, et.al., (1976) noticed a curious effect of consecutive subculturing of Salmonella typhimurium. Pyrograms prepared after each subculture showed variations in the relative concentrations of pyrolysis products. This may have been due to a slow degradation of the analytical column rather than any microbiological factor.

Inter-laboratory reproducibility is an ideal which is obviously difficult to obtain because of the difference in the pyrolysis equipment and gas chromatographic technique. However, a preliminary evaluation of the products detected and identified by Meuzelaar & in't Veld (1972) and Quinn (1974) showed many to be identical despite the use of different pyrolysers and column systems. This suggest that pyrolysates of bacteria produced by different systems may be comparable and inter-laboratory reproducibility might eventually be obtained by relating pyrogram peaks to chemically identifiable internal standards.

Stack, et.al., (1977) tried a different approach to this problem by comparing data derived with their PGLC system with a number of analyses of the same strains carried out on different system by Meuzelaar et.al., (1975a). The results were compared using the same quantitative estimate of similarity. Self comparison value for Streptococcus mutans BHT and Streptococcus mutans C67-1 were 0.91% and 0.82% according to Stack, et.al., (1977)

and 0.91% and 0.85% according to Meuzelaar, et.al., (1975a). Comparison of the C67-1 strain with BHT strain produced similarities of 0.71% and 0.62% in the respective laboratories. It seems likely that if a standard methodology could be developed for handling PGLC data the differences between strains as revealed by pyrolysis may prove to be more reproducible between laboratories than are the actual pyrograms.

(B) Application in Microbiology

Wilson, et.al., (1961) and later Oyama (1963), table 1, part A and B, reported on their earlier studies of PGLC, but their crude pyrolysis technique employed then did not establish the potential of PGLC for reproducible finger-printing of microorganisms. The credit for this achievement goes to Reiner (1965), who has been the leading authority in this field ever since, and should be regarded as the "father" of PGLC in microbiology.

Table 1 summarizes the application of PGLC in bacteriology and provides a chronologically arranged list of authors, methods (part A) specimens and chief results (part B), as derived from the available literature up until 1982.

The role of PGLC in mycology has been emphasized by several worker who have used this

Table 1. Part A: Application of Pyrolysis in bacteriology

Year	First Author	Pyrolyser		Pyrolysis Conditions			Sample Weight (µg)	COLUMN			Temp.program		Pyrogram Evaluation
		Type	Make	TRT (Sec)	Temp (°C)	Time (Sec)		Type	Length +Bore	Coating	Range (°C)	Rate (°C/min)	
1961	Wilson,E.M.	oven	own	?	500	600	?	packed glass	3.6m ?	PEG 20M 5%	66	n.a.	Visual
1963	Oyama,V.I.	oven	own	45	500	180	10-200	capill.	90m 0.25mm	DEGS (?)	?	?	?
1965	Reiner,E.	filam.	Barber Colman (?)	?	850	10	200-800	packed copper	2.4m ?	PEG 20M 15%	50-240	12	Visual
1967	Reiner,E.	ribbon	Barber Colman (?)	?	850	10	?	packed copper	6m 0.76m	PEG 20M 10%	0-200	12	Visual
1967	Oyama,V.I.	oven	own	45	450	240	10-000	capill.	66m 0.25mm	PEG 20M 6%	50-150	4	peak height measure-ment
1968	Reiner,E.	ribbon	Barber Colman (?)	?	850	10	100-275	packed copper	6m 0.76m	PEG 20M 10%	0-200	12	Visual
1969	Reiner,E.	ribbon	Barber Colman (?)	?	840	10	100-125	packed	6m	PEG 20M	0-180	12	peak height measure-ment
1969	Reiner,E.	ribbon	Barber Colman (?)	?	840	10	100-125	packed copper	6m 076mm	PEG 20M (TPA) 10%	0-180	12	peak height measure-ment
1970	Cone,R.D.	filam.	Hewlett-Packard	?	1200	12	900-1100	packed copper	1.8m ?	EGA 5%	50-240	10	Visual

Table 1, Part A: Continued

1971	Reiner,E.	ribbon	Barber Colman (?)	?	850	10	100-125 ?	packed copper	6m 1.7mm	PEG 20M (TPA) 5%	30-175	12	Visual
1972	Menger,F.M.	ribbon (?)	?	?	850 (?)	10 (?)	90-100	packed copper	6m 1.7mm	PEG 20M (TPA) 5%	20-175	12	Peak area measure-ment
=	Meuzelaar H.L.C.	Curie-point	own	0.08	770	0.2	10-15	capill. S.S.	50m 0.25mm	PEG 20M 10%	75-165	6	Visual
=	Reiner,E.	ribbon	Barber Colman	?	850 (?)	10 (?)	80	packed copper	6m 1.7mm	PEG 20M (TPA) 5%	20-175	12	Visual
=	Sekhon,A.S.	filam.	Carlo-Ebra	?	800	20	1300-1800	packed	2m (?)	DEGS 6%	50-200	7.5	peak height measure-ment
=	Haddadin, J.M.	Filam.	Pye	?	800	5	?	packed S.S.	3.6m ?	PEG 20M ?	50-145	12	Visual
1973	Meuzelaar H.L.C.	Curie-point	own	0.1	610	1	50	capill. (SCOT) S.S.	32m 0.5mm	PEG 20M 10%	70-140	5	peak height measure-ment
=	Huis in't Veld J.H.J.	Curie-point	own	0.08	770	0.6	30-40	Capill. (SCOT) S.S.	32m 0.5mm	PEG 20M 10%	70-140	5	peak height measure-ment
1974	Quinn, P.A.	filam.	Barber Colman	?	600	8	80	capill.	152m 0.76mm	Carbowax 20M 4%	50-200	10	Visual
1977	Oxborrow G.S.	oven	own	?	800	10	10	packed glass	6m 3mm	Carbowax 20M 10%	50-180	6.5	Visual

Table 1, Part A: Continued

1977	Ericsson I.	Filam.	own	?	Various	8	6	packed stainless steel	3m 2mm	Apiezon L 15%	100	?	peak area measurement
1978	Stack,M.V.	Curie-point	Pye	1	770	15	500	packed glass	3m 3mm	Carbowax 20M 15%	70-230	12	peak height measurement
1978	Macfie.H.Y.	Filam.	?	?	850	10	80	packed glass	≈3m 5mm	Carbowax 20M 15%	75-200	10	peak height measurement
1980	Gutteridge,C.	Curie-point	?	?	610	2	50-100	Capill.	40m 0.5mm	Carbowax 20M ?	70-180	5	peak height measurement
=	French,G.L	Curie-point	Pye	?	770	7.5	100	packed glass	1.5m 4mm	Carbowax 20M 5%	75-200	6	peak area measurement
=	Gutteridge,C	filam.	?	?	850	10	80	packed glass	3m 5mm	Carbowax 20M 15%	75-200	10	peak height measurement
=	O'Donnell, A.G.	Curie-point	Pye	?	610	4	80-100	packed glass	2.6m 5mm	Carbowax 20M 5%	75-200	8	peak height measurement
1981	French,G.L.	Curie-Point	Pye	?	770	8	100	packed glass	1.5m	various solid phases	100	n.a	peak area measurement
1982	Hudson,J.R.	Ribbon	Hew-lett pac-kard	?	800	1	100	Capill	20m 0.2mm	Carbowax 20M	50-190	15	peak area measurement

n.a. = not applicable, ? = no data given, (?) presumed data

Table 1, Part B:

Year	Microorganisms Analysed Genus or Species ()= no. of strains	Successful Differentiation of Genera	Species	Sub-species	Antigenic Types	Special Features and Remarks
1961	Hydrogenomonas (1) Chlorella (1) Neurospora (1)	yes	n.a.	n.a.	n.a.	-design of fully automated system -first p.g.l.c. of microorganisms
1963	"Soil organisms" (3)	?	?	?	?	-influence of pyrolysis temperature
1965	E.coli (18) Shigella (1) Mycobacterium (10) Streptococcus (4)	yes	yes	yes	yes	-first systematic study of coded samples
1967	Mycobacterium (2) + list of other genera (200)	yes (?)	yes (?)	yes (?)	yes (?)	-incompletely documented results
1967	Azobacter (3) Pseudomonas (2) Bacillus (3)	yes	yes	n.a.	n.a.	-influence of culture medium -first computer processing of pyrograms
1968	E.coli (7) Shigella (2) Proteus (1) Providencia (1) Enterobacter (1)	yes	yes	yes	yes	-differentiation of closely related antigenic types
1969	Mycobacterium (5)	n.a.	yes	yes	n.a.	-influence of culture medium and age of culture
1969	Mycobacterium (8)	n.a.	yes	yes	n.a.	-differentiation of drug resistant and non-resistant strains
1970	Clostridium botulinum (8) (3 types)	n.a.	n.a.	yes	n.a.	-differentiation of spores -exceptionally high pyrolysis temperature
1971	Mycobacterium 9 () (5 species)	n.a.	yes	yes	n.a.	-use of dual recorder tracing at different amplication
1972	Salmonella (6) (6 types)	n.a.	yes	yes	n.a.	-first computer matching of pyrograms
=	Neisseria sicca (1) Neisseria meningtides (3) (3 types)	n.a.	yes	yes	n.a.	-splitless coupling of pyrolyser to capillary column
=	Salmonella (47) (47 antigenic types)	n.a.	yes	yes	no	-correlation between serotype and pyrogram found
=	Nannizia (7) Arthroderma (1) Microsporum (3)	yes	no	no	n.a.	-bad GLC column -computer matching
=	Vibrio cholerae (45) (3 biotypes) E.coli (7)	yes	yes	yes	no	-all strains only differentiated from Vibrio cholerae

Table 1, Part B: Continued

1973	Streptococcus (5 species) (10)	n.a.	yes	yes	yes	-computer matching -fully automated system -samples directly taken from blood agar plates
1973	Streptococcus (4 antigenic types) (4)	n.a.	yes	n.a.	yes	-identification of type antigen in whole cells. -differentiation of of purified type antigens
1974	Mycoplasma (1) Sachromyces (1) Bacillus (1) Micrococcus (1)	yes	yes	n.a.	n.a.	-comparison of two types of pyrolyser, SSS and filament
1977	Bacillus (6)	yes	yes	yes	yes	-influence of growth media on pyrograms
1977	Bacteroides fragilis (1)	n.a.	n.a.	n.a.	n.a.	-influence of pyrolysis parameter on volatile organic product
1978	Streptococcus (oral strains) (50)	yes	yes	n.a.	n.a.	-results shows intercolum reproducibility
=	Moraxella (5) Pseudomonas (7) Lactobacillus (5) Micrococcus (5)	yes	n.a.	n.a.	n.a.	-computer matching
1980	E.coli (1) Pseudomonas putida (1) Staphylococcus aureus (1)	n.a.	yes	n.a.	n.a.	-computer matching -influence of variable growth conditions on discrimination of microorganisms
1980	Pseudomonas (5) Acinetobacter (2)	n.a.	yes	no	n.a.	-disadvantages of Carbowax 20M
=	Clostridium (41) related strains (24)	yes	yes	yes	yes	-computer matching -successful discrimination of heterogenous strains
=	Bacillus thuringiensis (18) B.mycoides (8) B.cereus (9)	yes	yes	n.a.	n.a.	-use of dual recorder at different amplication -Statistical discussion
1981	E. coli (1) Pseudomonas aeruginosa (1) Klebsiella aerogenes (1) Staphyloccus aureus (1) Streptococcus mutans (10)	yes	yes	n.a.	n.a.	-isothermal p.g.l.c.
1982	T.glabrato (1) S.cerevisiae (2) Candida species (3) K.pneumoniae P.vulgaris E.cloacae E.coli Streptococcus (2) Staphylococcus (2) P.acnes	Yes	Yes	n.a.	n.a.	-pyrolysis mass-spectrometry -Measured the level of acetamide and propionamide in different bacterial species

See footnotes, part A.

technique for fungal indentification such as; Vincent & Kulik (1970); Sekhon & Carmicheal (1972); Kulik & Vincent (1973); Carmicheal, Sekhon & Singler (1973); Seviour, et.al., (1974); Sekhon & Carmicheal (1975); Stretton, et.al., (1976); Burns, et.al., (1976); Taylor (1976) and Brosseau & Carmicheal (1978).

Pyrolysis has not been widely used in phycology; only few studies on algae have been reported (Nichols, et.al., (1968); Sprung & Wujek (1971); Derenbach & Ehrhardt (1975) and Gunnison & Alexander (1975)).

(c) Detection of Disease

The characteristics of p.g.l.c. namely speed, reliability, specificity and reproducibility have proved to be useful in biomedical studies. Reiner & Hicks (1972a) were able to distinguish normal white blood cells from leukaemic cells using pyrolysis. In a second paper (1972b) Reiner & Hicks reported a prospective method for confirming inborn errors of metabolism in foetuses, as well as detecting genetic disease such as Wilson's disease, Hurlers syndrome, Huntingdon's chorea and Cytinosis. In addition Reiner (1977) reported differences between pyrograms of a normal cell derived from Swiss mice and the profiles of malignant cells. Myers & Watson (1969) pyrolysed normal and

infected plant leaves and a clear distinction was apparent between the two.

In their abstract, Morgan, et.al., (1981) reported on a more direct approach for determination of infection using gas chromatography mass spectrometry for the detection of bacterial constituent (cell wall) in human tissue and fluids without prior isolation and cultivation. Moreover, they are investigating the use of pyrolysis for identification of bacterial infection.

1.6 Bacteroides Experimental Infections

Infection can be defined as an interaction between two or more species, with the infective organism surviving and establishing residence in the host tissues. Such a definition includes both disease producing interactions and non-pathogologic situations such as "latent" or "dormant" infections.

Development of a suitable animal infection model provides a simple quantitative method for evaluating pathogenicity, synergism between species and potential therapeutic agents.

Bacteroides experimental infections in animals was a relatively neglected subject until the 1960's and following the introduction of improved culture and identification techniques numerous studies on the pathological and clinical

importance of bacteriodes were carried out. The earliest work was that of Weiss, in 1937, where he demonstrated the pathogenicity of Bacterium melaninogenicus and its serological types. The pathogenicity of two clinical isolates was tested by injecting dogs intrabronchially, and white mice subcutaneously. Two dogs were injected with large doses of B.melaninogenicum suspended in a menstruum of starch. One animal developed a febrile reaction whereas the other had increased bronchial marking. Mice were inoculated subcutaneously in the groin with 0.05 - 0.1 ml of broth culture with the result that oedema and inflammation developed which persisted for 24 hours. Control animals injected with heat killed cultures showed no demonstrable lesions. Weiss also reported on the presence of two serologically different types in the isolates tested.

Different aspects of anaerobic infection were studied by Hite, et.al., (1948) who studied the synergism in experimental infections with non-sporing anaerobe. The strains studied were Streptococcus liquefaciens; St.mitis; Staphylococcus albus; Bacterium fusiformis; Bacterium melaninogenicum and other bacteroides. White mice were injected subcutaneously in the abdominal wall with pure mixed cultures. Their results showed that the anaerobic Bacteroides, especially Bacterium necrophorum exhibited synergistic

activity to a greater degree than did anaerobic streptococci. They also concluded that synergistic activity is exhibited in infections with a variety of non-sporulating anaerobic bacteria, in combination with each other or with aerobic organisms and that the property of enhancing bacterial virulence is intimately associated with cell substance.

Rosebury, et.al., (1950) studied the distribution of bacterial types in gingival scraping, by direct examination, guinea pig inoculation and anaerobic cultivation. The most prominent organisms isolated were Spirochetes, Vibrios, and Fusiformis bacilli. Subcutaneous inoculation of guinea pigs with gingival scraping from 40 subjects with various gingival or peridontal diseases resulted in typical fuso-spirochetal infection in 75 - 92% of trials. They also found that certain microorganism obtained from gingival scraping were not found in cultures from guinea pig exudates. These micro-organism (Leptotrichia; Actinomyces; Veillomella and unidentified Gram-negative thread-form organisms) seem in consequence to be unessential to the pathogenic fusospirochetal complex.

In a later study on bacterial mechanisms in periodontal disease, Macdonald, et.al., (1960 and 1963) used pure cultures of 17 organisms isolated from single infection, recombined,

ground with broth and inoculated into guinea pigs in 1 ml dose. After a typical transmissible lesion was obtained, the organisms were systematically deleted from the recombination mixtures to distinguish essential from non-essential component of this anaerobic infection. A minimum combination of 4 organisms was found to be essential to produce a typical infection. Two of them were found to be Bacteroides species, one of which was B.melaninogenicus.

In addition they found the organisms of the latter species produced hyaluronidase, protease, gelatinase and collagenase.

Gupta & Bhata (1970) found from their studies on strains isolated from various clinical sites that infection, due to anaerobes, were more common than is generally realized. 7% of total positive cultures were anaerobic bacteria. The strains isolated were: anaerobic cocci, dipheroids, clostridia and strains of B.convexus. They also used animal models to test for the pathogenicity of Clostridium welchii, where 1 ml of 24 hour cooked meat broth culture of this strain was injected intramuscularly into Swiss white mice and guinea pigs. The animals were sacrificed 10 days after the injection. No presence of local or systemic lesion was found on macroscopic examination, but Cl.welchii was successfully cultured from the site of injection, viscera and heart blood.

Further studies by Hill, et.al., (1974) on experimental anaerobic infection, describe a method for the production of liver abscess in mice using five strains of B.fragilis group, B.melaninogenicus, Fusobacterium nucleatum, Fusobacterium necrophorum and Peptostreptococcus. They injected mice intraperitoneally with 0.5 ml of 10^8 cell suspension of a 20 hour broth culture mixed in equal volumes with sterile mucin. Different inocula were tested consisting of: single species, all possible two-fold combinations of the five B.fragilis strains mixed with other non-spore forming anaerobes, and finally all two-fold combination between the species exclusive of B.fragilis. Incubation in the experimental animals were for 4, 8 and 12 weeks depending on the experiment. Their results showed that although some species which had been injected singly produced progressive liver abscesses, certain combinations of two species, generally gave more severe, reproducible and progressive disease.

Weinstein, et.al., (1974) studied the evolution of intra-abdominal abscesses. Gelatin capsules containing pooled colonic content and barium sulphate were prepared in an anaerobic chamber and implanted into the pelvic region of Wistar rats. The course of the disease was studied in various groups according to the source of the

inoculum and sex of the recipient. Their results showed that the colonic contents derived from rats fed on a grain diet produced highly lethal disease with an 80% mortality rate for males and 100% for female. Most death occurred within three days of implantation and autopsies showed generalized peritonitis.

In 1976 Walker & Wilkins were successful in initiating infection in mice from pure cultures of B.fragilis. The infection was initiated by injection of pure cultures grown in semisolid agar medium. The infection produce a large subcutaneous abscess at the site of injection which was observed seven days after animal inoculation. The inoculum used was 0.1 - 0.2 ml of 18 - 20 h cultures in chopped meat broth and the strains studied were, B.fragilis, B.distasonis, B.vulgatus and B.thetaiotaomicron. They have shown their method (using B.fragilis) to produce a localized abscess in 90% or more of the mice B.distasonis, B.ovatus and B.vulgatus produced abscesses in 30% or more of the mice injected, whereas B.thetaiotaemicron did not produce abscesses in any of the mice tested.

Microbial synergy in experimental intra-abdominal infection was studied by Onderdonk, et.al., (1976) using four microbial species: E.coli, enterococci, B.fragilis and Fusobacterium varium. They injected these organisms into the peritoneal cavity singly and in all possible dual combinations.

The results showed that mortality was restricted to recipients of E.coli, thus implicating coliforms in the acute lethality associated with this experimental model. Intra-abdominal abscesses were produced in 94% of the animals that received the combination of an anaerobe and facultative organisms.

Further investigation on the virulence of B.fragilis was carried out by Onderdonk, et.al., (1977) who compared the pathogenic potentials of encapsulated and unencapsulated strains of B.fragilis using a rat model of intra-abdominal sepsis. They showed that injection of encapsulated B.fragilis alone resulted in abscesses in most recipient whereas unencapsulated strains seldom produced this effect unless they were combined with other organisms. They also found that injection of heat killed, encapsulated B.fragilis also resulted in abscess formation.

Slots & Hausmann (1979) studied the relationship between microflora and alveolar bone loss in experimentally induced periodontal disease in monkeys with the result showing Haemophilus and B.melaninogenicus strains constituting a major part of monkey periodontal microflora. They also showed that B.asaccharolyticus strains were found to increase in proportion from a few per cent to about 66% of the total isolates concommitant with the development of a significant loss of alveolar bone mass, thus implicating B.asaccharolyticus and closely related

strains to be important pathogens in actively destructive periodontal disease. A more recent study on Bacteroides infection in mice was carried out by McConvile, et.al., (1981). They evaluated two clinical isolates of B.fragilis for their capability to cause intra-abdominal abscesses in Swiss albino mice. Each mouse received a 2 ml intra-peritoneal injection containing various combination of bacterial suspension, sterile faecal suspension and 1 ml of anaerobic dilution blank. The animals were sacrificed 15 days after inoculation and abscesses not at the site of inoculation were measured. They concluded that the intraperitoneal inoculation of sterile mouse faeces into mice produced intra-abdominal abscesses and that the addition of B.fragilis increased the incidence and the number of abscesses.

Bacteroides infectivity has been attributed to a number of factors that appear to help the organism invade tissues such as: presence of surface antigens (Hofstad, et.al., 1972 and 1977; Meisel-Mikolajczyk & Dworczynski, 1973; Mansheim & & Kasper, 1977; Mansheim, et.al., (1978), presence of capsules (Onderdonk, et.al., 1977), its ability to inhibit phagocytosis (Ingham, et.al., 1977) and production of enzymes such as hyaluronidase (Nolte, 1968), mucopolysaccharidases (Reid, et.al., 1945; Gesner & Jenkin, 1961) proteases (Macdonald, et.al., 1962) and collagenase (Gibbons & Macdonald, 1960).

In 1960 Gibbons & Macdonald surveyed the oral strains of B.melaninogenicus for the production of different enzymes, one of which was shown to be collagenase. Later on they carried out a quantitative study on the degradation of collagenous substrate by B.melaninogenicus with their results showing this enzyme to be intimately associated with the cell, but released during autolysis(Macdonald & Gibbons, 1962).

Rudek & Haque (1976) studied the extracellular enzymes of the genus Bacteroides by examining 33 organisms belonging to this genus; fifteen belonged to B.fragilis and related groups. Their results showed that the extracellular production of hyaluronidase and chondroitin sulphatase was demonstrated in all B.frigilis group except B.vulgatus. Additional enzymes were also found to be produced by certain members of this genus such as fibrinolysin; pencillinase, lysozyme, lecithinase, deoxyribonuclease phosphatase, protease and lipase.

Further studies were carried out surveying intestinal Bacteroides for the production of enzymes by Slayer, Vercellotti & Wilkins (1977) who studied the ability of 10 intestinal strains to ferment mucin and plant polysaccharides. Their results showed that a number of these strains fermented mucopolysaccharides (heparin, hyaluronate and chondrotin sulphate.) and ovomucoid. In addition, many of these strains fermented a variety of plant polysaccharides.

Finally Steffen, et.al., (1981) in their

abstract on the selected enzymes of clinical anaerobic bacteria suggested that B.melaninogenicus was the most active Gram-negative rods, usually degrading all protein and polysaccharide substrates except heparin. B.fragilis and closely related species, showed hyaluronidase, chondroitin sulphatase and heparinase activity, but were generally non-proteolytic.

1.7 Aims of Present Study

The purpose of the present study was to evaluate the use of GLC and PGLC in characterization and identification of Bacteroides species; to investigate the pathogensis of Bacteroides infections and to illustrate the isolation and identification of Bacteroides from clinical specimens.

CHAPTER TWO

MATERIALS AND METHODS

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SECTION ONE

MATERIALS

2.1.1 Organisms

Representatives of the following species and subspecies were used experimentally. In addition all organisms were used for both GLC analysis and pyrolysis GLC. These were:

Bacteroides fragilis	NCTC 8560 NCTC 9343
Bacteroides distasonis	ATCC 8503
Bacteroides ovatus	ATCC 8483
Bacteroides vulgatus	WB137
Bacteroides thetaiotaomicron	NCTC 10582
Bacteroides ruminicola ss.*brevis*	NP333
Bacteroides melaninogenicus ss. *melaninogenicus*	VPI 9085
Bacteroides melaninogenicus ss. *intermedius*	T588
Bacteroides melaninogenicus ss. *macacea*	ATCC 33141
Bacteroides asaccharolyticus	B536,,B537 &W50

The organisms used in experimental infection study were:

Bacteroides fragilis	NCTC 8560
Bacteroides melaninogenicus ss. *macacea*	ATCC 33141
Bacteroides asaccharolyticus	W50
Escherichia coli	NCTC 9001
Streptococcus faecalis	NCIB 6783
Clostridium sporogenes	NCIB 532

The following changes in nomenclature of organisms used in this study have occurred:

1. B.asaccharolyticus W50 is now B.gingivalis (1),(7),(10).

2. B.ruminicola ss. brevis NP333 is now B.pentosaceus (this is similar to B.buccae and B.capillus) (1),(2).

3. B.melaninogenicus ss. melaninogenicus VPI 9085 is now B.loeschii (this has also been referred to as B.oralis),(4),(10).

Other recently described species were not available when this work was commenced.

2.1.2 Media

BM Medium

This liquid medium was derived from that of Williams, et.al., (1975). It contained ($g.l^{-1}$):

Tryptone (Oxoid)	10
Special peptone (Oxoid)	10
Yeast extract (Oxoid)	5
Glucose (BDH)	5
Sodium chloride (Fisons)	5
Distilled water	1l

The medium was adjusted to pH 7.4 and autoclaved at 121°C for 15 min. 1 ml filter-sterilized haemin - menadione solution was added aseptically (Dowell & Hawkins, 1976) to give a final concentration of 5 $\mu g.ml^{-1}$ of haemin and 0.5 $\mu g.ml^{-1}$ of menadione.

Haemin-menadione solution

Haemin stock solution was prepared by dissolving 50 mg haemin (Sigma Chemical Co., Dorset) in 1 ml 1N NaoH and diluting to 100 ml with distilled water.

Menadione stock solution was prepared by dissolving 100 mg menadione (Sigma Chemical Co., Dorset) in 20 ml of 95% ethyl alcohol. Both solutions were sterilized by membrane filtration.

The haemin - menadione solution was prepared by adding 1 ml sterile menadione to 100 ml sterile haemin solution.

Bacteroides Enriched Medium

This medium consists of BM liquid medium supplemented with 5% w/v sterile defibrinated horse blood (Oxoid, Basingstoke, England) and 2% w/v agar (Oxoid).

Reinforced Clostridial Medium (Oxoid CM 151)

This medium was prepared according to manufacturer's instructions (Oxoid)

Nutrient Broth Medium

This medium contained ($g.l^{-1}$):

Lab-lemco (Oxoid)	10
Proteose peptone (Oxoid)	10
Sodium chloride (Fisons)	5
Distilled water	1l

The medium was adjusted to pH 7.5 and autoclaved at 121°C for 15 minutes.

Nutrient agar was prepared by adding 1.5% agar (Oxoid) to nutrient broth.

Nutrient agar glucose medium was prepared by adding filter sterilized glucose to give a final concentration of 1%.

Blood agar was prepared by adding sterile defibrinated horse blood (Oxoid) to a rapidly

cooled medium (50°C) of nutrient agar to give a final concentration of 5% v/v.

Selective Medium (Bacteroides Recovery Medium)

Bacteroides species were reisolated, when necessary, using Bacteroides recovery medium which contained ($g.l^{-1}$):

Lab-lemco (Oxoid)	10
Proteose peptone (Oxoid)	10
Sodium chloride (Fisons)	5
Technical agar (Oxoid)	20
Distilled water	1 l

This medium was adjusted to pH 7.5 and autoclaved at 121°C for 15 min. All additives were filter sterilized and added to a rapidly cooled medium. Additives were:
10 ml of haemin-menadione solution per litre of medium; Vancomycin (Sigma) to give a final concentration of 5 $\mu g.ml^{-1}$; Kanamycin sulphate (Sigma) to give a final concentration of 1000 $\mu g.ml^{-1}$, and finally 50 ml of sterile defibrinated horse blood (Oxoid) per litre of medium. This medium was always prepared fresh.

Motility (Thioglycollate) Medium

This medium contained ($g.l^{-1}$):

Yeast extract (Oxoid)	5
Tryptone (Oxoid)	15
Glucose (Fisons)	5.5

Sodium thioglycollate	0.5
Sodium chloride (Fisons)	2.5
L-Cystine (BDH)	0.5
Resazurin (Sigma)	0.001
Oxoid agar No. 2	2
Distilled water	1 l

This medium was adjusted to pH 7.1 and autoclaved at 121°C for 15 min. Filter-sterilized haemin-menadione solution was added aseptically (10 ml per liter of medium) to give a final concentration of 5 $\mu g.ml^{-1}$ and 0.5 $\mu g.ml^{-1}$ respectively.

Egg Yolk Agar Medium

This medium contained ($g.l^{-1}$):

Tryptone (Oxoid)	40
Disodium hydrogen phosphate	5
Sodium chloride (Fisons)	2
Magnesium sulphate (5% w/v solution)	0.2 ml
Glucose (Fisons)	2
Agar (Oxoid)	25
Distilled water	1 l

The medium was adjusted to pH 7.4 and autoclaved at 121°C for 15 min. Two fresh egg yolks were added to a rapidly cooled medium (50°C), plates were poured and used immediately.

Gelatin Medium

The medium contained ($g.l^{-1}$):

Special peptone (Oxoid)	10
Yeast extract (Oxoid)	10
Glucose (Fisons)	1
Resazurin solution (Sigma)	4 ml
Salts solution (Sigma)	40 ml
Vit. K_1 (Sigma)	0.2 ml
Cystine hydrochloride (BDH)	0.5
Distilled water	1 l

All constituents were boiled, cooled then Vit K_1, haemin and cystine were added. The medium pH was adjusted to 7.2. Gelatin (Difco, Surrey, England) was added to give a final concentration of 15%w/v The medium was dispersed in 5 ml aliquots in test tubes and autoclaved at 121°C for 15 min. For preparation of resazurin solution, salt solution, Vit K_1 and haemin solutions refer to PYG medium.

Cooked Meat Medium (CM)

Cooked meat medium was made according to manufacturers instructions (Oxoid) Basingstoke England). The medium was autoclaved at 121°C for 15 min. Filter-sterilized aesculin was added to CM medium at 1% (w/v) concentration to test for hydrolysis of aesculin by the bacteria whereas cooked meat glucose medium (CMG) which was widely used throughout the study was prepared

by the addition of filter-sterilized glucose to BM medium to give a final concentration of 1%. The pH at inoculation was 6.9 ± 0.1.

Peptone Yeast Glucose Medium (PYG)

The preparatory steps for pre-reduced PYG, are described by, Holdeman, et.al., (1977). The medium contained ($g.l^{-1}$):

Tryptone (Oxoid)	5
Special peptone (Oxoid)	5
Yeast extract (Oxoid)	10
Resazurin solution	4 ml
Salts solutions	40 ml
Vit K_1 solutions	0.2 ml
Haemin solution	10 ml
Cystine hydrochloride (BDH)	0.5
Distilled water	1 l

The Vit.K_1 and haemin solutions and the cystine were added after the medium had boiled, but before it was dispensed and autoclaved. The medium was autoclaved at 12°C for 15 min. Filter-sterilized glucose was added to give a final concentration of 1%. The pH at inoculation was 7.1 ± 0.1.

Salts solution contained ($g.l^{-1}$)

Calcium chloride (anhydrous)	0.2
Magnesium sulphate (anhydrous)	0.2
Dipotassium hydrogen phosphate	1
Potassium dihydrogen phosphate	1

Sodium bicarbonate	10
Distilled water	1 l

Resazurin solution was prepared by dissolving 25 mg resazurin (BDH) in 100 ml distilled water. Haemin solution was prepared by dissolving 50 mg of haemin (Sigma) in 1 ml 1N NaoH, made up to 100 ml with distilled water. Vit K_1 solution was prepared by dissolving 0.15 ml of Vit K_1 (Sigma) in 30 ml of 95% ethanol.

PYG-Starch Medium

This is PYG medium to which starch was added to give a final concentration of 1% (w/v).

Dextran Medium

This medium was made up in nutrient agar base to which the following constituents were added:

Dextran (Sigma)	0.5% w/v
Blue dextran (Sigma)	0.5% w/v
Glucose (Fisons)	0.2% w/v
Yeast extract (Oxoid)	0.25% w/v

Filter sterilized haemin menadione solution was added to give a final concentration of 5 $\mu g.ml^{-1}$ and 1 $\mu g.ml^{-1}$ respectively.

Bile Salts Medium

This medium was made up in nutrient agar

base to which the following constituents were added:

Bile salts (Oxoid)	20% w/v
Yeast extract (Oxoid)	1% w/v

The medium was adjusted at pH 7.4 and autoclaved at 121°C for 15 min. Filter-sterilized haemin and menadione solution was added to give a final concentration of 5 $\mu g.ml^{-1}$ and 1 $\mu g.ml^{-1}$ respectively.

Carbohydrate Fermentation Medium

This medium is a modification from Loesche, et.al., (1964). The test medium was fresh thioglycollate medium to which test substrate was separately added. Substrates included glucose, lactose, maltose, sucrose, rhamnose, trehalose, mannitol, arabinose and cellobiose to give a final concentration of 1% w/v. All sugars were of analytical reagent grade (BDH Ltd., Poole, U.K.).

2.1.3 Reagents

(i) Nitrate Test Reagent

Solution A was prepared by dissolving 8g of sulphanilic acid in one litre of 5N acetic acid. Solution B was prepared dissolving 5g of α-naphthylamine in one litre of 5N acetic acid.

(ii) Ehrlich Reagent was prepared by dissolving 0.25% w/v of --dimethylaminobenzaldehyde in 1N Hcl.

(iii) Ringer Solution (¼ strength)

This solution contain $g.l^{-1}$)

Sodium chloride	2.25
Potassium chloride	0.105
Calcium chloride	0.12
Bicarbonate	0.05
pH was adjusted to	7.00.

SECTION TWO

GENERAL PROCEDURES

2.2.1 Anaerobic Cultivation Procedures

Test strains were received in a freeze-dried form. Each strain was grown on the following media: 2 x 10 ml BM broth, two blood agar plates and two BM agar plates. All media were incubated anaerobically at 37°C in a vented gaspak jar (BBL, Middlesex, England) which was immediately evacuated and refilled with a mixture of 90% carbon dioxide and 10% hydrogen (British Oxygen Company, Derby, England). The flushing procedures were repeated three times. The jar also contained a gaspak generator envelope (BBL) which released hydrogen and carbon dioxide when distilled water was added immediately prior to sealing the jar and before commencing the flushing procedure. The released hydrogen reacts with any residual oxygen to form water in the presence of a Palladium catalyst (BBL).

2.2.2 Clostridum sporogenes Cultivation Procedure

This organism was received in a freeze dried form. It was then inoculated onto the following media: two plates of reinforced clostridial agar (Oxoid) and 2 x 20 ml of CMG

medium and incubated anaerobically at 37°C in vented Gaspak jar containing two Gaspak generator envelopes, which alone provided a sufficiently anaerobic atmosphere for excellent growth of this organism. Stock cultures were stored in the dark at room temperature in tightly closed vials. Subsequent subcultures were always carried out using CMG broth.

2.2.3 Aerobic Cultivation Procedure

Esherichia coli and *Streptococcus faecalis* lypholized suspension was culture onto the following media: 2 x 10 ml nutrient agar plates for *E.coli*; 2 x 10 ml nutrient broth and two blood agar plates for *St.faecalis*. Both organisms were incubated aerobically at 37°C. Subsequent subculture was carried out using either nutrient agar slopes (*E.coli*) or nutrient agar slopes enriched with 1% glucose (*St. faecalis*).

2.2.4 Maintenance of Organisms

Bacteroides species were maintained by weekly subcultures on freshly prepared blood agar. Broth cultures were checked for purity by plating on 5% blood agar followed by aerobic and anaerobic incubation. Anaerobic culture identity was then confirmed by Gram-strain and GLC profile.

Clostridium sporogenes was maintained by subculturing at six monthly intervals into CMG

medium.

E.coli was maintained by monthly subculture onto nutrient agar slopes.

Streptococcus faecalis was maintained by monthly subculture onto nutrient agar glucose (1%) slopes. Purity checks on Cl.sporogenes, E.coli and St.faecalis were confirmed by Gram staining and colonial morphology.

2.2.5 Preservation (Long Term Storage)

Bacteroides strains were inoculated onto blood agar and incubated anaerobically for 4 days at 37°C. Then colonies of each strain were inoculated into freshly prepared CMG broth and incubated anaerobically for 48 hours at 37°C. After purity checks, cultures were then ready to be transferred into sterile plastic vials with screw capped tops and to be stored under liquid nitrogen (-196°C). Under these conditions viability was retained for up to one year.

Cl.sporogenes, E.coli and St. faecalis were preserved by routine subcultures.

2.2.6 Preparation of Experimental Inocula

In all bacteroides experimental procedures, a master culture was set up using blood agar which was incubated typically for 48 hours for the non-pigmented species, or, for 72 hours (except for experimental infection and pyrolysis) when working

with black pigmented species. One loopful of the master culture (5-6 isolated colonies) was inoculated into appropriate media. Incubation was always at 37°C. When working with Bacteroides species, media were always prepared fresh, cooled rapidly and inoculated.

SECTION THREE

Specific Experimental Methods

2.3.1 Tests for Characterisation of Bacteroides Species

Colonial morphology: This was usually studied after growth for 48 hours, or, 72 hours on blood agar.

Cellular morphology: Gram-stained smears from cultures grown 48 hours or 72 hours (i) on blood agar (ii) in CMG medium, were examined microscopically (Brightfield, Carl Zeiss, Jena, Amplival) Haemolysis on blood agar was observed after incubation for 4 days.

Pigment production: Plates were observed for the production of black-pigmented colonies on blood agar after set time intervals at 37°C.

Motility: A wet-film prepared from a 24 hour CMG broth was examined by phase-contrast microscopy (Amplival, Carl Zeiss, Jena). Strains were also stab-inoculated into semi-solid agar (motility test medium) which was then incubated anaerobically until growth was visible.

Lipase activity: Cultures were grown on egg yolk agar and incubated anaerobically at 37°C for 72 hours. Lipase activity was indicated by the production of a pearly-layer effect.

Oxidase production: A positive reaction was indicated by a blue colour rapidly developed in the colony after a freshly prepared 1% solution of tetramethyl-p-phenylene-diamine dihydrochloride in distilled water had been poured onto 7 days culture, on supplemented egg-yolk medium.

Catalase production: A 7 days culture on supplemented egg-yolk agar was exposed to oxygen for 30 min then flooded with 10% hydrogen peroxide; a stream of bubbles arose from catalase-producing colonies.

Gas production: A cool molten PYG agar was inoculated with 4-5 drops of 24 hour culture in CMG medium. After 48 hours of anerobic incubation at 37°C, the production of gas by cultures was determined by bubble formation and splitting of the agar.

Nitrate reduction: This test was carried out in thioglycollate medium with yeast extract, heamin-menadione (refer to motility medium). The presence of nitrite ions was indicated by a deep red colour when 0.5 ml of nitrate solution A and 0.5 ml of nitrate solution B (Cruickshank, 1970) were added to 48 hour cultures.

Indole production: Two ml of 18 hour culture (CM medium) was pipetted into a sterile tube. One ml of xylene was added and left to stand for about 2 min, after which 0.5 ml of Ehrlich's reagent

was added. The xylene layer turned pink if indole was present.

Gelatinase test: A charcoal-gelatin disc in CM medium culture was observed for digestion of the disc during one week of incubation. However difficulties were encountered using these discs because they gave irregular results. So a gelatinase test was carried out using methods of Holdeman, et.al., 1977) Pre-reduced gelatin medium was inoculated from 48 hour CMG medium and incubated anaerobically at 37°C for one week. Controls in duplicate were also included. The cultures and controls were chilled in a refrigerator until the controls solidified, then they were left at room temperature. Failure of cultures to solidify was interpreted as positive gelatinase production.

Aesculin hydrolysis: Development of black discolouration on adding 0.5 ml of a 1% aqueous solution of ferric ammonium citrate to a 48 hour-culture in CM medium containing 1% aesculin indicated a positive reaction.

Starch hydrolysis: A one-week culture on PYG starch medium (Holdeman & Moore, 1974) was flooded with Gram's iodine; and results were recorded immediately. No colour was interpreted as positive, blue-black colour was recorded as negative.

Dextran hydrolysis: Strains were grown for one week on dextran medium. Hydrolysis was detected by the development of a zone of clearing around colonies growing on the blue medium.

Growth in 20% Bile : Cultures were incubated anaerobically in bile salt medium for 48 hours at 37°C. Growth was compared with that of the control plates (test medium without bile salts). Growth results were regarded as negative when strains failed to grown on test medium or positive if growth occurred. If growth on test medium was better than control then the presence of bile was regarded to have stimulatory effect on growth.

2.3.2 Characterization of Other Strains:

Escherichia coli, *Streptococcus faecalis* and *Clostridium sporogenes* were characterized as described by Cowan & Steel (1974).

2.3.3 Carbohydrate Fermentation Tests:

The test medium was inoculated and incubated anaerobically for 48 hours at 37°C. The final pH was measured with pH meter (Pye Unicam model 9W9409). Controls included cultures of the test strains in sugar free thioglycollate medium and tubes of uninoculated (but incubated) 1% sugar medium. The test was

regarded as positive if the pH fell > 0.5 unit below that of the uninoculated control and > 0.5 pH unit below that recorded for the inoculated, sugar-free thioglycollate medium.

In general, pH 5.5-6 was regarded as weak acid pH below 5.5 as strong acid.

2.3.4 Biochemical Tests (API 20 A)

API 20A is a standardised system which allows the rapid and reliable performance of a large series of biochemical tests coupled with computer assisted identification. It comprises plastic strips with 20 microtubes each containing dehydrated substrates. These were glucose, mannitol, lactose, sucrose, maltose, salicin, D-xylose, L-arabinase, glycerol, cellobiose, mannose, melezitose, raffinose, sorbitol, rhamnose and trehalose. The strips also help to test for; production of indole, urease and catalase, as well as hydrolysis of gelatin, aesculin and motility.

A cell suspension of all species was prepared, with cell density of $10^6.ml^{-1}$ and inoculated into the microtubes. The strips were incubated anaerobically at 37^oC for 72 hours before recording the results.

SECTION FOUR

CHROMATOGRAPHY

2.4.1 Instrumentation

A Pye-Unicam, series 104, gas chromatograph (Cambridge - England) fitted with dual flame ionization detectors was operated isothermally at 137°C. The instrument was fitted with two identical glass columns (1.5 m x 4 mm). The columns were treated with acid dichromate solution, washed with water and then rinsed thoroughly with acetone followed by ether.

The columns contained Celite, 80/100 mesh, (BDH, Poole, England) which was treated with 2% phosphoric acid and then coated with 10% DEGA, diethylene glycol adipate, (Phase Separation Ltd., England). After air-drying the resin overnight, the columns were packed by the normal vacuum-vibration technique. The columns were thoroughly vibrated with an electric vibrator along the whole column length while the resin was very slowly poured to ensure a very close packing. The N_2 flow rate was adjusted to 50 ml.min^{-1} and the air flow to a rate of 400 ml.min^{-1}. The hydrogen flow rate of both detectors was adjusted for optimum sensitivity (40 ml.min^{-1}). Both sample and reference columns were conditioned by flowing nitrogen through them for 24 hours at an oven

temperature of 180°C. This was generally sufficient to establish a stable base line at the working attenuation of 100 to 300. The recorder was a Tarkan recorder, model W600 (W + W Electronic Inc.) set at the 1 mv range with a chart speed of 30 $cm.h^{-1}$. Nitrogen, hydrogen and air were supplied by British Oxygen (London, England).

2.4.2 Sample Preparation

The amount of fatty acids (volatile and non-volatile) produced by thirteen strains of the genus Bacteroides were estimated over 7 days of incubation. All strains were surveyed for level of fatty acids produced in CMG broth whereas for estimation of fatty acids level in PYG broth clinically important species were used, namely: B.fragilis (NCTC 8560 and 9343), B.melaninogenicus ss. macacea (ATCC 33141) and B.asaccharolyticus (B536, B537 and W50).

48 hrs incubated blood agar cultures (non-pigmented bacteroides) or 72 hours (black pigmented bacteroides) served as a master culture for all experiments. A loopful (4-5 colonies) was emulsified into 15 ml of the appropriate broth and incubated anaerobically at 37°C for periods of time which ranged from 12 - 168 hours. The experiments for each strain were performed in duplicate and repeated three times except for B.asaccharolyticus where three representative

strains of this species were examined. The experiment was performed in duplicate and repeated twice. Fatty acid analysis, as well as growth and pH measurement, were done simultaneously for a few selected species in CMG and PYG media. These were B.fragilis (NCTC 8560), B.melaninogenicus ss. macacea (ATCC 33141) and B.asaccharolyticus (W50).

Growth was measured using Pye-Unicam model SP 500, Series 2 spectophotometer and the absorbance was read at 600 nm in 1 cm cuvettes.

2.4.3 Analysis

The procedures used were derived from those of Holdeman, et.al., (1977).

(a) Volatile fatty acids (VFAs): 1 ml of culture was acidified with 0.2 ml of 50% H_2SO_4, then 0.4 g of sodium chloride was added followed by 1 ml of diethyl ether (distilled and sodium dried) which contained the internal standard (methyl salicylate). The mixture was gently mixed and the ether-culture emulsion was broken by centrifugation at 600 r.p.m. for 10 min. The ether layer was pipetted off into small vials until analysed. 1 µl of the sample was injected directly onto the analysing column using 5 µl syringe (Scientific Glass Engineering, U.K. Ltd).

(b) Non-volatile fatty acids (n-VFAs): To 1 ml of cultures, a 1 ml internal standard

(glutaric acid) and 2 ml of triboronofluoride methanol (Sigma) were added. Cultures were then acidified with 0.4 ml of 50% H_2SO_4 and the mixture heated at 60°C for 45 min in a stoppered universal bottle in a water bath. To the above mixture 1 ml of distilled water and 0.5 ml of chloroform (distilled and dried) were added, gently mixed and the chloroform-culture emulsion was broken by centrifugation at 600 r.p.m. for 10 min. 1 µl of the sample was injected into the analysing column using a 5 µl syringe.

2.4.4 Standards

The VFAs standards solution contained 0.03M acetic (A), and 0.01M of propionic (P), isobutyric (IB), butyric (B), isovaleric (IV) and valeric (V)acids (BDH analar) and it was made up in CMG or PYG media thus representing the fatty acids present in the media. Due to the insolubility of methyl salicylate (internal standard) in water, 0.006 M solution of the internal standard was made up in diethylether which was used for extraction.

The n-VFAs standards solution contained 0.03M lactic (L), 0.02M oxalic (O), 0.007M malonic (M), 0.004M succinic (S) and 0.003M glutaric acids (BDH analar) and it was made up in CMG or PYG media. The internal standard (glutaric acid) dissolved readily in both media and thus underwent the

normal extraction procedure for n-VFAs.

Both internal standards (methyl salicylate and glutaric acid) were used for determination of relative retention time as well as for quantitative purposes.

Samples of uninoculated (sterile) PYG and CMG media were included as controls in every batch of each of the two types of analysis. A media control and a combined acid standard were used to monitor retention times and the sensitivity settings of the instrument as part of the basic routine.

2.4.5 Calculation

Calibration with an internal standard was performed by the addition of a known amount of the internal standards to the acids standards mixtures. The formula used for calculating the quantity ($\mu m.ml^{-1}$) of a sample unknown, containing an internal standard is given by:

$$\frac{Au}{Ci} / RRF \times Ai$$

where Au = area of the unknown component in a sample.

Ai = area of the internal standard

Ci = concentration of internal standard (μmole/ml)

RRF is the relative response factor which is the ratio of $A_s C_i$ to $A_i C_s$

where A_s = area of the standard fatty acid mixtures

C_s = concentration of the standard fatty acid mixtures.

2.4.6 Metabolism of ^{14}C-acetate

B.distasonis (ATCC 8503) and B.asaccharolyticus (W50) were tested for their ability to use acetic acid in the form of ^{14}C-labelled sodium acetate as either 1-^{14}C or 2- ^{14}C (Radiochemical Centre, Amersham, U.K.). A suspension in nutrient broth was prepared of each strain at a cell density of approximately 10^{10} cell.ml^{-1} from blood agar plate. The two differently labelled sodium acetate solutions was diluted to give a final concentration 1 µCi.ml^{-1}. The specific activity for 1-^{14}C and 2-^{14}C sodium acetate was 718 µCi.mg^{-1} and 665 µCi. mg^{-1} respectively.

The medium chosen to carry out the experiment was CMG with addition of 0.2% sodium acetate. Cooked meat particles were removed after sterilization and the broth was dispensed into 20 ml quantity into universal bottles to which 75 mm x 10 mm sterile durham tubes were placed with the opening upwards carefully and slowly, so avoiding getting any broth into them. The durham tubes were then filled 3/4 with hyamine (Sigma Chemical Co. U.K.), Peng,(1977),to trap the released CO_2. Bottles were then inoculated with 4 drops of cell suspension and incubated anaerobically under oxygen free nitrogen at 37°C for 72 hours. After incubation 1 ml of hyamine

was pipetted and added to 15 ml of scintillation fluid, Unisolve E, (KoCH Light-U.K.), mixed and then counted for radioactivity (counts.min^{-1}) using LKB Wallace Rack Beta, UK, Scintillator counter.

The experiment was performed in triplicate for each species and repeated four times.

SECTION FIVE

PYROLYSIS GAS CHROMATOGRAPHY

2.5.1 Sample Preparation

At 72 hours, master cultures on blood agar, of all strains were inoculated onto a freshly prepared enriched medium (BM blood agar) and incubated anaerobically at 37°C for 48 hours. Cultures were then inoculated onto freshly prepared BM agar (without blood) and plates were incubated anaerobically for 96 hours, after which samples were ready for pyrolysis. Each culture was plated in duplicate and the experiment was repeated 3 - 6 times.

2.5.2 Pyrolysis conditions

A Philips, Curie-point pyrolyser was used with flame-cleaned pyrolysis wire (0.5 mm in diameter) to which 2 µl of internal standard (hydroxy proline) was added and air dried; samples were then added. Sample size could not be accurately controlled. but was maintained within a range of 0.5 - 1 mg which was transferred to the terminal 4 mm of the ferromagnetic wire. Pyrolysis was carried out at 770°C for 10 sec. After each analysis the Curie-point wires were discarded. The quartz tubes were cleaned after every third pyrolysis using concentrated sulphuric acid and

reused. Baseline noise was sometimes seen if quartz tubes were replaced less frequently. The quality of the baseline was checked from time to time by blank firing; "peak ghosts" were not observed.

2.5.3 Gas chromatography

The pyrolyzer delivered the products from bacterial cells to a Pye series 104 (Pye-Unicam, Cambridge, U.K.) model 64. All analysis were run on dual 3-m glass column (ID 3mm) containing 4% carbowax 20M (Phase Separation Ltd., Flintshire) on gas-chrom Q, 100 - 120 mesh, (Applied Science Laboratories Inc. Pennasillvan). The columns were conditioned overnight at 230°C and the column was brought to optimum performance within 5 - 6 weeks of commissioning. Pure nitrogen was used as the carrier gas at a flow rate of 50 ml.min^{-1}. Both flame ionization detectors were supplied with 40 ml.min^{-1} of hydrogen and 400 ml.min^{-1} of air. The attenuator range was set at 100 or 1000. A dual recorder (Omniscribe, Wouston Instrument) was set up at 1 mv and 10 mv with a chart speed of 0.2 ins.min^{-1}. The separations were temperature programmed for an initial temperature of 65°C up to 190°C at 2.5^{o}C.min^{-1} and with indefinite hold at 190°C. Each cycle of pyrolysis, column heating, and cooling was completed within 2 hours.

2.5.4 Column rejuvenation

To maintain column performance the silinised glass wool and the 10 cm length of the column substrate located within the injection zone of the instrument, were replaced every 6 months using fresh portions of the coated support material. The re-juvenated columns were held at 230°C overnight before re-use. The flame ionization detectors and their associated inlet connection were cleaned at the same time to remove accummulated high-boiling point residues.

2.5.5 Data handling

The conversion of a pyrogram into a quantitative data requires the setting of a standard baseline which was set to each program in the same manner. Two criteria were applied to peak selection:

(i) Choose the maximum number of peaks possible

(ii) Ensure that all chosen peaks are present and reproducibly resolved on each pyrogram.

Peak heights were measured from the baseline to the nearest mm. Peak height data was punched onto cards for submission to the computer. Before any computer analyses, each batch of data was normalized to remove variation due to sample size. Computer programmes were written in GENSTAT and run on ICL 4/70 system at Rothamsted Experimental Station.

Multivariate analysis:

Data from pyrolysis gas liquid chromatography (PGLC) consists of a number of different variates (peak heights) describing a number of samples (pyrogram) and each batch can be represented as a matrix of pyrograms vs. peak heights. This matrix (often termed an input matrix) would contain n rows and P columns, where, n = the total number of pyrograms in a study and P = the total number of peaks measured on each pyrogram. If only two peaks were measured it would be easy to obtain a two-dimensional picture of the variation between pyrograms by plotting one peak height against another. For large number of peaks this is impossible and multivariate analysis is used (Blackith & Reyment, 1971; Marriott, 1974). From a mathematical viewpoint each peak height is seen as a dimension or axis in graphical terms, and the starting point for all these techniques is to consider the sequence of normalised peak heights which describe a pyrogram as defining the co-ordinates of a single point in multidimensional space. The techniques of multivariate analysis aims at extracting the pertinent information from an n x P input matrix and presenting it in an

easily understandable form.

Multivariate techniques can be divided into two types:

(i) Those that try to classify or partition the samples into homogeneous sub-sets, e.g. cluster analysis.

(ii) Those which try to give a visualisation of the results in a low-dimensional space, e.g. principal components analysis, canonical variate analysis.

For each group of pyrograms the vectors of the mean heights of each peak give the co-ordinates of the group mean in multidimensional space. In testing whether groups are separated, ordinary distance measures are of little use because they do not take into account the observed scatter of samples around the means. To overcome this, samples are scaled so that the average within group scatter is equal and a generalised distance known as Mahalanobis D^2 (Mahalanobis, 1936) is defined which is independent of the scale of the peak heights and takes account of the correlations between them. This implicity defines a new set of co-ordinates. The aim of <u>canonical variates analysis</u> is to recover this set of co-ordinator relative to a set of co-ordinates relative to a set of canonical variate axes which are expressions of the original scatter of the pyrograms in multidimensional space. Each canonical variate is related to the mean heights of the groups by a set of coefficients known as loading. Plotting the

means relative to the first two canonical axes displays as much of the generalised inter-distances as is possible in two dimensions.

Further canonical axes can be considered but with PGLC data 80 - 90% of the variation is usually expressed by the first two. When the same scale is used on each axis, circular confidence regions may be placed around each mean (Seal, 1966). In this thesis 95% confidence intervals have been imposed on the populations and these are circular with radii equal to the square root of the chi square value with two degrees of freedom ($\sqrt{5.99} = 2.45$).

SECTION SIX

EXPERIMENTAL INFECTION

2.6.1 Suspension Preparation

A 24 hour culture of B.fragilis and 48 hour culture of B.melaninogenicus ss. macacea and B.asaccharolyticus grown on blood agar were harvested separately and each culture was then emulsified in 5 ml sterile 1/4 strength Ringer's solution. The cell density was then adjusted to the required level using Wellcome opacity tubes to give approximately 10^{10} cells. ml^{-1}.

A 24 hour cultures of E.coli and St.faecalis grown separately on nutrient agar were harvested and emulsified in 5 ml sterile 1/4 strength Ringer solution. The cell density was adjusted as before.

Cl.sporogenes suspension was prepared by inoculation of CMG broth (without the meat particles) from a fresh young culture (24 h), after 24 hours of anaerobic incubation at 37°C the cells were centrifuged at 3000RPM for 10 minutes and washed twice with sterile distilled water. The pellet was then re-suspended in sterile 1/4 strength Ringer's solution and adjusted to the required level (10^{10} cells.ml^{-1}) as previously mentioned.

2.6.2 Animals and Injection Methods

Female Dutch rabbits weighing 1.5 - 2 kg were used throughout. 0.5 ml of the required suspension was injected into the rabbit in the thigh region subcutaneously (s.c.) and intramuscularly (i.m.).

In all cases 0.5 ml of the suspension was used unless otherwise stated. Rabbits were sacrificed on the seventh day after injection, except in cases where death occurred within 60 - 72 hours after injection.

At autopsy all infected areas or organs were excised and fixed immediately in 40% formalin for histopathological examination.

2.6.3 Experiments

The experimental work carried out was divided into three sections:

(i) Pathogenic capability of selected strains

The test strains were *B.fragilis* (NCTC 8560), *B.melaninogenicus* ss. *macacea* (ATCC 33141) and *B.saccharolyticus* (W50). The same inoculum level was used in all cases, i.e. 10^8cell.ml^{-1} which was injected s.c. and i.m. Each rabbit received 0.5 ml of 10^8cell.ml^{-1} s.c. in one thigh and 0.5 ml i.m. in the other thigh, of the same strain. Control animals were injected s.c. with 0.5 ml

of heat killed suspension of B.fragilis (20 min at 60°C) at a concentration of 10^8 cell.ml^{-1} the other thigh was injected S.C. with 0.5 ml of sterile 1% kaolin suspension. The experiment was repeated three times.

(ii) The effect of variation in inoculum size on abscess formation

The experiment was in two parts:

(a) The test organism was B.asaccharolyticus, different levels of inoculum were used, namely, 10^2, 10^5, 10^8 and 10^{10} cells.ml^{-1}; of which 0.5 ml was injected S.C. in one thigh and 0.5 ml in the other. Control animals were injected S.C. with 0.5 ml of heat killed (20 min at 60°C) suspension of B.asaccharolyticus at a concentration of 10^{10} cells.ml^{-1}. The other thigh was injected S.C. with 0.5 ml of sterile 1% kaolin suspension. The experiments were repeated 3 times except for 10^8 cells.ml^{-1} where results from the previous experiment were used.

(b) The test strains were B.fragilis and B.asaccharolyticus, the inoculum size used was 10^{10} cells.ml^{-1}; of which 0.5 ml of B.asaccharolyticus was injected S.C. in the thigh region followed by 0.5 ml i.m. injection of the same

culture into the same thigh. The other thigh of the same rabbit received 0.5 ml of 10^{10} cells.ml^{-1} of B.fragilis subcutaneously and intramuscularly in the above manner. Control animals were injected with 0.5 ml of heat killed (20 min at 60°C) suspensions of B.fragilis at a concentration of 10^{10} cells.ml^{-1} S.C. and 0.5 ml i.m. in the same thigh. The other thigh was injected with 0.5 ml s.c. and 0.5 ml i.m. of heat killed (20 min at 60°C) suspension of B.asaccharolyticus at a concentration of 10^{10} cells.ml^{-1}. This experiment was repeated three times.

(iii) Mixed infection

Test strains were E.coli, St.faecalis, Cl.sporogenes and B.asaccharolyticus (W50). A suspension was prepared from each culture containing 10^{10} cells.ml^{-1}. All suspensions were mixed at 1:1 ratio, the resulting suspension was used for animal inoculation where the final concentration of each strain would be 10^5 cells.ml^{-1}. 0.5 ml was injected s.c. in one thigh and 0.5 ml i.m. into the other. Control animals were injected S.C. with 0.5 ml of heat killed mixed suspension (90 mins at 80°C), the other thigh was injected S.C. with 0.5 ml of 1% kaolin suspension. This experiment was repeated twice.

2.6.4 Morphological and Biochemical Characterization

E.coli, St. faecalis and Cl.sporogenes, when isolated from lesions, were re-identified as described in Bergy's Manual (1974).

B.fragilis, B.melaninogenicus ss. macacea and B.asaccharolyticus, when isolated from lesions, underwent a set routine for their identification (Fig. 1).

The pus was made into a homogenous suspension sterile 1/4 strength Ringer's solution by shaking with sterile glass beads. This suspension was treated as a "culture" and subjected to the identification test shown in Fig. (1), except for pyrolysis for which a sample of the actual pus material was used.

At autopsy, all organs such as heart, liver, spleen and kidney were surveyed for the presence of Bacteroides species. The routine adopted is shown in Figure (2).

For direct plating, two media were always used, nutrient agar supplement with 5% defibrinated horse blood and BM agar supplemented with haemin, menadione and 5% horse blood.

2.6.5 Enzymes assays

B.fragilis (NCTC 8560) and B.asaccharolyticus (W50) were assayed for the presence of certain enzymes using a simple series of qualitative tests. The test medium was

BM medium. Cell suspensions of B.fragilis and B.asaccharolyticus was prepared to give 10^{10} cells.ml^{-1}. Suspensions of each of the different substrates, fibrin blue, chondroitin sulphate sodium salt, hyaluronic acid potassium salt, collagen and hide powder azure (Sigma Chemical Co. U.K.) was prepared in sterile distilled water. To 8 ml of double strength BM medium, 1 ml of culture was added as well as 1 ml of the appropriate substrate to give a final concentration of 1%. In addition a control tube was included for each substrate. All tubes were incubated anaerobically under CO_2 and H_2 at $37^{o}C$ for seven days before the results were recorded.

The experiment was performed in triplicate and repeated three times.

Fig. (1)

Identification procedures for Bacteroides group of organisms re-isolated from experimentally infected animals

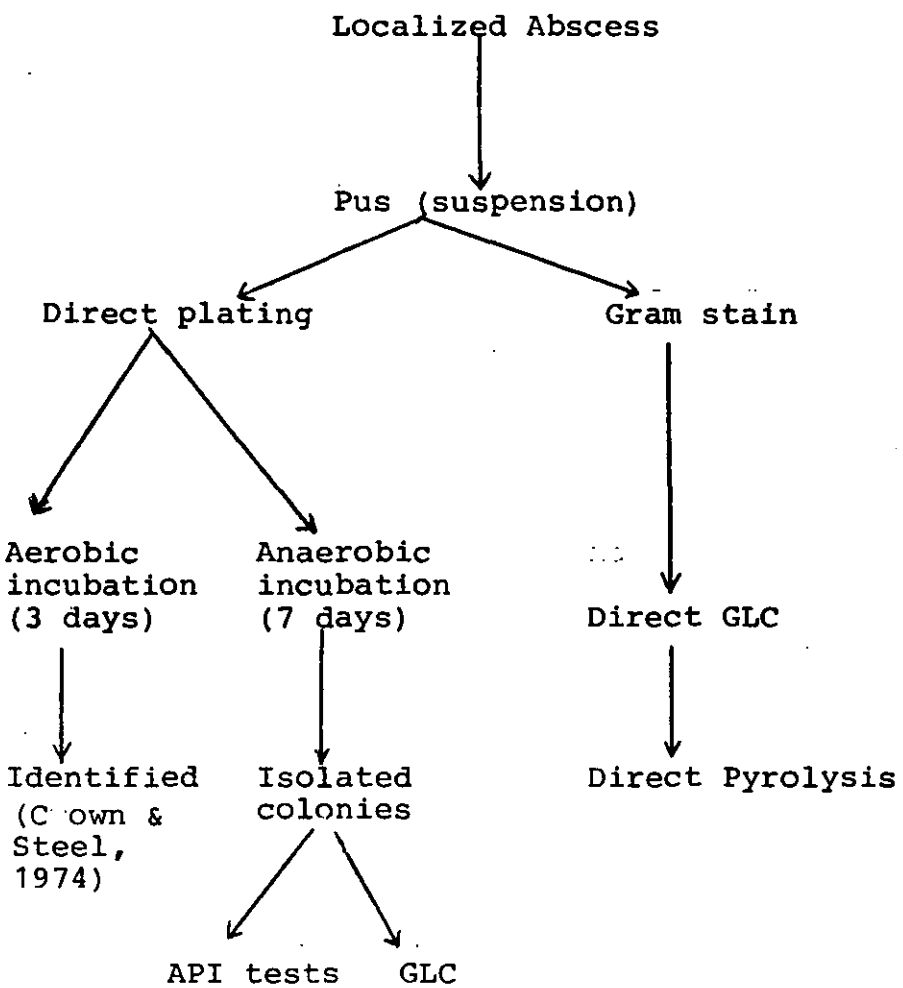
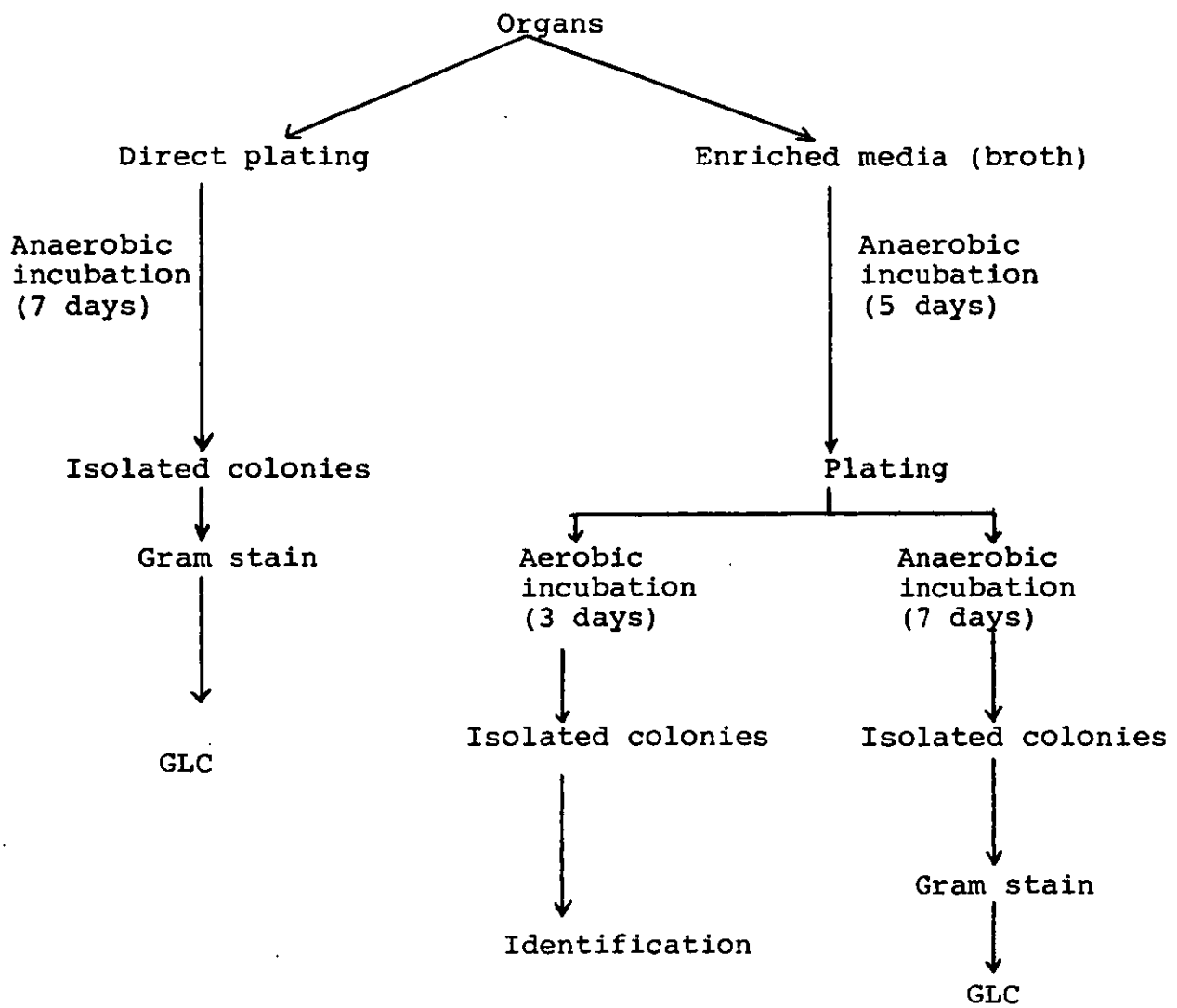


Fig. (2)

Identification procedures of all organisms isolated from internal organs of experimentally infected animals



CHAPTER 3

RESULTS

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RESULTS

All the strains studied were divided into the following two main groups on the basis of pigment production:

Group I (non-Pigmented bacteroides):

All strains that failed to produce black pigment when grown on blood agar. These include: B.fragilis, B.thetaiotaomicron, B.distasonis, B.ovatus, B.vulgatus and B.ruminicola SS. brevis.

Group II (black Pigmented bacteroides):

All strains that produced a black pigment when grown on blood agar. These include: B.melaninogenicus ss. melaninogenicus, B.melaninogenicus SS. intermedius, B.melaninogenicus SS. macacea and B.asaccharolyticus

3.1 Morphology

All the species studied were Gram-negative, non-sporing obliqately anaerobic bacteria. Many strains were highly pleomorphic; cell shape varied from filamentous to coccobacillary, often in the same smear. Some strains formed chains. Some had pointed ends and others had rounded ends, cellular morphology had little discriminatory value but certain points were noted.

Group I organisms:

B.fragilis (NCTC 8560 & 9343)

These were short bacilli with rounded ends, sometimes occurring singly, in pairs or in short chains. Cells from (48 h) colonies on blood agar were seen as medium rods (0.5 by 3-5μm) of relatively regular shape and length with rounded ends. (Fig.5b). Cells from broth cultures frequently have one or more vacuoles which swell the cells and some cells may be more than five times as long as others. On blood agar plates incubated for three days, colonies were 1-2 mm circular, entire convex, white-grey and semi opaque (Fig.3b).

B.thetaiotaomicron (NCTC 10582)

Cells from colonies on blood agar (48 h) were straight rods with rounded ends and occurred singly or in pairs. In CMG they were 2 by 1-5μm, pleomorphic and often stained irregularly. Surface colonies on blood agar incubated for three days were punctiform, circular, entire, convex, and semiopaque.

B.distasonis (ATCC 8503)

Cells from blood agar (48 h) occurred singly occasionally in pairs. Cells were smaller than those from CMG broth and were 2 by 1.5-5μm, with rounded ends. (Fig.5a).

Colonies on blood agar incubated for three days were 0.5-1 mm in diameter, circular, entire convex, translucent-opaque, grey-white, soft and smooth.

B.ovatus (ATCC 8483)

Cells from CMG broth were evenly stained with rounded ends, oval forms, they usually occur singly and occasionally in pairs.

Surface colonies on blood agar incubated for three days were 1-2 mm in diameter circular, entire convex pale in colour and semi-opaque (Fig. 3c).

B.vulgatus (WBM 137)

Cells examined from CMG broth were 1 by 2-7μm with rounded ends. They were pleomorphic with swelling or vacuoles. Cells occurred singly, in pairs and in short chains.

Surface colonies on blood agar incubated for 48 h were 0.5- 1mm in diameter, circular, entire, convex greyish and semi-opaque (Fig. 3a).

B.ruminicola SS. brevis (NP 333)

Cells from CMG broth were medium rods with rounded ends and contained densely stained areas. Surface colonies on blood agar incubated for 72 h, were pinpoint to 0.5 mm in diameter, circular, convex, greyish-white, (Fig. 3d).

Group II Organisms:

Strains were coccobacillary sometimes forming short chains. No clear distinction was possible between subspecies on the basis of microscopic appearances.

Pigmentation started as a light tan (3-5 days) increasing through brown to shiny black (7-14 days). (Fig.6a & b). The colour is seen first in the centre of the colonies.

B.melaninogenicus SS. melaninogenicus (VPI 9085)

Cells from colonies on blood agar (48 h) were coccobacillary 0.5 by 0.5-1μm straight or slightly bent rods; cells from broth cultures were of similar size with those from solid media.

Surface colonies (72 h) were circular, entire, convex and 1-2 mm in diameter. Brown-black pigment occurred in 5-7 days, (Fig.4a).

B.melaninogenicus SS. intermedius (T588)

Cells from colonies on blood agar (48 h) were 1 by 0.5-3μm rods with rounded ends. Cells from CMG broth were larger and highly vacuolated. Surface colonies on blood agar incubated for 72 h were 0.5-2 mm; circular, entire, rough, dry, becoming dark brown-black in 3-5 days (Fig.4d)

B.melaninogenicus SS. macacea (ATCC 33141)

Cells from colonies on blood agar (48 h) and from CMG broth were of the same size and shape. Cells were 0.5 by 1-1.5μm bacilli to coccobacilli with rounded ends (Fig.5c). Surface colonies on blood agar incubated for 3 days were 0.5-1.5 mm in diameter, circular, convex, entire, opaque, initially greyish turning black on prolonged incubation (7-14 days) (Fig.4c).

B.asaccharolyticus (B536, B537, W50)

Cells from colonies on blood agar (48 h) were 0.5 by 0.5-1μm coccobacilli with vacuoles and some area of dense staining (Fig.5d). Surface colonies on blood agar incubated for 72 h were smooth, shiny and convex 2-4 mm in diameter with brown pigment turning black on prolonged (6-7 day) incubation (Fig.4b).

Fig. 3: Colonial morphology of four, non-Pigmented Bacteroides species grown anaerobically on blood agar for 48 h at 37°C.

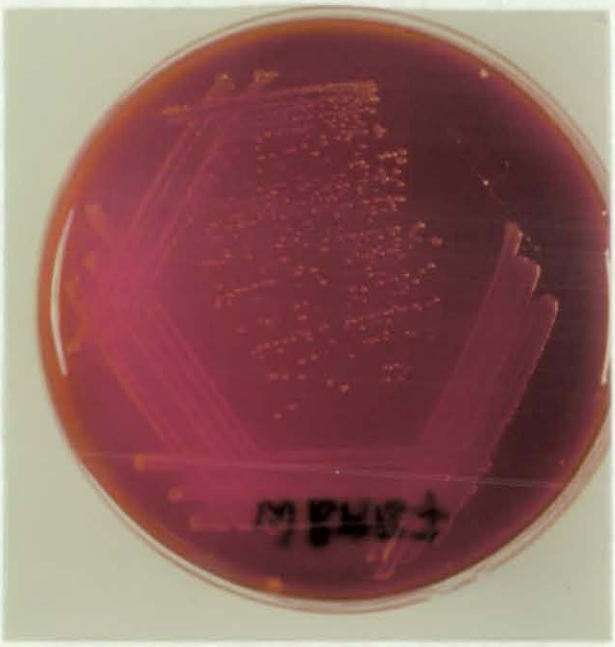
(a) B.vulgatus (WBM137)

(b) B.fragilis (NCTC 8560)

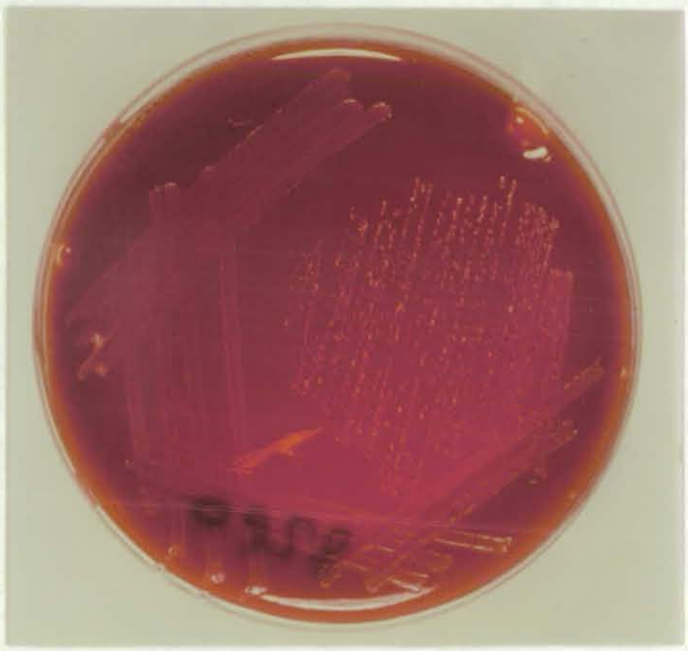
(c) B.ovatus (ATCC 8483)

(d) B.ruminicola ss. brevis (NP333)

Due to lighting conditions 'high light' effects are apparent on Figures 3, 4 and 6.



(a)



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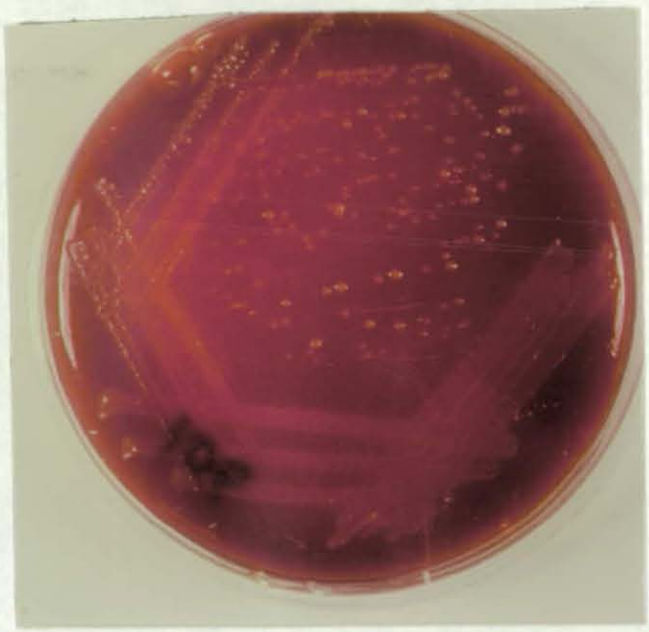
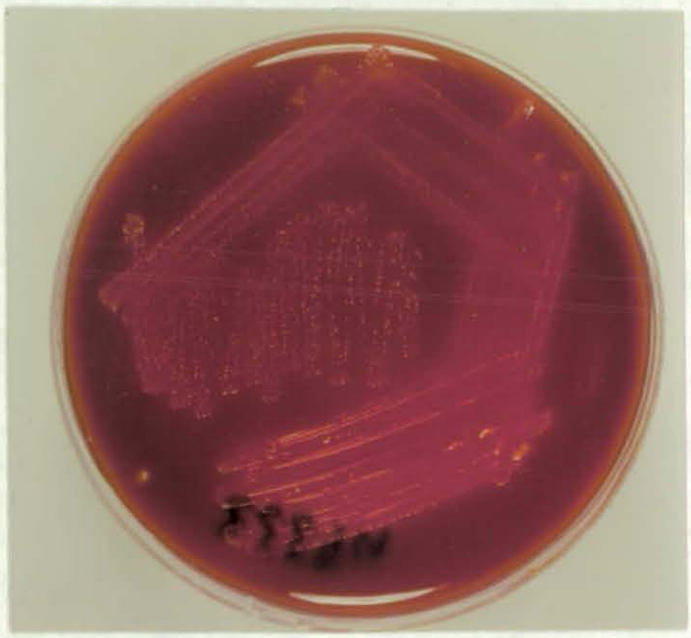


(c)



(d)

Fig. 4: Colonial morphology of four, black pigmented Bacteroides species grown anaerobically on blood agar for 72 h at 37°C.

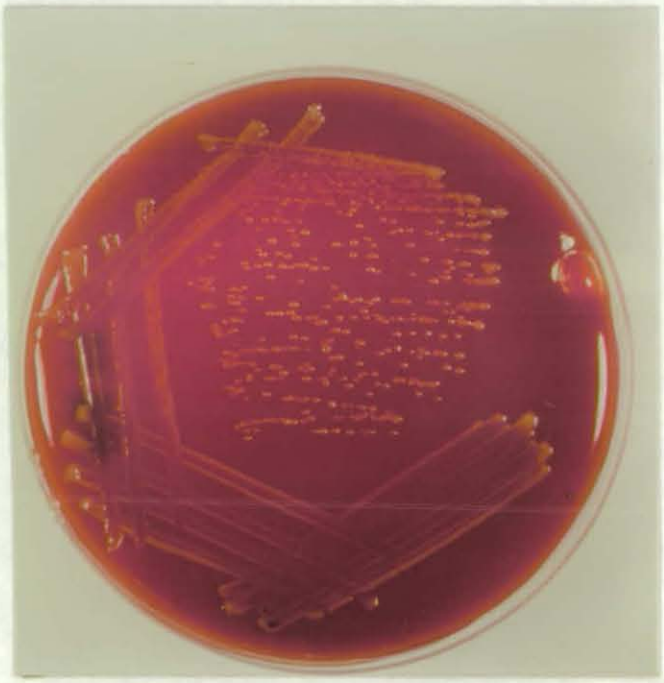
(a) B.melaninogenicus ss. melaninogenicus (VPI 9085)

(b) B.asaccharolyticus (W50)

(c) B.melaninogenicus ss. macacea (ATCC 33141)

(d) B.melaninogenicus ss. intermedius (T588)

(a)



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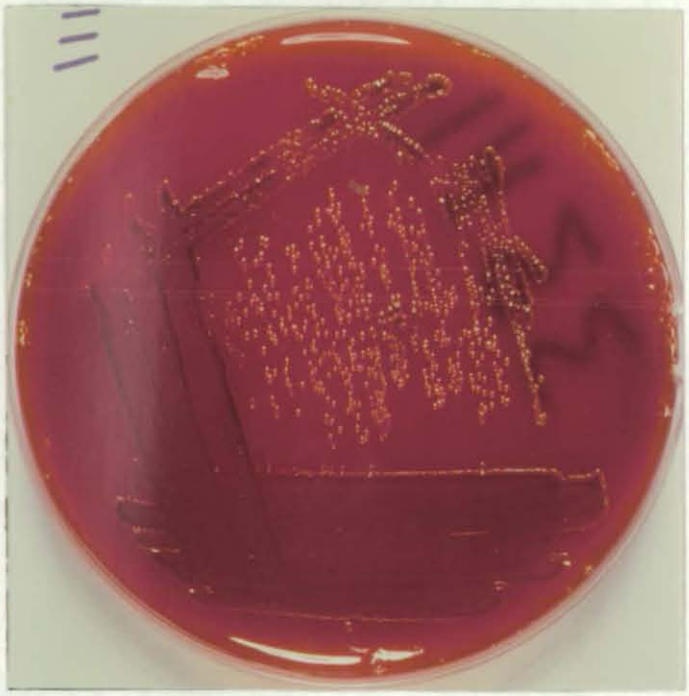


(c)

(d)

Fig. 5: Microscopic appearance of four Bacteroides species grown anaerobically on blood agar for 72 h at 37°C. (X 400)

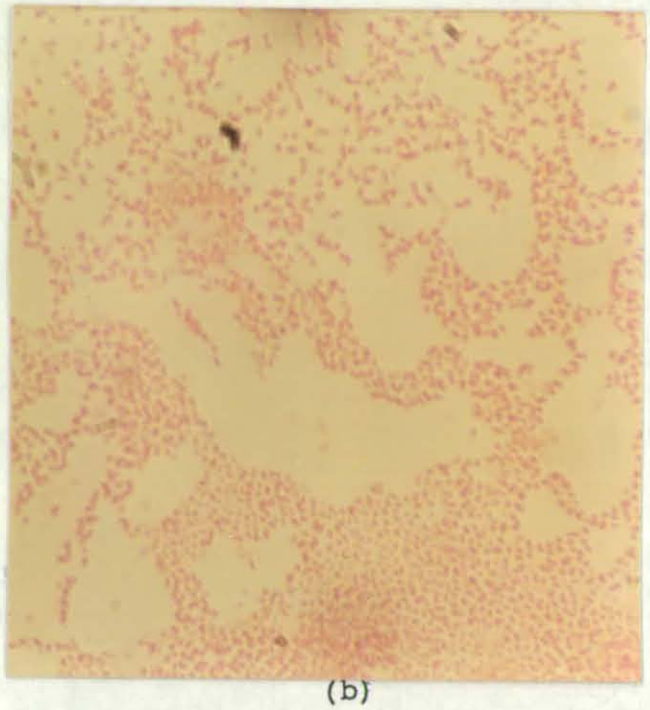
(a) B.distasonis (ATCC 8503)

(b) B.fragilis (NCTC 9343)

(c) B.melaninogenicus ss. macacea (ATCC 33141)

(d) B.asaccharolyticus (W50)

(a)



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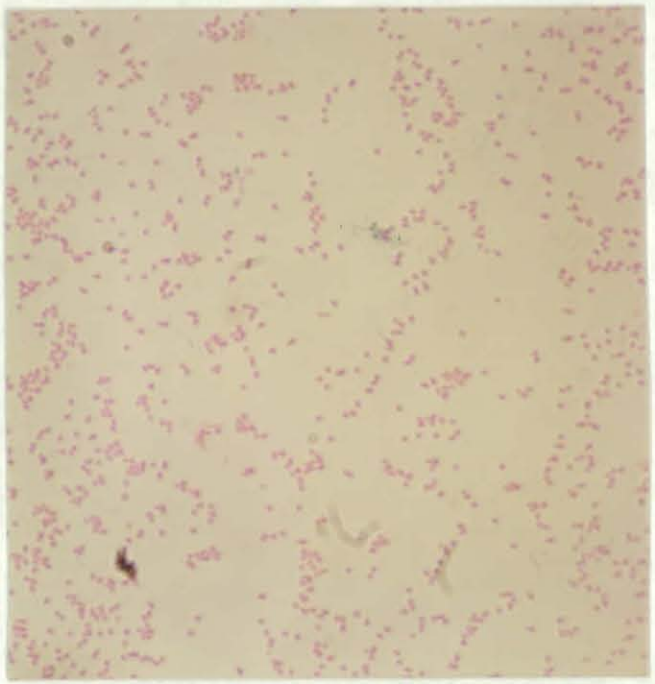
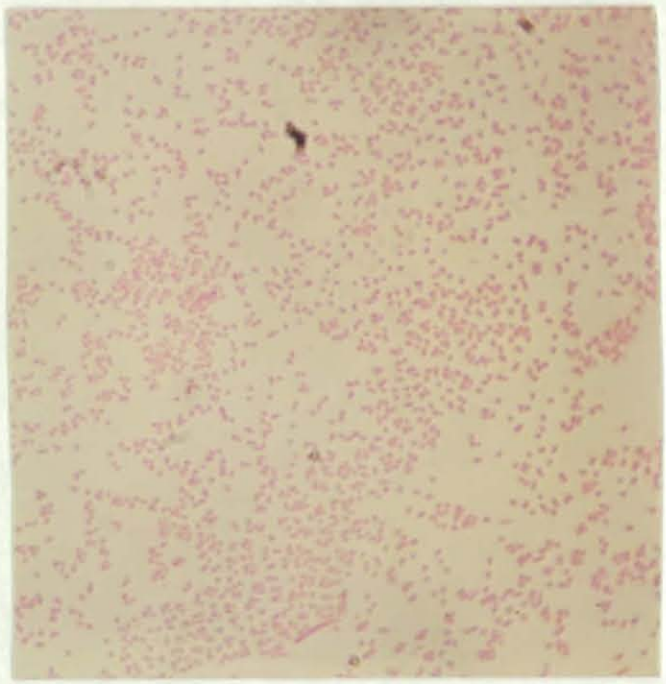


(c)

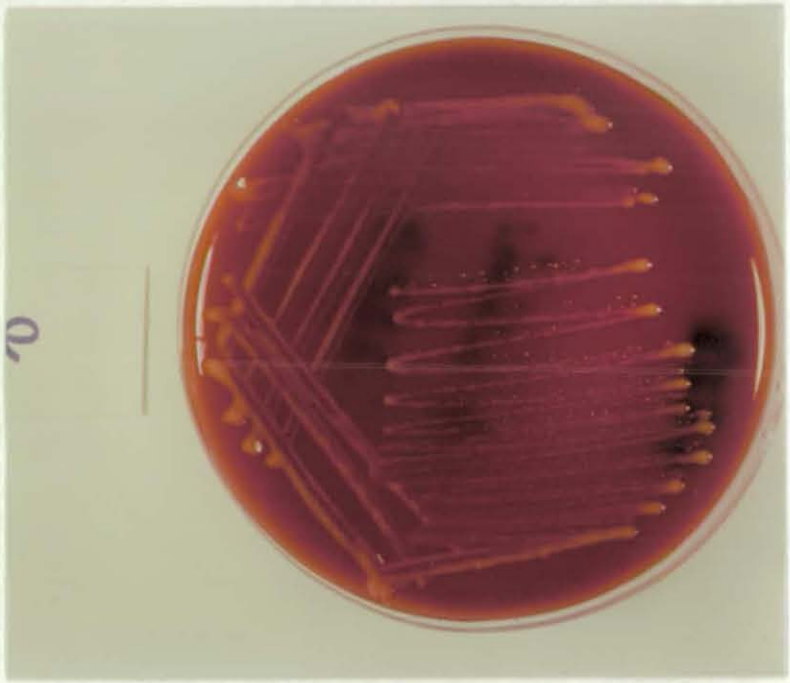


(d)

Fig. 6: Increase in pigmentation of B.asaccharolyticus (B536) on increase in anaerobic incubation from 3-7 days at 37°C.

(a) B.asaccharolyticus (B536) after 3 days of incubation

(b) B.asaccharolyticus (B536) after 7 days of incubation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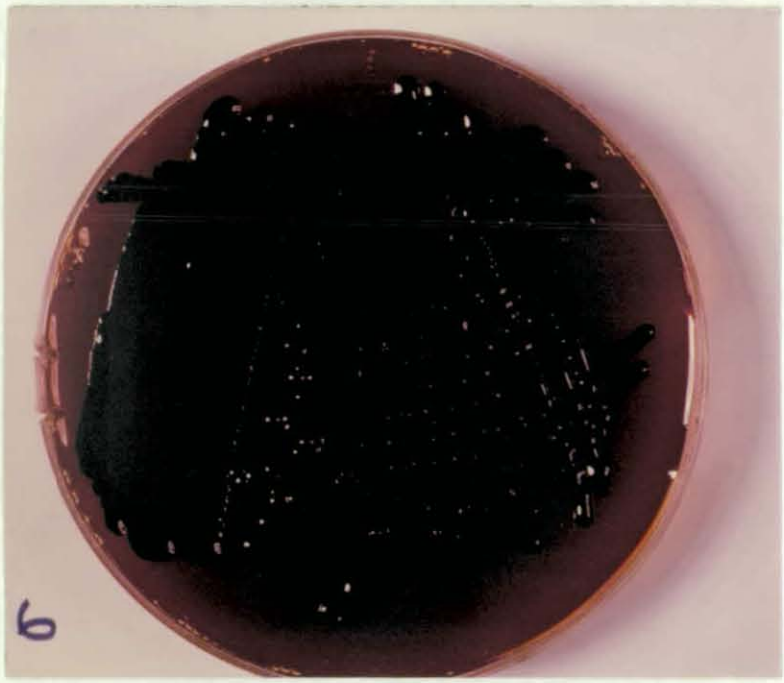
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3.2. Biochemical Tests

The results given in this section were derived from both conventional tests and the API 20A system of identification.

The overall results of the conventional biochemical results of all strains is presented in Table 2 and 3. The overall results of the biochemical tests using the API 20A system of identification, for all strains is presented in Table 4 and 5.

Group I Organisms: Overall results are in Table 2 and 4. All strains were non-motile and non-pigmented. Some were non-haemolytic. Gas was produced by all strains. The presence of 20% bile salts in the growth medium enhanced the growth of most strains except for B.ruminicola SS. brevis (NP 333). All strains were able to ferment glucose, lactose, sucrose, maltose, raffinose and Xylose and to hydrolyse aesculin and starch. In addition catalase was produced by all species except B.ruminicola SS. brevis and B.vulgatus. Individual species gave rise to the following characteristics:

B.fragilis (NCTC 8560 and 9343)

Both strains gave identical patterns of results characterized by their inability to ferment rhamnose, trehalose, mannitol and arabinose. They also failed to produce indole or to reduce nitrate. Fermentation of cellobiose was variable.

B.thetaiotaomicron (NCTC 10582)

The pattern of results obtained was characterized by production of indole, fermentation of rhamnose, trehalose, arabinose, cellobiose, mannose, melezitose and hydrolysis of dextran. It failed to ferment mannitol glycerol and to digest gelatin.

B.distasonis (ATCC 8503)

The pattern obtained was distinguished by failure to produce indole, to hydrolyse dextran and to ferment mannitol, glycerol and arabinose. Acid was produced from the fermentation of rhamnose, trehalose, cellobiose, melezitose, salicin and mannose.

B.ovatus (ATCC 8483)

The results obtained with this strain were characterized by the production of indole, hydrolysis of dextran and fermentation of rhamnose, trehalose, cellobiose and mannitol.

B.vulgatus (WBM137)

The pattern of results obtained was characterized by failure to produce indole, to hydrolyse dextran, and to ferment trehalose, mannitol, cellobiose and salicin. Gelatin was however digested and the organism fermented rhamnose, arabinose and mannose.

B.ruminicola ss. brevis (NP333)

The pattern obtained differed drastically from those of the rest of the strains in the group, by the organism's failure to produce catalase and the inhibitory effect of 20% bile salts on the growth of this strain. In addition the organism failed to produce indole, to digest gelatin or to ferment trehalose, mannose, melezitose and mannitol. Acid was produced from fermentation of salicin, rhamnose, arabinose and cellobiose.

Group II Organisms: Overall results is given in table 3 and 5. All species were non-motile, haemolytic (except for B.melaninogenicus SS. macacae (ATCC 33141) rods which produce black pigmentation when grown on blood agar. All strains produced H_2S, failed to ferment mannitol, rhamnose, salicin, xylose, arabinose, glycerol, melezitose, cellobiose and sorbitol. The presence of 20% bile salts in growth media had an inhibitory effect on the growth of all strains. Catalase was produced only by B.melaninogenicus SS. macacae, ATCC 33141. In addition, none of the strains produced urease but all were able to digest gelatin. Individual characteristics were as follows:

B.melaninogenicus SS. melaninogenicus (VPI 9085):

The pattern of results obtained was characterized by the failure to produce indole. The results also shows the saccharolytic ability of this strain. Glucose,

lactose, maltose, sucrose, raffinose and aesculin were fermented. Dextran and starch were also hydrolysed.

B.melaninogenicus SS. Intermedius (T588)

The pattern of results shows the limited saccharolytic ability of this strain where only glucose was fermented. Lactose, sucrose, maltose, mannose were not fermented. The results also show the ability of this strain to produce indole.

B.melaninogenicus SS. Macacea (ATCC 33141)

Production of catalase by this strain differentiates it from the rest of the group. This strain, also produced indole and fermented glucose, lactose and mannose. Other carbohydrates such as maltose, trehalose, aesculin and raffinose were not fermented. In addition this organism was unable to hydrolyse starch and dextran.

B.asaccharolyticus (Strains B536, B537, W50)

All three strains gave identical patterns of results characterized by the absence of any saccharolytic activity as they were unable to ferment a wide variety of carbohydrates such as glucose, lactose, maltose, sucrose, rhamnose, trehalose, mannitol, arabinose and cellobiose. They also failed to hydrolyse aesculin, dextran and starch but they were able to produce indole.

Table 2

Results of Conventional Biochemical Tests for Group I Organisms

Tests	B.fragilis NCTC 8560	B.fragilis NCTC 9343	B.thetaiomicron NCTC 10582	B.distasonis ATCC 8503	B.ovatus ATCC 8483	B.vulgatus WBM 137	B.ruminicola SS.brevis NP 333
Pigment	-	-	-	-	-	-	-
Motility	-	-	-	-	-	-	-
Haemolysis on blood agar	V	V	-	-	-	-	-
Growth in presence of 20% bile	+	+	+	+	+	+	-
Gas Production	+	+	+	+	+	+	-
Indole	-	-	+	-	+	-	-
Lipase	-	-	-	-	-	-	-
Aesculin hydrolysis	+	+	+	+	+	+	+
Dextran	-	-	+	-	+	-	+
Starch	+	+	+	+	+	+	+
Digestion of gelatin	-	-	-	+	+	+	+
Nitrate reduction	-	-	-	-	-	-	-
Catalase	+	+	+	+	V	-	-
Fermentation of:							
Glucose	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+
Rhamnose	-	-	+	+	+	+	+
Trehalose	-	-	+	+	+	-	-
Mannitol	-	-	-	-	+	-	-
Arabinose	-	-	+	-	+	+	V
Cellobiose	+	+	+	V	+	-	$+^{W}$

+ = Positive reaction in carbohydrate culture, pH < 5.5.

$+^{W}$ = weak positive reaction, pH between 5.5 - 5.7

- = negative reaction, pH > 5.7

V = variable

Table 3

Result of Conventional Biochemical Tests for Group II Organisms

Tests	B.mel.SS.mel VPI9085	B.mel. SS Intermedius T588	B.mel.SS macacea ATCC33141	B.asaccharolyticus B536	B537	W50
Pigment at 72h	brown	black	grey	brown/green	Brown/green	brown/green
Motility	-	-	-	-	-	-
Haemolysis on blood agar	+	+	+	+	+	+
Growth in presence of 20% bile	-	-	-	-	-	-
Gas production	+	+	+	+	+	+
Indole	-	+	+	+	+	+
Lipase	-	V	-	-	-	-
Aesculin hydrolysis	+	-	-	-	-	-
Dextran	+	-	-	-	-	-
Starch	+	+	-	-	-	-
Digestion of Gelatin	+	+	+	+	+	+
Nitrate reduction	-	-	-	-	-	-
Catalase	-	-	+	-	-	-
Fermentation of:						
Glucose	+	+	$+^{W}$	-	-	-
Lactose	+	-	$+^{W}$	-	-	-
Maltose	+	-	-	-	-	-
Sucrose	+	V	-	-	-	-
Rhamnose	-	-	-	-	-	-
Trehalose	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-
Arabinose	$+^{W}$	-	-	-	-	-
Cellobiose	V	-	-	-	-	-

+ = Positive reaction in carbohydrate culture, pH < 5.5

$+^{W}$ = weak positive reaction, pH 5.5 - 5.7

- = negative reaction pH > 5.7

V = variable

Table 4

Results of the API 20A System of Identification for Group I Organisms

Test	B.fragilis NCTC 8560	B.fragilis NCTC 9343	B.thetaiomicron NCTC10852	B.distasonis ATCC8503	B.ovatus ATCC8483	B.vulgatus WBM137	B.ruminicola SS.brevis NP333
Indole	-	-	+	-	+	+	-
Urease	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	+
Mannitol	-	-	-	-	+	-	-
Lactose	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+
Salicin	-	-	+	+	+	-	+
Xylose	+	+	+	+	+	+	-
Arabinose	-	-	+	-	+	+	V
Gelatin	-	-	-	-	-	+	+W
Aesculin	+	+	+	V	+	V	+
Glycerol	-	-	-	-	-	-	+W
Cellobiose	+	+	+	V	+	-	+W
Mannose	+	+	+	+	+	+	-
Melezitose	-	-	+	+W	V	-	-
Raffinose	+	+	+	+	+	+	+
Sorbitol	-	-	-	-	-	-	-
Rhamnose	-	-	+	+W	+	+	+
Trehalose	-	-	+	+	V	-	-
Catalase	+	+	+	+	+	+	-

+ = positive reaction in carbohydrate culture, pH < 5.5

+W = weak positive reaction, pH 5.5 - 5.7

- = negative reaction, pH > 5.7

V = variable

Table 5

Results of the API 20A System of Identification for Group II Organisms

Test	B.mel. SS. mel VPI9085	B.mel. SS. inter. T588	B.mel. SS. maca. ATCC33141	B.asaccharolyticus		
				B536	B537	W50
Indole	-	+	+	+	+	+
Urease	-	-	-	-	-	-
Glucose	+	+	$+^{W}$	-	-	-
Mannitol	-	-	-	-	-	-
Lactose	+	-	$+^{W}$	-	-	-
Sucrose	+	V	-	-	-	-
Maltose	+	-	-	-	-	-
Salicin	-	-	-	-	-	-
Xylose	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-
Gelatin	+	+	+	+	+	+
Aesculin	+	-	-	-	-	-
Glycerol	-	-	-	-	-	-
Cellobiose	V	-	-	-	-	-
Mannose	+	-	$+^{W}$	-	-	-
Melezitose	-	-	-	-	-	-
Raffinose	+	V	-	-	-	-
Sorbitol	-	-	-	-	-	-
Rhamnose	$+^{W}$	V	-	-	-	-
Trehalose	-	-	-	-	-	-
Catalase	-	-	+	-	-	-

+ = positive reaction in carbohydrate culture, pH < 5.5

$+^{W}$ = weak positive reaction, pH 5.5 - 5.7

- = negative reaction pH > 5.7

V = variable

3.3. Discriminatory Biochemical Tests for Identification of Bacteroides species

Table 6 represents a summary of all biochemical tests performed using conventional methods (Table 2 and 3) and API 20A system of identification (Table 4 and 5) and found to be of discriminatory value in the identification of Bacteroides species.

Pigment production and enhancement of growth in presence of 20% bile divide the species studied into two groups. Group one includes the non-pigmented Bacteroides in which growth is enhanced by the presence of bile salts in growth medium, this include, B.fragilis, B.thetaiotaomicron, B.ovatus and B.vulgatus. The second group includes the black pigmented species which growth is inhibited by the presence of bile salts in growth medium and includes B.melaninogenicus SS. melaninogenicus, B.melaninogenicus SS. intermedius, B.melaninogenicus SS. macacae and B.asaccharolyticus. The only exception noted is the species B.ruminicola SS. brevis which belongs to the non-pigmented species but its growth was inhibited by the presence of bile salts in growth medium. Individual species were further characterized as follows:

B.fragilis was characterized by its ability to ferment glucose, lactose, mannose, cellobiose, and aesculin, and its failure to ferment arabinose, rhamnose, trehalose, salicin and mannitol. In addition it failed to produce indole but is found to produce catalase and hydrolyse starch.

B.thetaiotaomicron was characterized by its ability to ferment glucose, lactose, mannose, cellobiose, trehalose, rhamnose, arabinose, aesculin and salicin and production of indole and catalase. Starch was hydrolysed by this species but mannitol was not fermented.

B.distasonis was characterized by its ability to ferment glucose, lactose, mannose, salicin, trehalose and rhamnose and its failure to ferment mannitol, arabinose and produce indole. It hydrolysed starch and produced catalase. Tests for fermentation of cellobiose and aesculin were variable.

B.ovatus was characterized by its ability to ferment glucose, lactose, mannose, mannitol, salicin, cellobiose, trehalose, rhamnose, arabinose and aesculin and its production of indole. Starch was hydrolysed by this strain. Results for production of catalase were variable.

B.vulgatus was characterized by its ability to ferment glucose, lactose, mannose, rhamnose and arabinose and its failure to ferment mannitol, salicin, cellobiose and trehalose. Indole and catalase was not produced but starch was hydrolysed. Tests for fermentation of aesculin were variable.

B.ruminicola SS. brevis was characterized by its ability to ferment glucose, lactose, salicin, rhamnose and aesculin and its failure to ferment mannose, mannitol, trehalose and

to produce indole and catalase. It hydrolysed starch but gave variable results with arabinose and cellobiose fermentation.

B.melaninogenicus SS. melaninogenicus was characterized by its ability to ferment glucose, lactose, mannose and aesculin and its failure to hydrolyse mannitol, salicin and rhamnose and to produce indole and catalase. It hydrolysed starch but variable results was recorded for cellobiose, trehalose and arabinose fermentation.

B.melaninogenicus SS. intermedius was characterized by its failure to ferment lactose, mannose, mannitol, salicin, cellobiose, rhamnose, arabinose and aesculin and to produce catalase. Glucose was fermented and starch hydrolysed by this strain. Indole was produced but results of trehalose fermentation were variable.

B.melaninogenicus SS. macacae was characterized by failure to ferment mannitol, salicin, cellobiose, trehalose, rhamnose, arabinose and aesculin and its ability to ferment weakly glucose, mannose and lactose. In addition, indole and catalase were found to be produced by this species.

B.asaccharolyticus was characterized by its failure to ferment all carbohydrates listed in Table 6 and to hydrolyse starch. Indole was found to be produced by this species.

Table 6

Summary of all Tests of Discriminatory Value for the Identification of Bacteroides Species

Tests	B. fragilis	B.thetaiotaomicron	B. distasonis	B. ovatus	B. vulgatus	B. ruminicola SS.brevis	B.mel. SS.mel.	B.mel SS. Inter.	B.mel SS. macacea	B.asaccharolyticus
Pigment at 72h	-	-	-	-	-	-	Brown	Black	Grey/ Green	Brown/ Green
Growth in 20% bile	+	+	+	+	+	-	-	-	-	-
Indole	-	+	-	+	-	-	-	+	+	+
Catalase	+	+	+	V	-	-	-	-	+	-
Glucose	+	+	+	+	+	+	+	+	$+^W$	-
Mannose	+	+	+	+	+	-	+	-	$+^W$	-
Mannitol	-	-	-	+	-	-	-	-	-	-
Starch	+	+	+	+	+	+	+	+	-	-
Salicin	-	+	+	+	-	+	-	-	-	-
Cellobiose	+	+	V	+	-	$+^W$	V	-	-	-
Trehalose	-	+	+	+	-	-	V	V	-	-
Rhamnose	-	+	+	+	+	+	-	-	-	-
Arabinose	-	+	-	+	+	V	V	-	-	-
Aesculin	+	+	V	+	V	+	+	-	-	-

+ = Positive reaction in carbohydrate culture, pH < 5.5

$+^W$ = weak positive reaction, pH between 5.5 - 5.7

- = negative reaction pH > 5.7

V = variable

3.4. Chromatographic Analysis of SCFA

Acetic (A) and succinic (S) acids were the principal end products encountered in all species, whereas propionic (P), isobutyric (IB), butyric (B), isovaleric (IV) and lactic (L) acids were detected in smaller quantity. The production of these acids by each species was as follows:

(1) Production of short chain fatty acid (SCFA) in cooked meat glucose (CMG) medium:

Group I Organisms:

B.fragilis (NCTC 8560)

The VFAs produced were A, P with B and IV, in trace amounts. The presence of these acids was detected very clearly after 12 h of incubation and it increased gradually until a maximum level was reached after 96 h. The ratio of A:P at 96 h was 2:1 (Fig. 7a).

The n-VFAs produced were S and L and their production profiles were similar to those of VFAs with maximum levels reached after 96 h was 2:1 (Fig.7b).

B.fragilis (NCTC 9343)

The VFAs and n-VFAs produced were similar to those produced by the previous strain, and included A, P, traces of B and IV, S and L. The production of these acids reached a steady level after 72 h of incubation followed by a slight decrease towards the end of the incubation period. After 96 h of incubation the

ratio of both A:P and of S:L was 2:1, Figs. (8a & b).

B.ovatus (ATCC 8483)

The VFAs produced were A and P. Their production reached its highest level after 96 h of incubation; this was followed by a decrease in concentration detected over the latter part of incubation. The ratio of A:P at 96 h was 1.8:1 (Fig.9a).

The n-VFAs produced were S and L. The concentration of L reached a maximum steady level after 96 h and remained constant until the end of the incubation period, while S showed a slight decrease after 96 h. The ratio of S:L was 4.8:1 at 96 h (Fig.9b).

B.distasonis (ATCC 8503)

The VFAs produced were A, P with traces of IV. A reached its maximum level after 72 h and remained so for the rest of the incubation preiod. P reached its maximum level at 96 h followed by a decrease in its concentration after 120 h until the end of the incubation period. The ratio of A:P at 96 h of incubation was 2:1 (Fig.10a).

The n-VFAs produced were S and L. The pattern of production for these acids reached a steady level after 96 h of incubation and remained constant until the end of the incubation time of 168 h. The ratio of S:L at 96 h was 7.5:1 (Fig.10b).

B.vulgatus (WBM137)

The VFAs produced were A and P. The pattern of production of both acids showed a gradual increase in detected levels throughout the incubation period. The ratio A:P at 96 h was 1.4:1 (Fig. 11a).

The n-VFAs produced as with all previous strains were S & L. The pattern of production for both acids was similar and reached a maximum level at 96 h after which the detected level of S showed a slight decrease, while L remained constant. The ratio of S:L at 96 h of incubation was 5.7:1 (Fig. 11b).

B.ruminicola SS. brevis (NP333)

The VFAs produced were A, in moderate amount, with traces of IV. The pattern of A production was found to reach its maximum level at 96 h followed by a slight decline towards the end of the incubation period. Although only produced in trace amounts, IV production followed a similar pattern (Fig. 12a).

The n-VFAs produced were S and L. The pattern of production of both acids was similar and it reached its maximum level after 96 h of incubation followed by a slight decrease towards the end of the incubation period.

The organism prdouced S and L at a ratio of 3.3:1 at 96 h of incubation (Fig. 12b).

B.thetaiotaomicron NCTC 10582)

The VFAs produced were A, P with a smaller amount of IV. The overall pattern of production for both acids A and P was showing maximum levels at 96 h. This was followed by a slight decrease towards the end of the incubation period. The ratio of A:P at 96 h was 1:1. The level of IV increased gradually reaching its maximum amount after 120 h. A slight decrease was detected after 168 h of incubation (Fig. 13a).

The n-VFAs produced were S and L. The production of S reached and maintained a maximum level from 48 h to 120 h of incubation followed by a slight decrease towards the end of the incubation period. The production of L gave rise to lower detectable level and was more steady after reaching its maximum level at 48 h. The ratio of S:L at 96 h was 3.6:1 (Fig.13b).

Group II Organisms:

B.melaninogenicus SS. melaninogenicus (VPI 9085)

The only VFA produced by this strain was A. Maximum level was attained at 96 h followed by gradual decrease until 168 h of incubation (Fig. 14a).

The n-VFAs produced were S and L and their production reached its maximum level at 96 h followed by a slight decrease until the end of incubation. The ratio of S:L at 96 h were 2.2:1 (Fig. 14b).

B.melaninogenicus SS. intermedius (T588)

The VFAs produced were A and IV as a major products and P and IB in trace amounts. The pattern of production for A and IV showed a gradual accumulation of these acids in the medium with a steady state reached between 96 - 120 h of incubation followed by decrease towards the end of the incubation period (Fig. 15a).

The n-VFAs produced were S and L with a maximum and steady level reached after 72 h; it remained so until the end of incubation period. The ratio of S:L at 96 h was 7.9:1 (Fig. 15b).

B.melaninogenicus SS. macacea (ATCC 33141)

The VFAs produced were A, P. IV, B and IB. The concentration of these acids reached a maximum level after 48 h which was maintained until the end of incubation period. The ratios of A:P:IV:B:IB at 96 h were 1:1.27:0.46:0.3:0.16 respectively (Fig. 16a).

The n-VFAs produced were S and L. Their production was slow resulting in gradual accumulation of these acids in the medium reaching its highest level after 144 h for S though L decreased slightly after 144 h. The ratio of S:L at 96 h was 1.3:1 (Fig. 16b).

B.asaccharolyticus (B536, B537 and W50)

(i) B536

The VFAs produced were A, P, IV, B and IB. The

overall pattern of production for all these acids was similar and reached a maximum level at 120 h, after which their level stayed constant until the end of the incubation period. The ratios of A:P:IV:B: IB at 96 h were 1:3.3:0.8:0.6:0.4 respectively (Fig. 17a).

The n-VFAs were S and L which reached and maintained a steady level after 96 h. The ratio of S:L at 96 h was 2:1 (Fig. 17b).

(ii) B537)

The VFAs production patterns were similar to those of strain B636. Acids detected were A, P, IV, B and IB. The maximum level of production was achieved approximately after 48 h and maintained throughout the incubation period. Though IV showed a slight decrease after 144 h. The ratios of A:P: IV:B:IB at 96 h were 1:1.2:0.67:0.5:0.26 respectively. (Fig.18a).

The n-VFAs produced were S and L. Concentration detected reached and maintained a maximum level after 72 h and until the end of the incubation period. The ratios of S:L at 96 h was 2.1:1 (Fig. 18b).

(iii) W50

The overall profile and pattern of the VFAs produced by this strain were similar to that of the previous 2 strains.

The ratios of A:P:IV:B:IB at 96 h were 1:1.20:0.68:0.43:0.29 respectively (Fig.19a).

The n-VFAs produced was S and L, with production patterns similar to those shown by strain B536. The ratio of S:L at 96 h was 1.82:1 (Fig. 19b).

(2) Production of SCFA in PYG

B.fragilis (NCTC 8560 and 9343)

The VFAs produced by both strains were A,P with traces of IV and B. Production reached maximum and steady levels at 72 h and remained so until the end of the incubation period. The ratio, in cultures of both strains, of A:P at 96 h was 2:1:1 (Figs.20a & 21a).

The n-VFAs produced by both strains were S and L and their pattern of production was similar to that of the VFAs. The ratio of S:L at 96 h was 2:1 (Figs.20b & 21b). This ratio and that of A:P were the same as those obtained for the same organisms using CMG medium.

B.asaccharolyticus (B536 and W50)

i) B536:

The overall profile and pattern of VFAs production (Fig. 22a) was similar to that of the same strain in CMG.

The ratio of A:P:IV:B:IB at 96 h were 1:1.20:0.62:0.43:0.21 respectively; these ratios show a slight decrease from those obtained in CMG medium (Fig. 17a).

The n-VFAs were S and L with a similar pattern of production as in CMG medium (Fig. 16b). The ratio of S:L at 96 h of incubation was 2:1 (Fig. 22b).

ii) W50

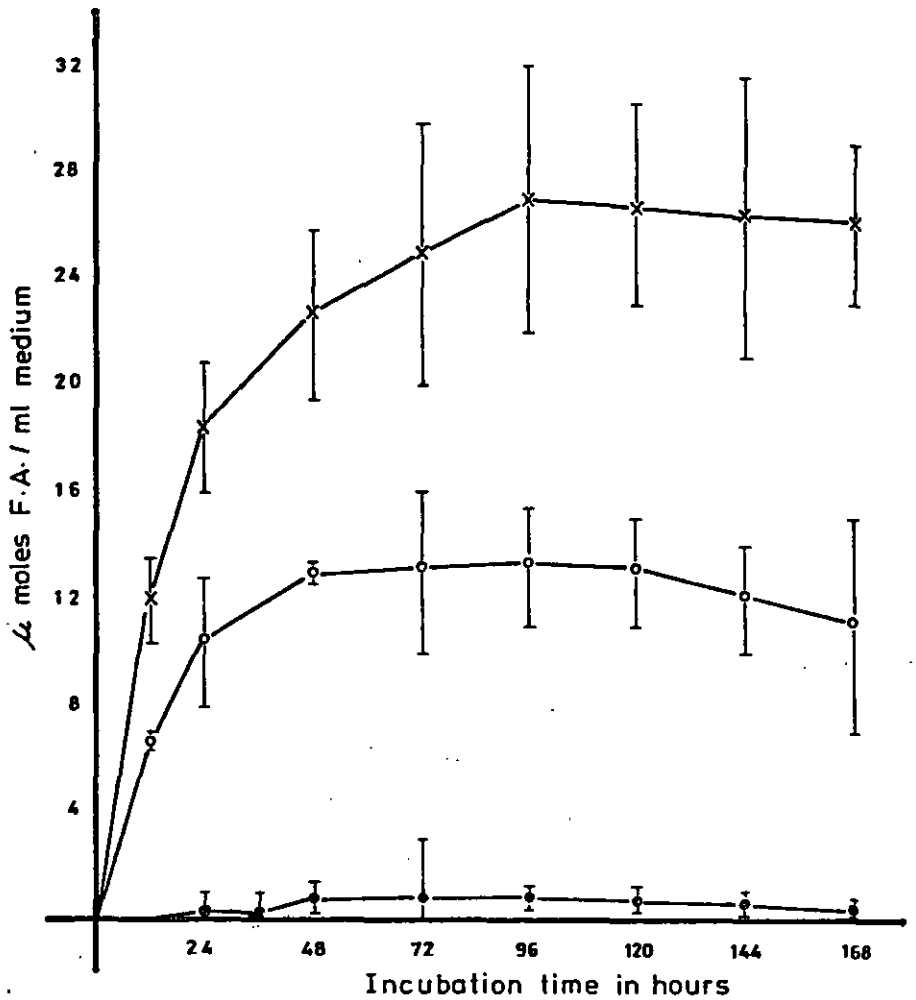
The VFAs detected were A, P. IV, B and IB. The production pattern of all acids reached its maximum level at 96 h and remained so until the end of the incubation period. The ratio of A:P:IV:B:IB at 96 h were 1:99:0.62:0.42:0.23 respectively (Fig. 23a).

The n-VFAs S and L followed a similar pattern to that of the VFAs; the ratio of S:L at 96 h of incubation was 1.90:1 (Fig. 23b).

B.melaninogenicus ss. macacea (ATCC 33141)

The VFAs produced were A,P.IV,B and IB, their pattern of production reached its maximum level at 96 h; it remained at this level until the end of incubation period. The ratios of A:P:IV:B:IB at 96 h were 1:1.21:0.40:0.21:0.108 respectively (Fig. 24a).

The n-VFAs were S and L, with their maximum level detected after 48 h. This level was maintained to the end of the incubation period. The ratio of S:L at 96 h was 1.23:1 (Fig. 24b).


32
28
24
20
16
12
8
4
µ moles F.A./ ml medium
24
48
72
96
120
144
168
Incubation time in hours

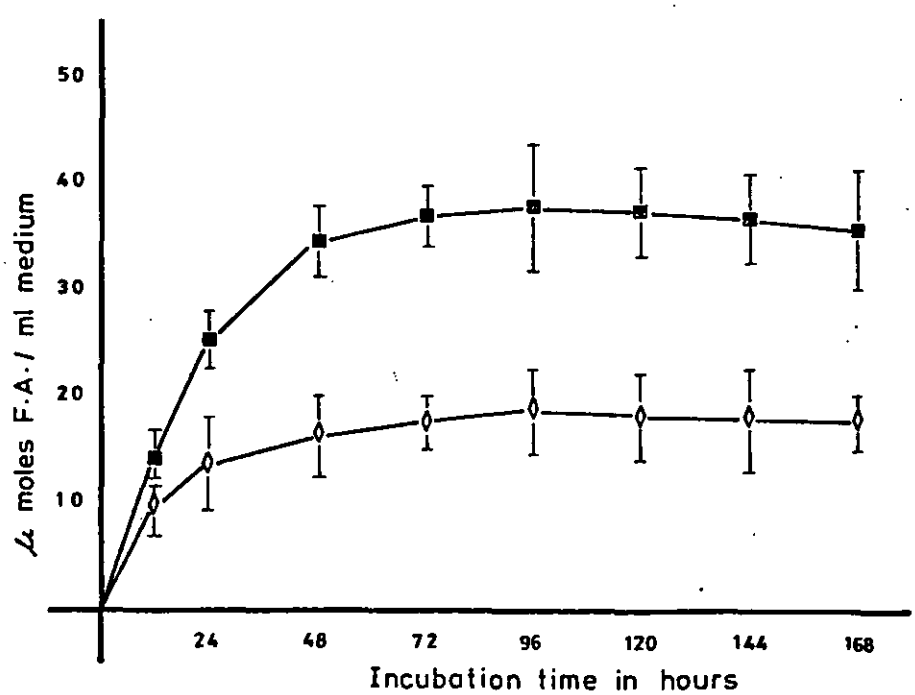

50
40
30
20
10
µ moles F.A./ ml medium
24
48
72
96
120
144
168
Incubation time in hours

Fig. 7(a): Production of VFAs by B.fragilis (NCTC 8560) in CMG medium over 7 days of anaerobic incubation at 37°C.

X——X Acetic acid (A)

o——o Propionic acid (P)

●——● Isovaleric acid (IV)

Fig. 7(b) Production of n-VFAs by B.fragilis (NCTC 8560) in CMG medium over 7 days of anaerobic incubation at 37°C.

■——■ Succinic acid (S)

◊——◊ Lactic acid (L)

Fig. 8(a): Production of VFAs by B.fragilis (NCTC 9343) in CMG medium over 7 days of anaerobic incubation at 37°C.

x—x Acetic acid (A)

o—o Propionic acid (P)

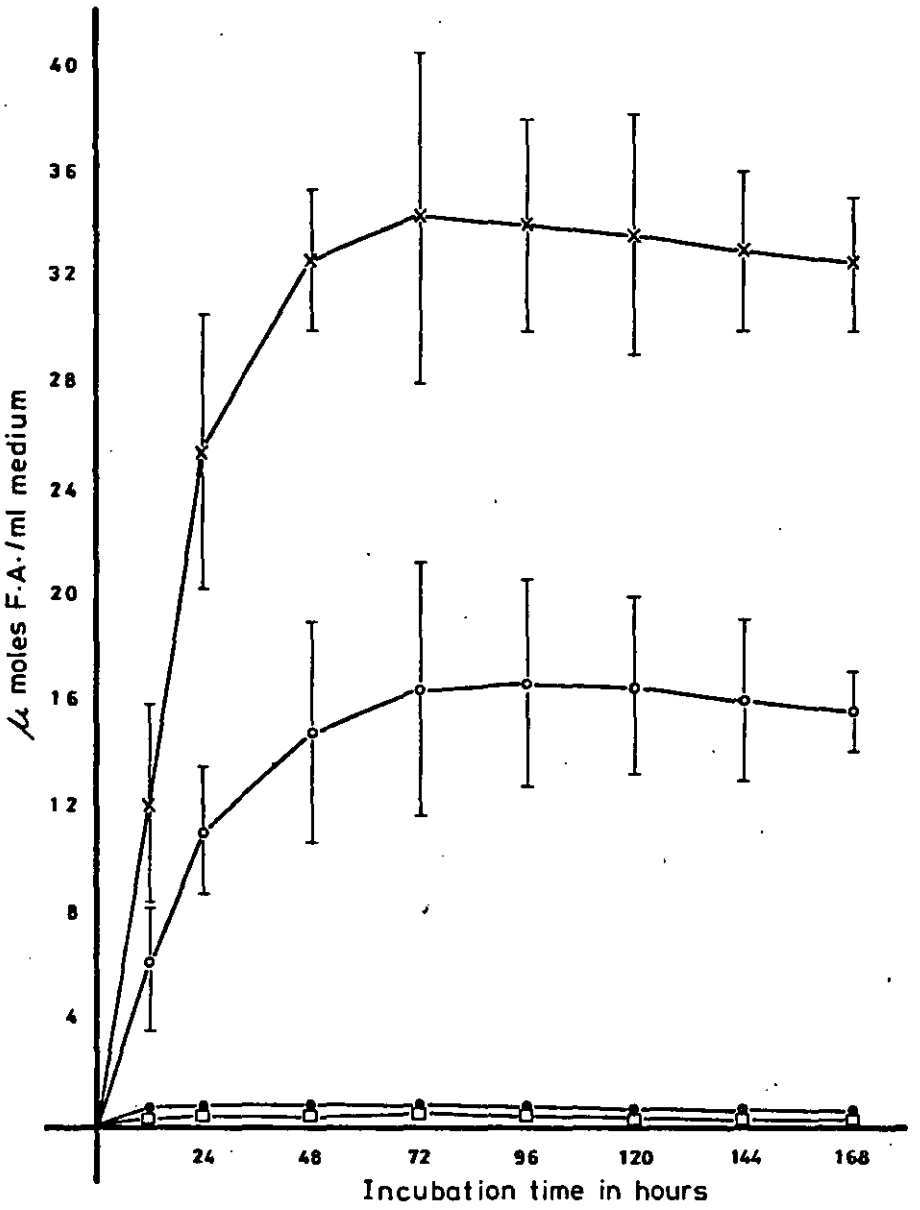
●—● Isovaleric acid (IV)

□—□ Butyric acid (B)

Fig. 8(b): Production of n-VFAs by B.fragilis (NCTC 9343) in CMG medium over 7 days of anaerobic incubation at 37°C.

■—■ Succinic acid (S)

◊—◊ Lactic acid (L)


40
36
32
28
24
20
16
12
8
4
μ moles F·A·/ml medium
24
48
72
96
120
144
168
Incubation time in hours

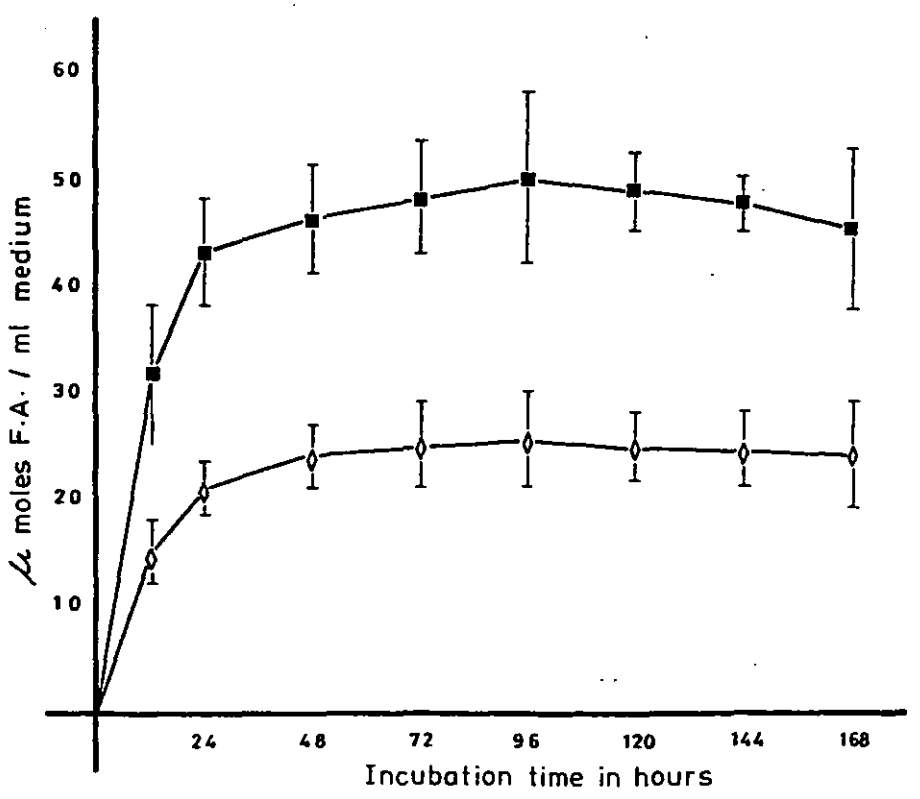

60
50
40
30
20
10
μ moles F·A· / ml medium
24
48
72
96
120
144
168
Incubation time in hours

Fig. 9(a): Production of VFAs by B.ovatus (ATCC 8483) in CMG medium over 7 days of anaerobic incubation at 37°C.

x——x Acetic acid (A)

o——o Propionic acid (P)

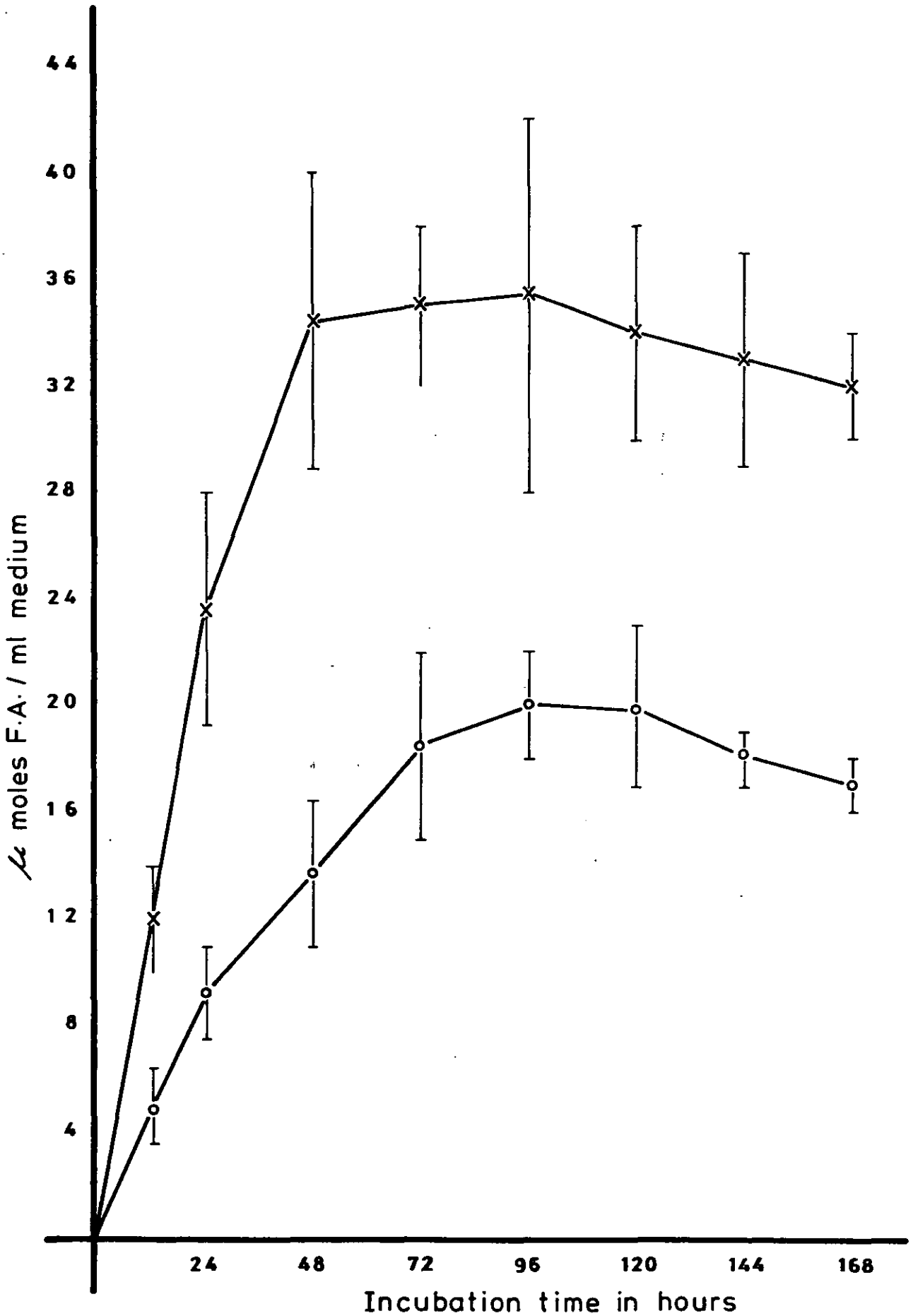

44
40
36
32
28
24
20
16
12
8
4
μ moles F.A. / ml medium
24
48
72
96
120
144
168
Incubation time in hours

Fig. 9(b): Production of n-VFAs by B.ovatus (ATCC 8483) in CMG medium over 7 days of anaerobic incubation at 37°C.

■—■ Succinic acid (S)

◊—◊ Lactic acid (L)

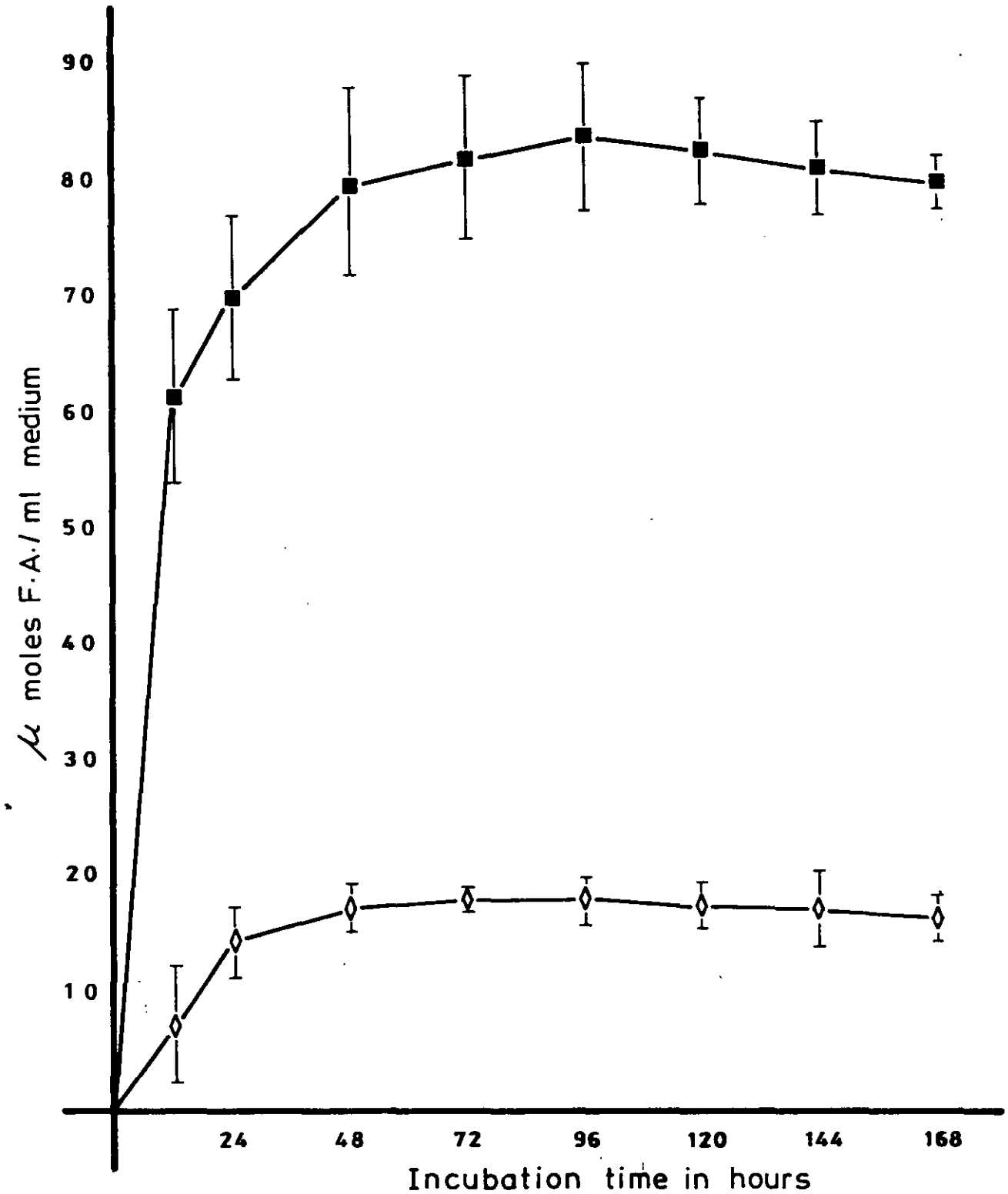

90
80
70
60
50
40
30
20
10
μ moles F.A./ml medium
24
48
72
96
120
144
168
Incubation time in hours

Fig. 10(a): Production of VFAs by B.distasonis (ATCC 8503) in CMG medium over 7 days of anaerobic incubation at 37°C.

x——x Acetic acid (A)

o——o Propionic acid (P)

●——● Isovaleric acid (IV)

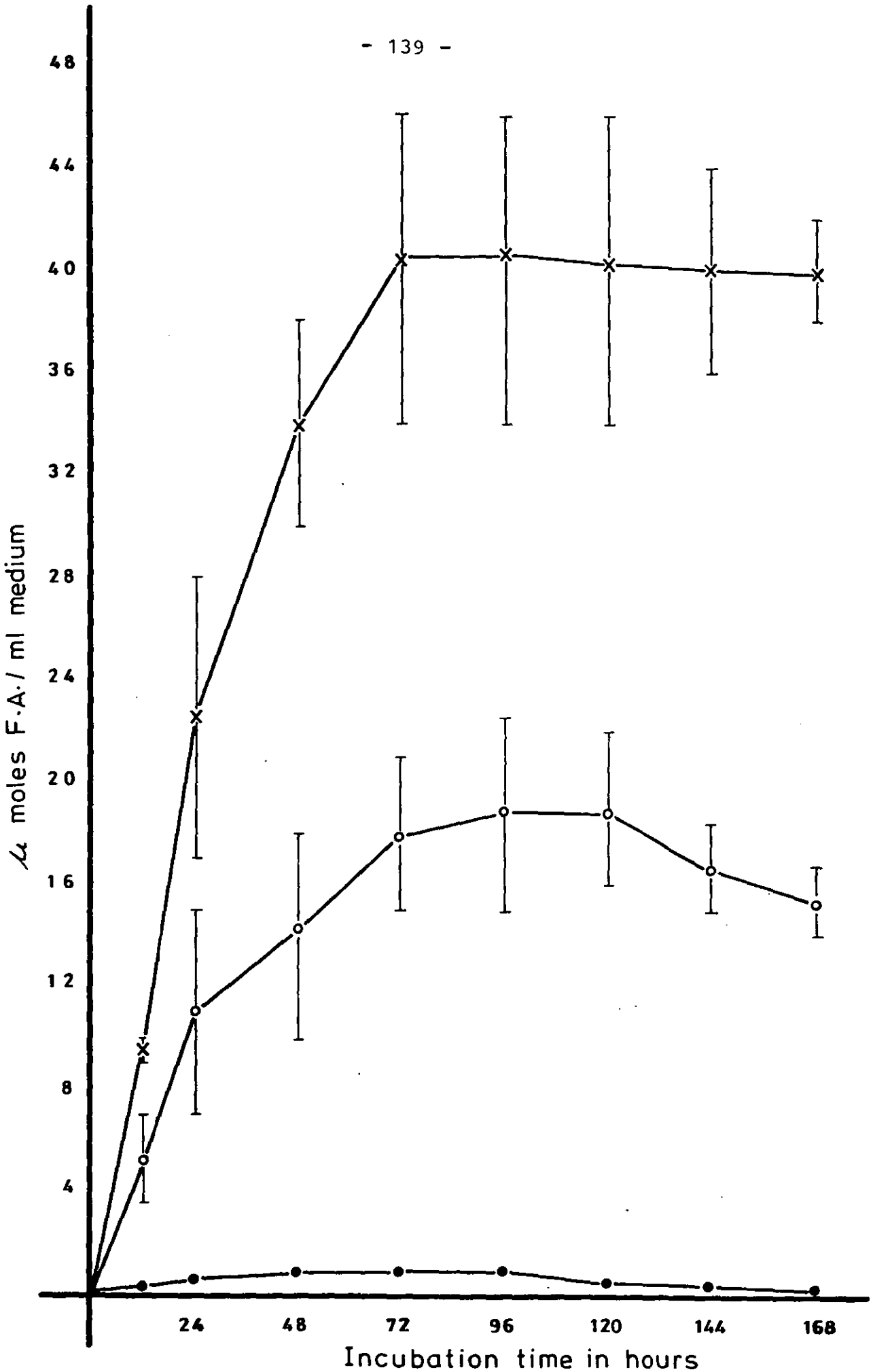

48
44
40
36
32
28
24
20
16
12
8
4
μ moles F·A·/ ml medium
24
48
72
96
120
144
168
Incubation time in hours

Fig. 10(b): Production of n-VFAs by B.distasonis (ATCC 8503) in CMG medium over 7 days of anaerobic incubation at 37°C.

■——■ Succinic acid (S)

◊——◊ Lactic acid (L)

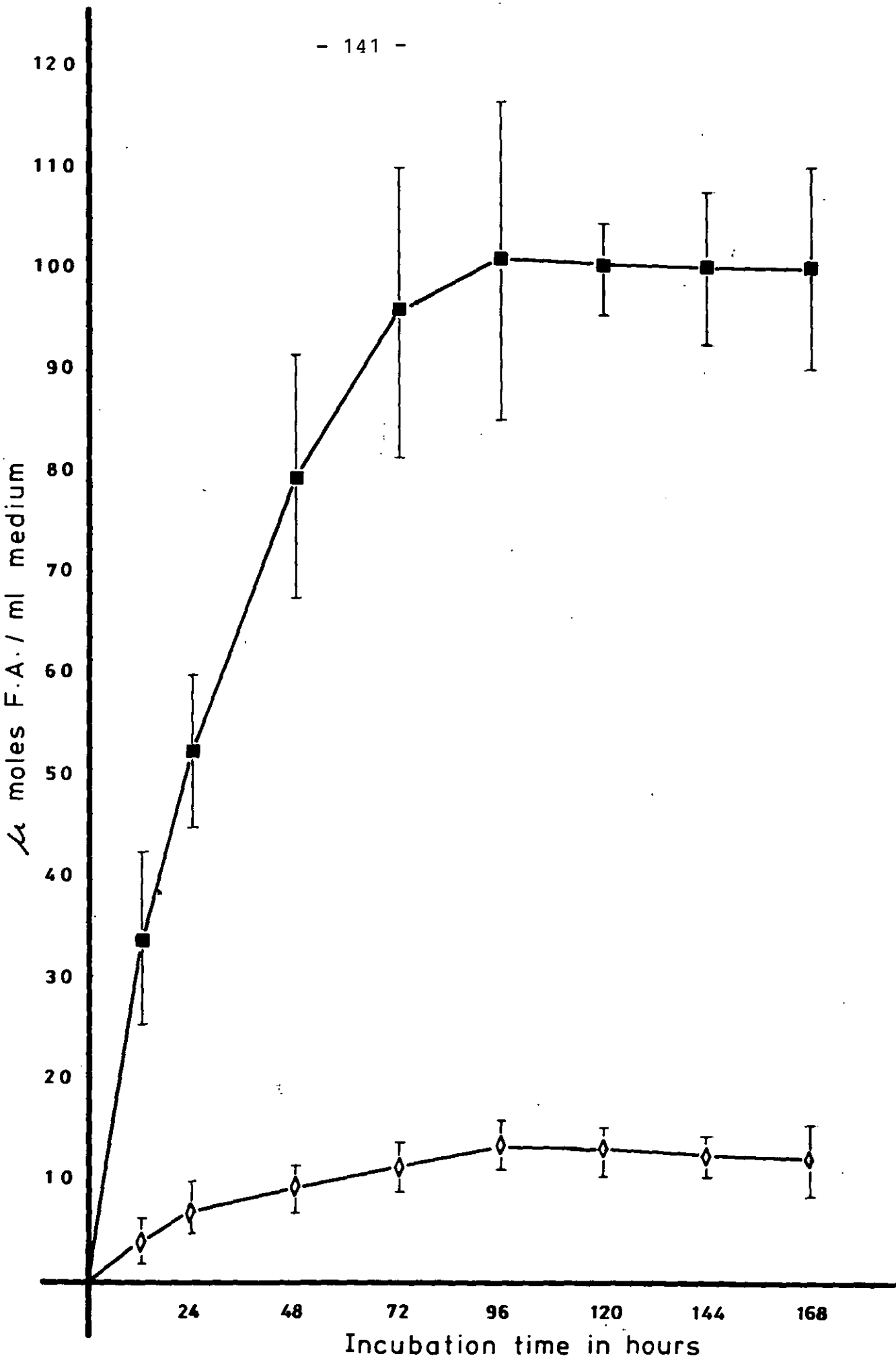

120
110
100
90
80
70
60
50
40
30
20
10
μ moles F.A. / ml medium
24
48
72
96
120
144
168
Incubation time in hours

Fig. 11(a): Production of VFAs by B.vulgatus (WBM137) in CMG medium over 7 days of anaerobic incubation at 37°C.

x——x Acetic acid (A)

o——o Propionic acid (P)

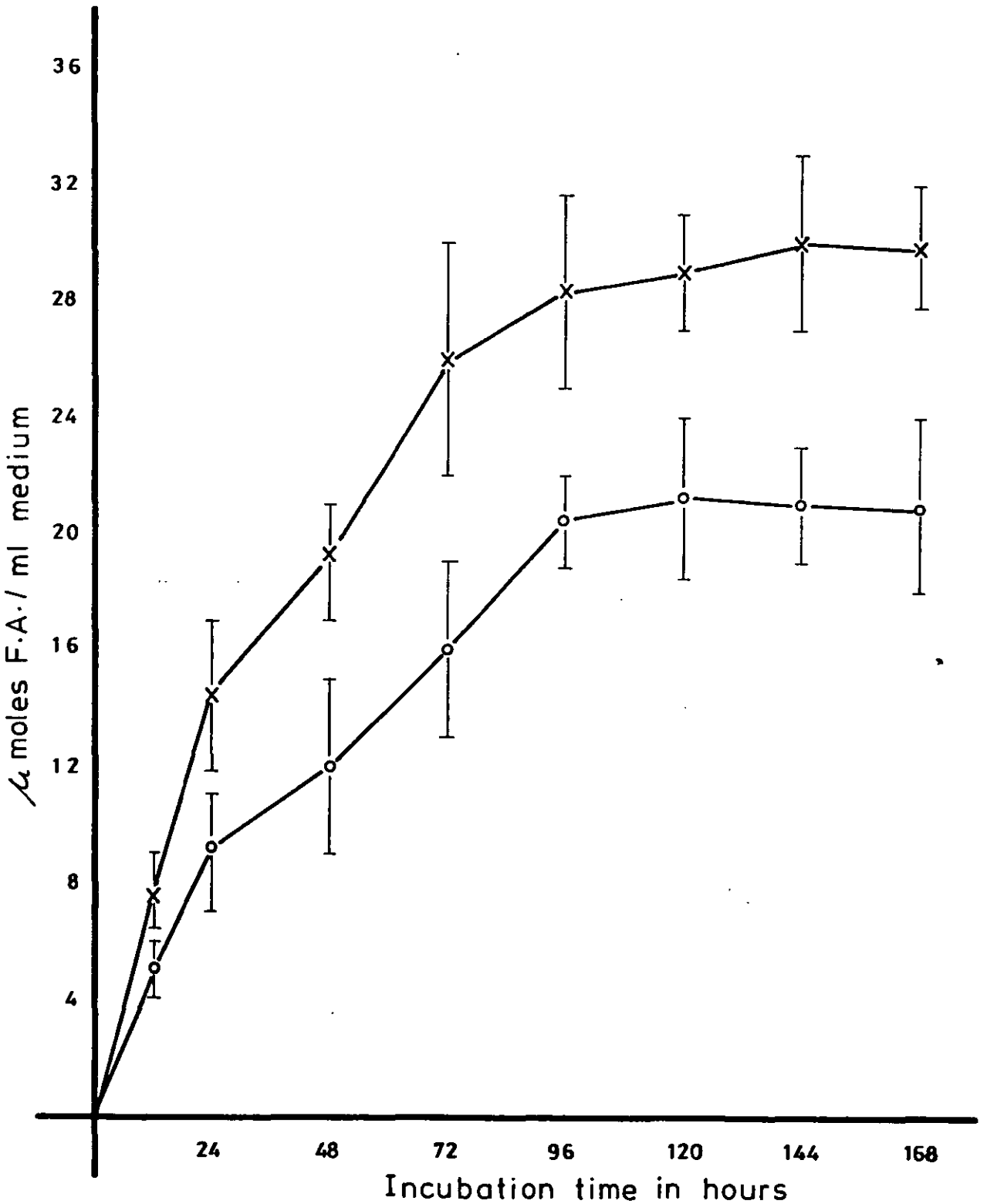

36
32
28
24
20
16
12
8
4
μ moles F.A./ ml medium
24
48
72
96
120
144
168
Incubation time in hours

Fig. 11(b): Production of n-VFAs by B.vulgatus (WBM137) in CMG medium over 7 days of anaerobic incubation at 37°C.

■—■ Succinic acid (S)

◊—◊ Lactic acid (L)

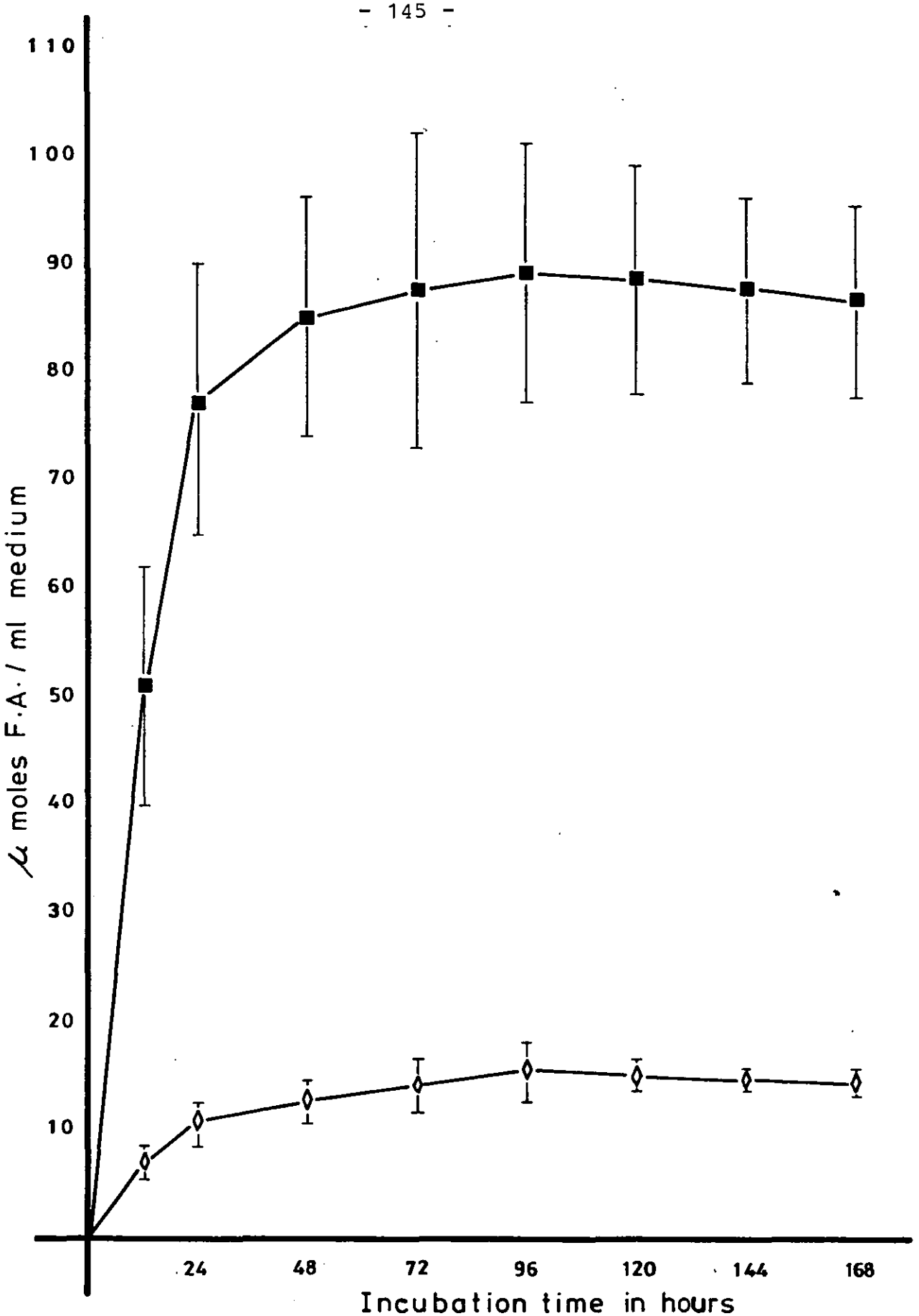

110
100
90
80
70
60
50
40
30
20
10
μ moles F.A. / ml medium
24
48
72
96
120
144
168
Incubation time in hours

Fig. 12(a): Production of VFAs by B.ruminicola ss. brevis (NP333) in CMG medium over 7 days of anaerobic incubation at 37°C.

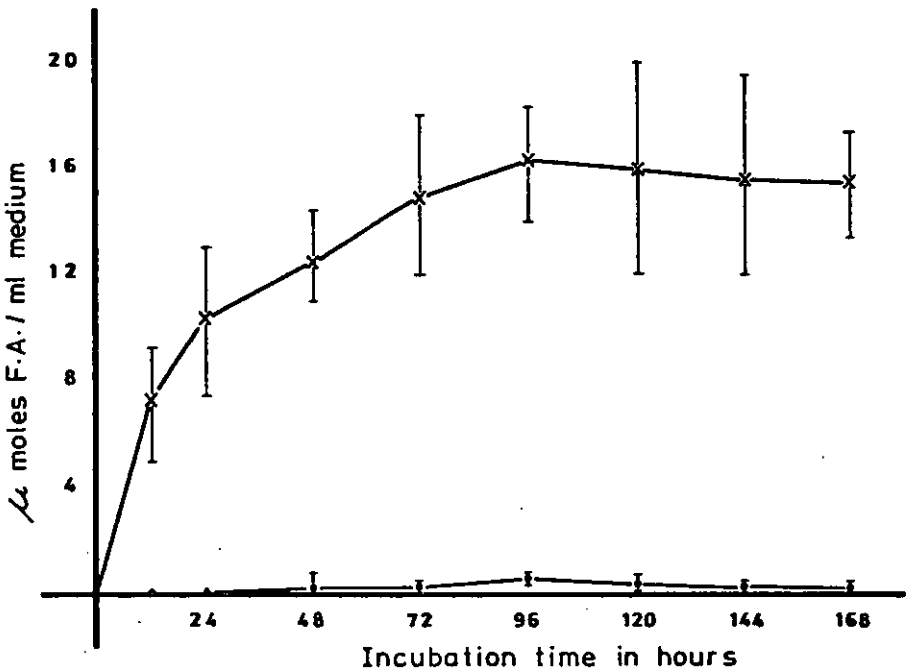
x——x Acetic acid (A)

●——● Isovaleric acid (IV)

Fig. 12(b): Production of n-VFAs by B.ruminicola ss. brevis (NP 333) in CMG medium over 7 days of anaerobic incubation at 37°C.

■——■ Succinic acid (S)

◊——◊ Lactic acid (L)


20
16
12
8
4
μ moles F.A. / ml medium
24
48
72
96
120
144
168
Incubation time in hours

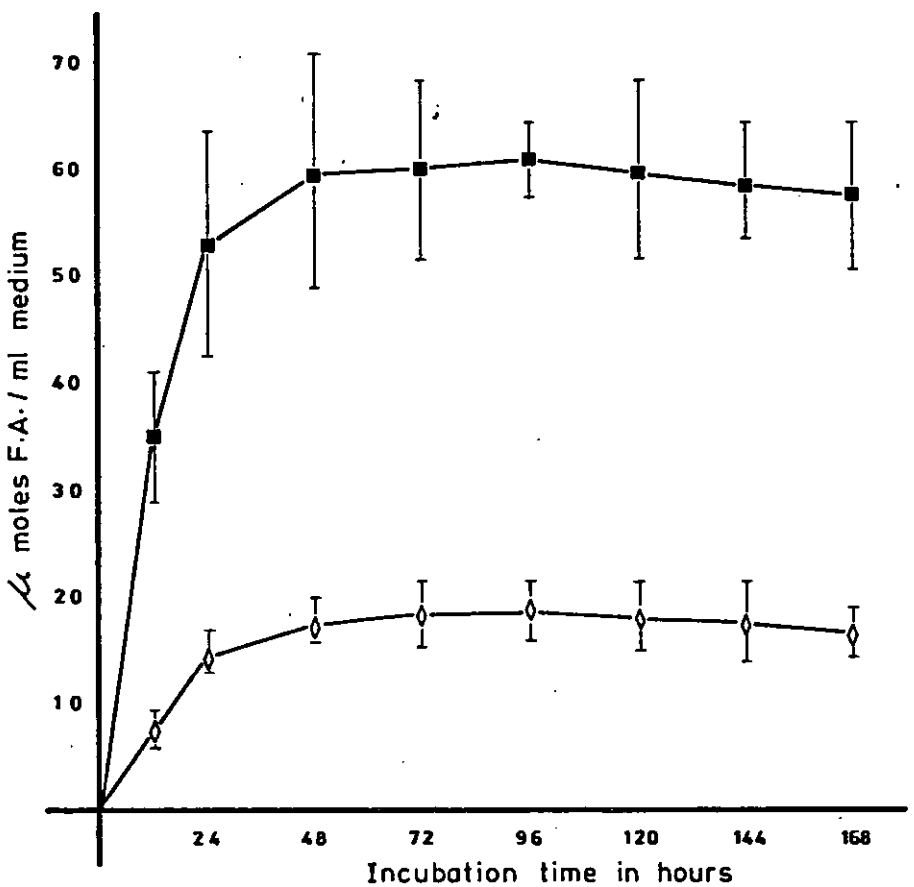

70
60
50
40
30
20
10
μ moles F.A. / ml medium
24
48
72
96
120
144
168
Incubation time in hours

Fig. 13(a): Production of VFAs by B.thetaiotaomicron (NCTC 10582) in CMG medium over 7 days of anaerobic incubation at 37°C.

Acetic acid (A)

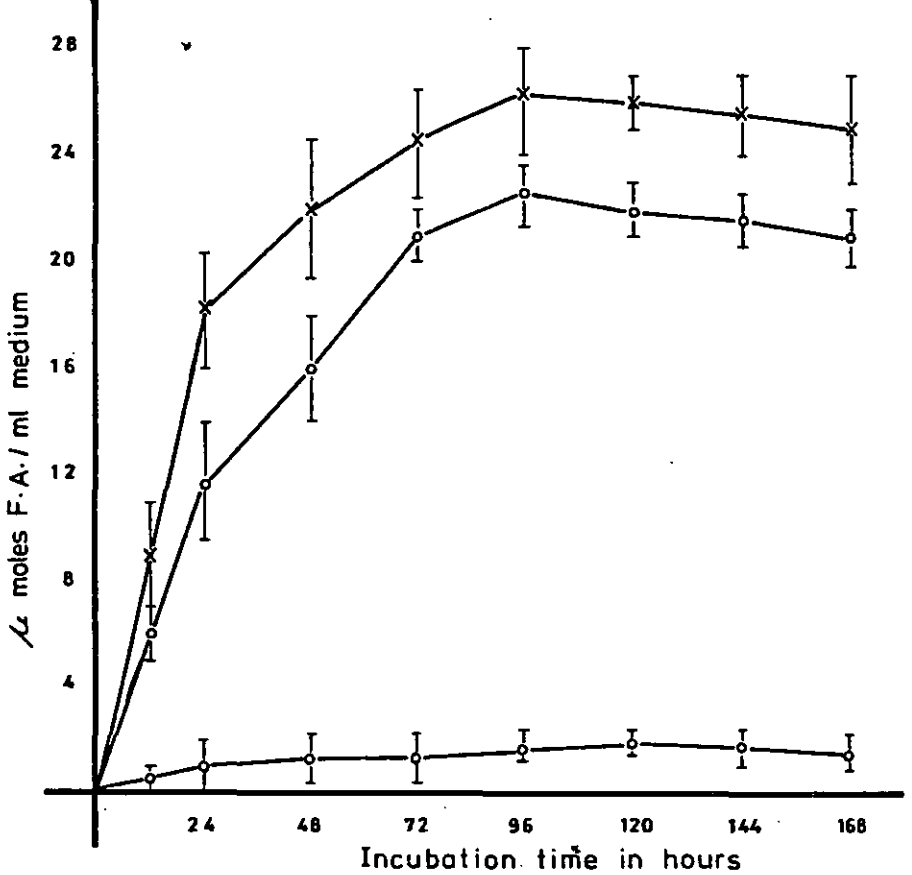
Propionic acid (P)

Isovaleric acid (IV)

Fig. 13(b): Production of n-VFAs by B.thetaiotaomicron (NCTC 10582) in CMG medium over 7 days of anaerobic incubation at 37°C.

Succinic acid (S)

Lactic acid (L)


28
24
20
16
12
8
4
μ moles F.A. / ml medium
24
48
72
96
120
144
168
Incubation time in hours

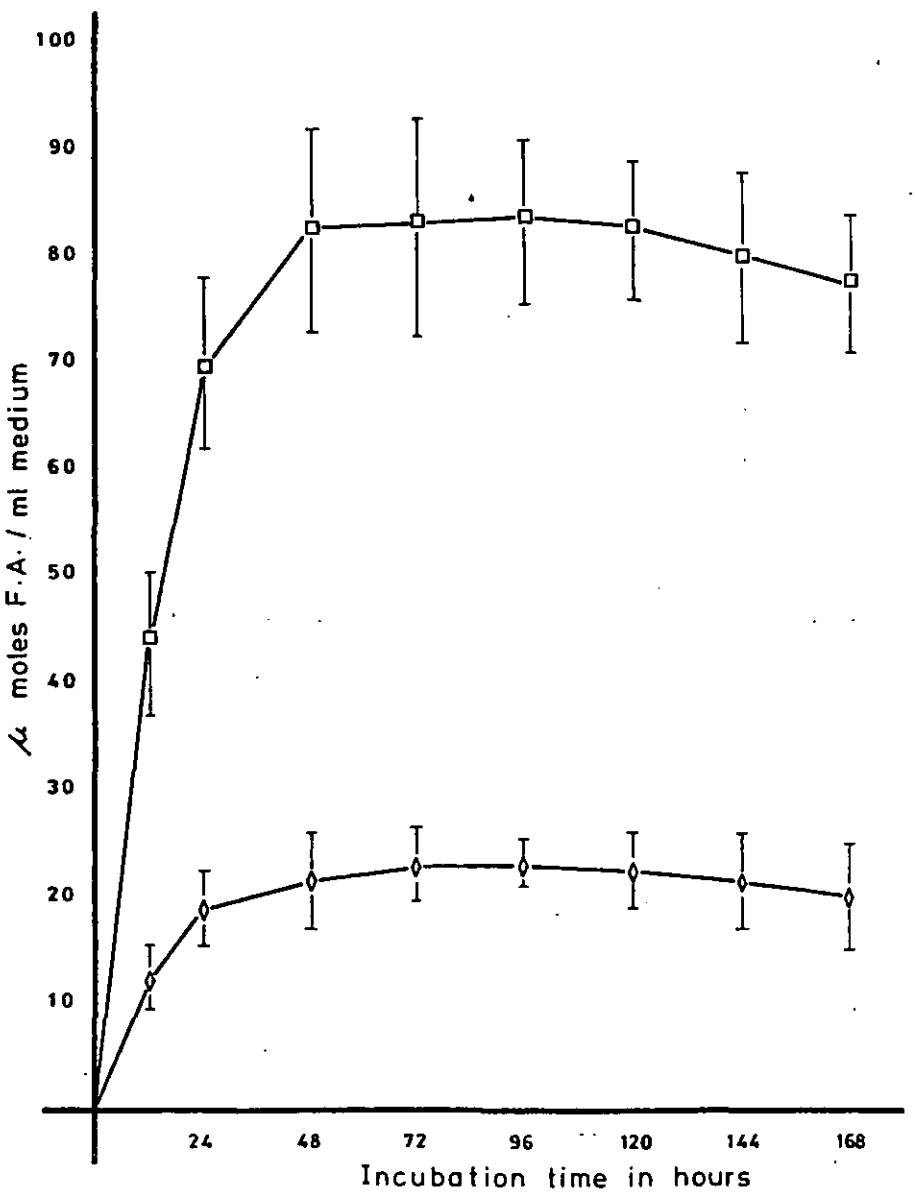

100
90
80
70
60
50
40
30
20
10
μ moles F.A. / ml medium
24
48
72
96
120
144
168
Incubation time in hours

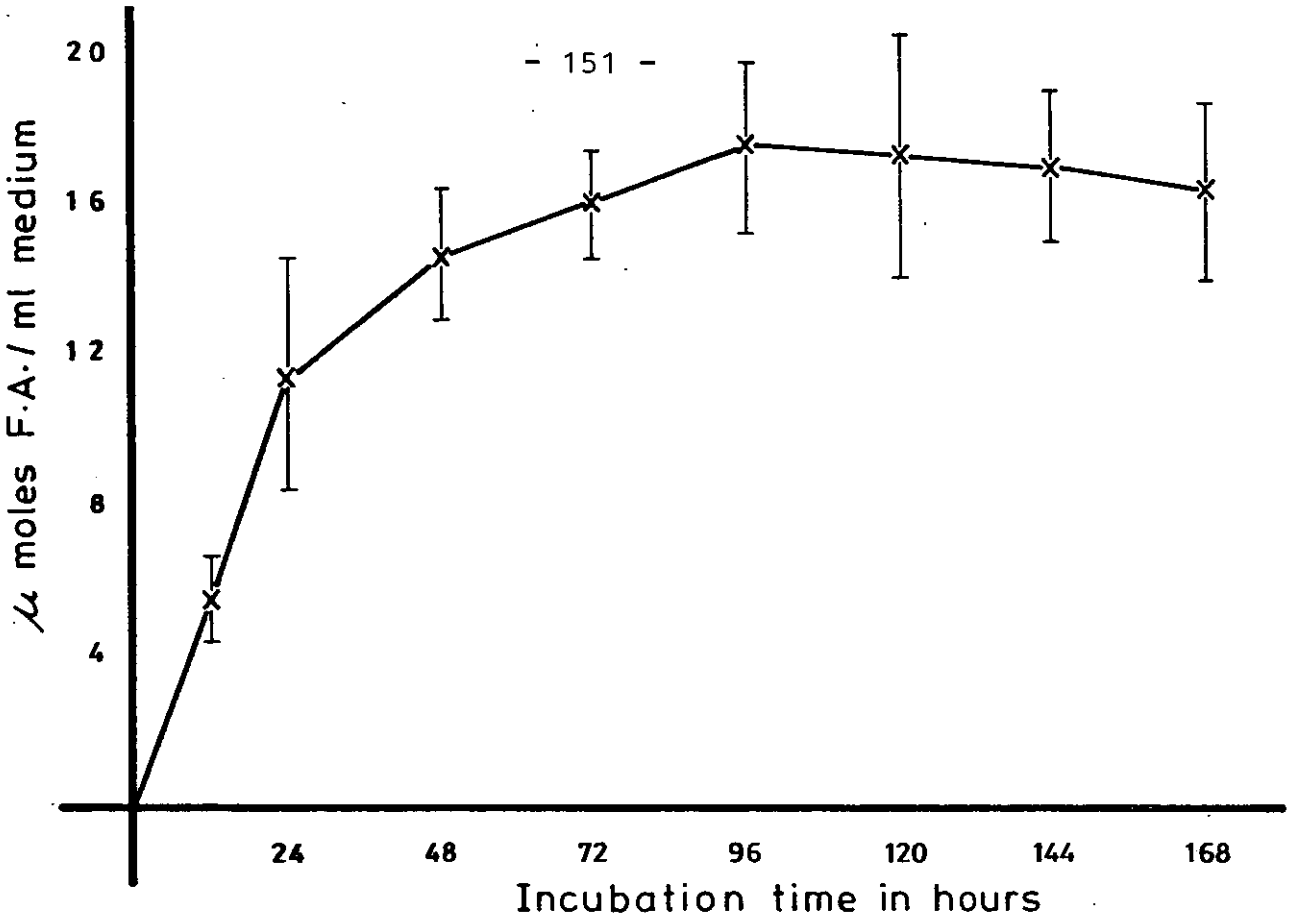
Fig. 14(a): Production of VFAs by B.melaninogenicus ss. melaninogenicus (VPI 9085) in CMG medium over 7 days of anaerobic incubation at 37°C.

x——x Acetic acid (A)

Fig. 14(b): Production of n-VFAs by B.melaninogenicus ss. melaninogenicus (VPI 9085) in CMG medium over 7 days of anaerobic incubation at 37°C.

■——■ Succinic acid (S)

◊——◊ Lactic acid (L)


20
16
12
8
4
μ moles F.A./ml medium
24
48
72
96
120
144
168
Incubation time in hours

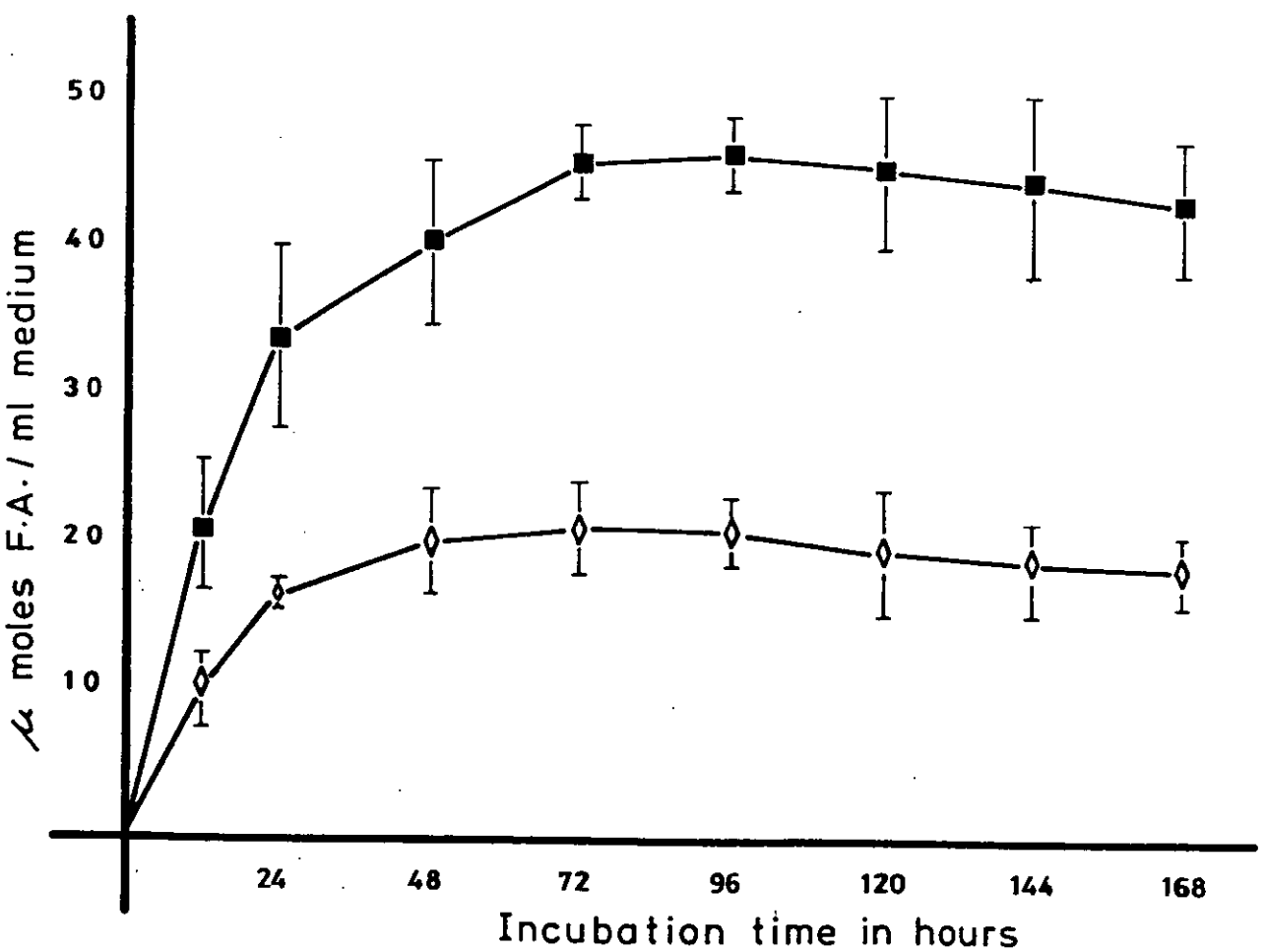

50
40
30
20
10
μ moles F.A./ml medium
24
48
72
96
120
144
168
Incubation time in hours

Fig. 15(a): Production of VFAs by B.melaninogenicus ss.intermedius (T588) in CMG medium over 7 days of anaerobic incubation at 37°C.

x——x Acetic acid (A)

●——● Isovaleric acid (IV)

Δ——Δ Isobutyric acid (IB)

o——o Propionic acid (P)

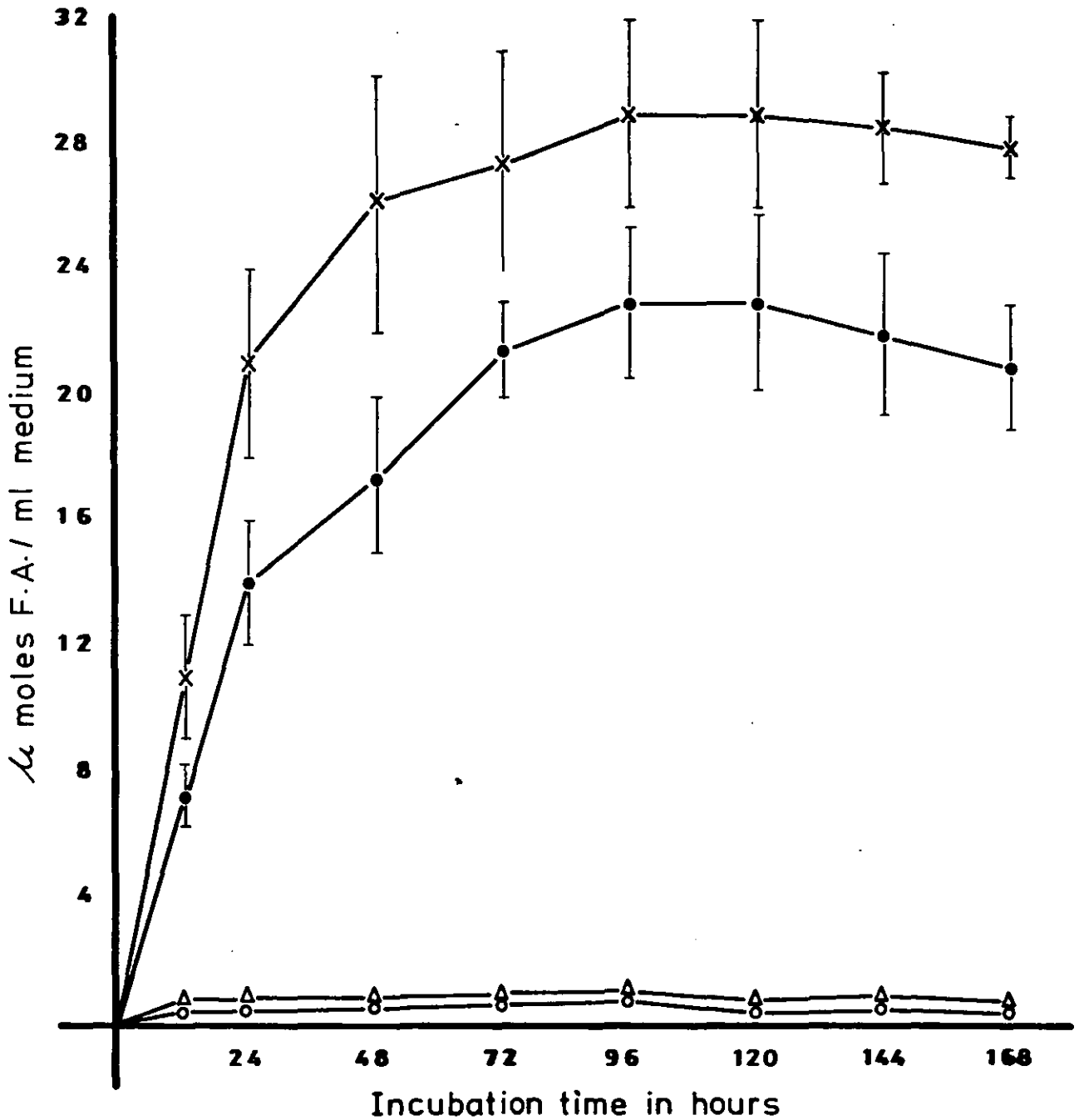
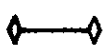

32
28
24
20
16
12
8
4
μ moles F.A./ ml medium
24
48
72
96
120
144
168
Incubation time in hours

Fig. 15(b): Production of n-VFAs by B.melaninogenicus ss.intermedius (T588) in CMG medium over 7 days of anaerobic incubation at 37°C.

Succinic acid (S)

 Lactic acid (L)

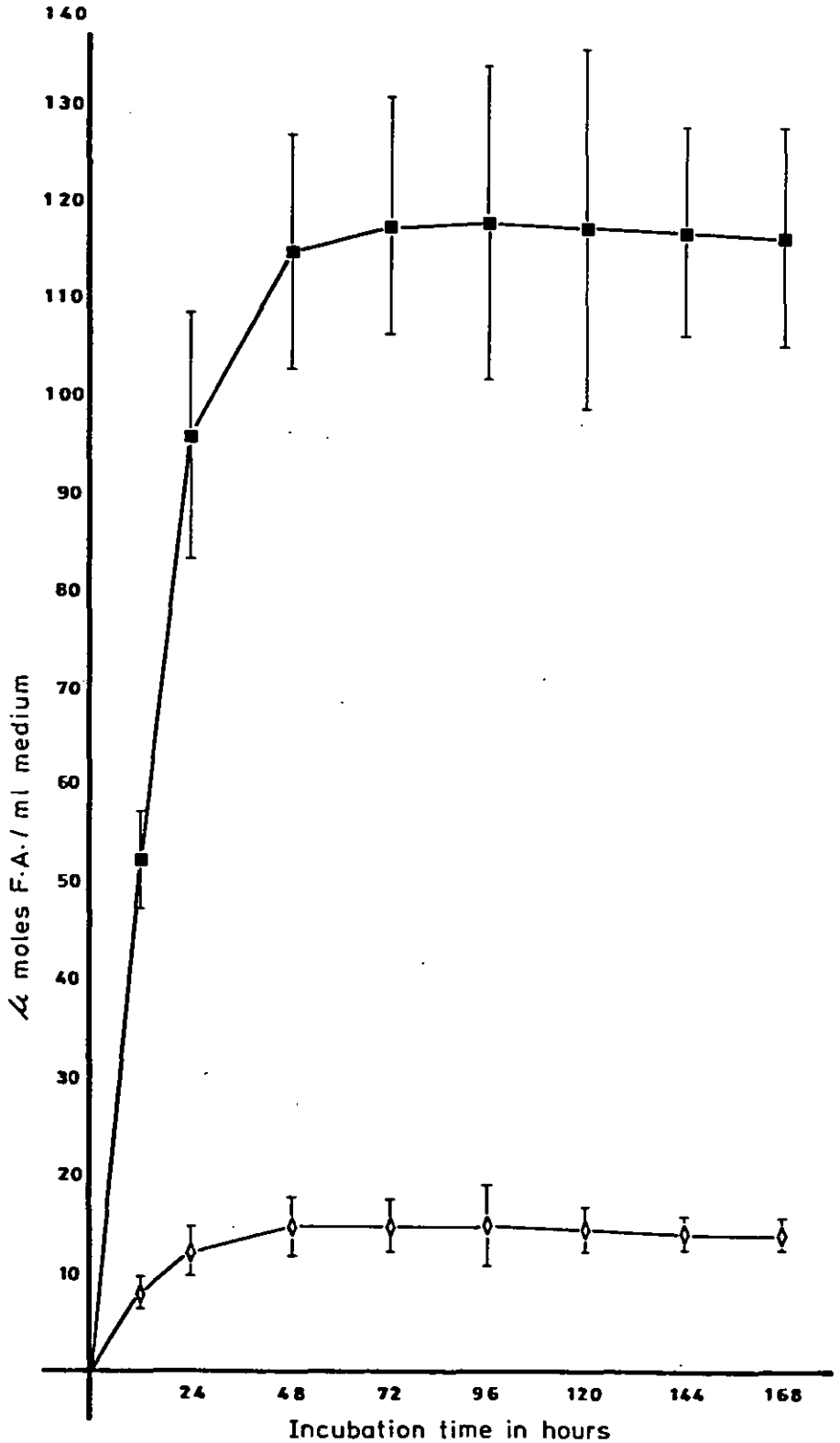

140
130
120
110
100
90
80
70
60
50
40
30
20
10
μ moles F·A·/ ml medium
24
48
72
96
120
144
168
Incubation time in hours

Fig. 16(a): Production of VFAs by B.melaninogenicus ss.macacea (ATCC 33141) in CMG medium over 7 days of anaerobic incubation at 37°C.

o——o Propionic acid (P)

x——x Acetic acid (A)

●——● Isovaleric acid (B)

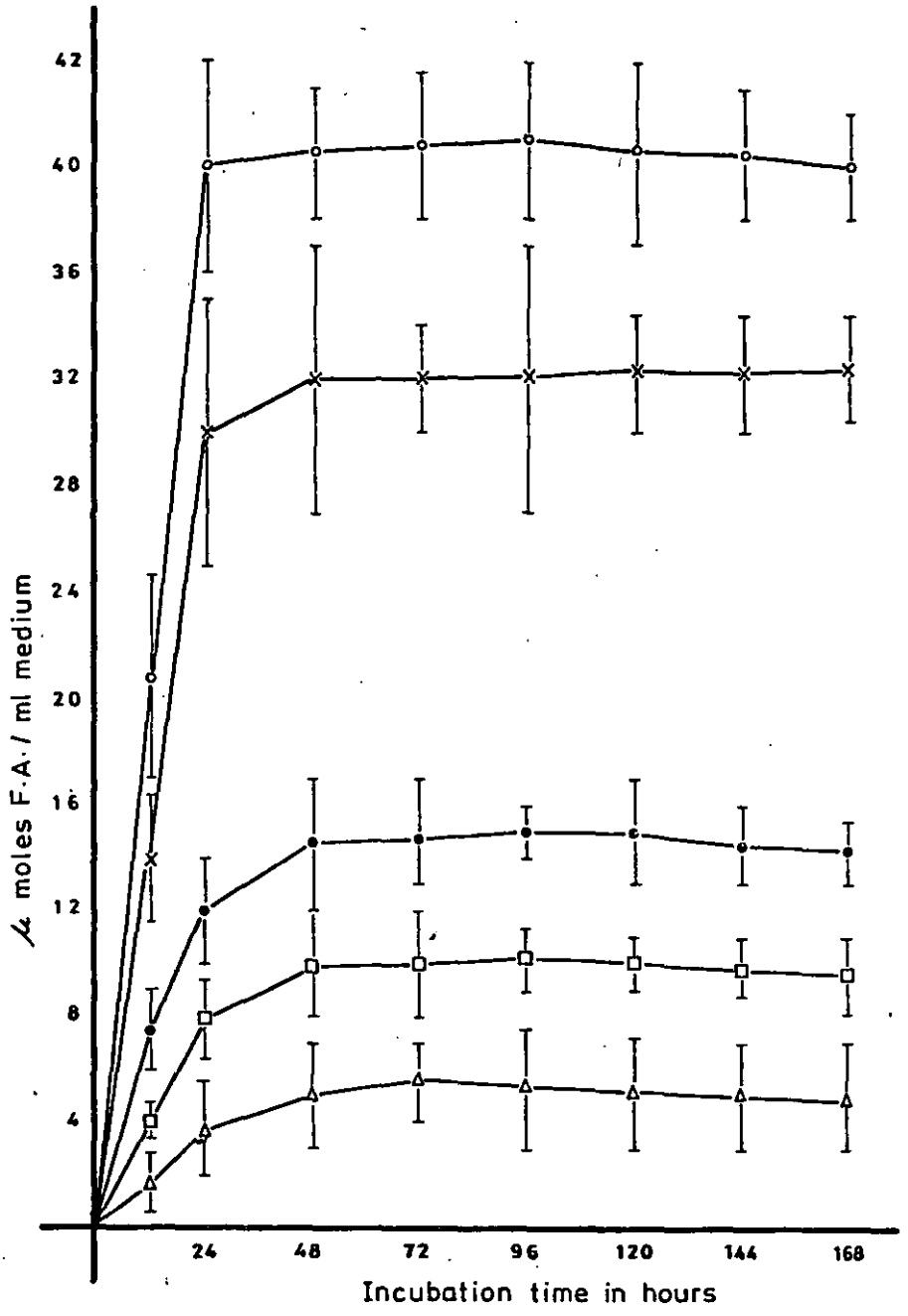
□——□ Butyric acid (B)

Δ——Δ Isobutyric acid (IB)

Fig. 16(b): Production of n-VFAs by B.melaninogenicus ss. macacea (ATCC 33141) in CMG medium over 7 days of anaerobic incubation at 37°C.

■——■ Succinic acid (S)

◊——◊ Lactic acid (L)


42
40
36
32
28
24
20
16
12
8
4
µ moles F.A. / ml medium
24
48
72
96
120
144
168
Incubation time in hours

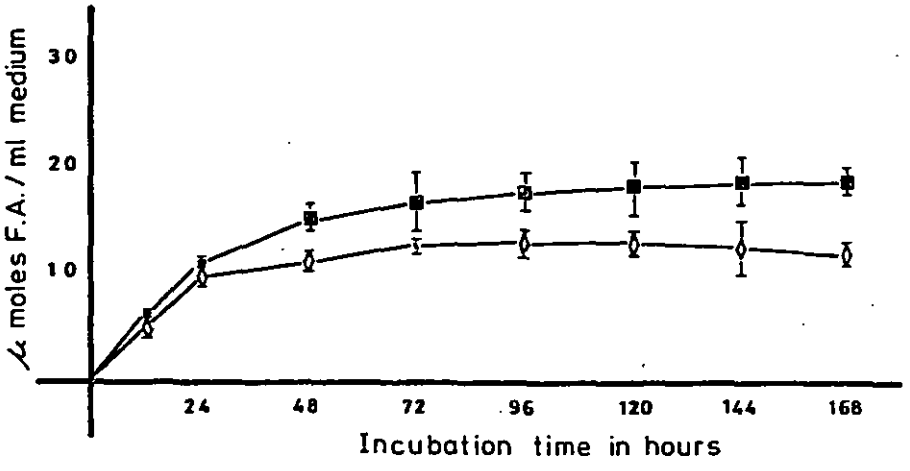

30
20
10
µ moles F.A. / ml medium
24
48
72
96
120
144
168
Incubation time in hours

Fig. 17(a): Production of VFAs by B.asaccharolyticus (B536) in CMG medium over 7 days of anaerobic incubation at 37°C.

o——o Propionic acid (P)

x——x Acetic acid (A)

●——● Isovaleric acid (IV)

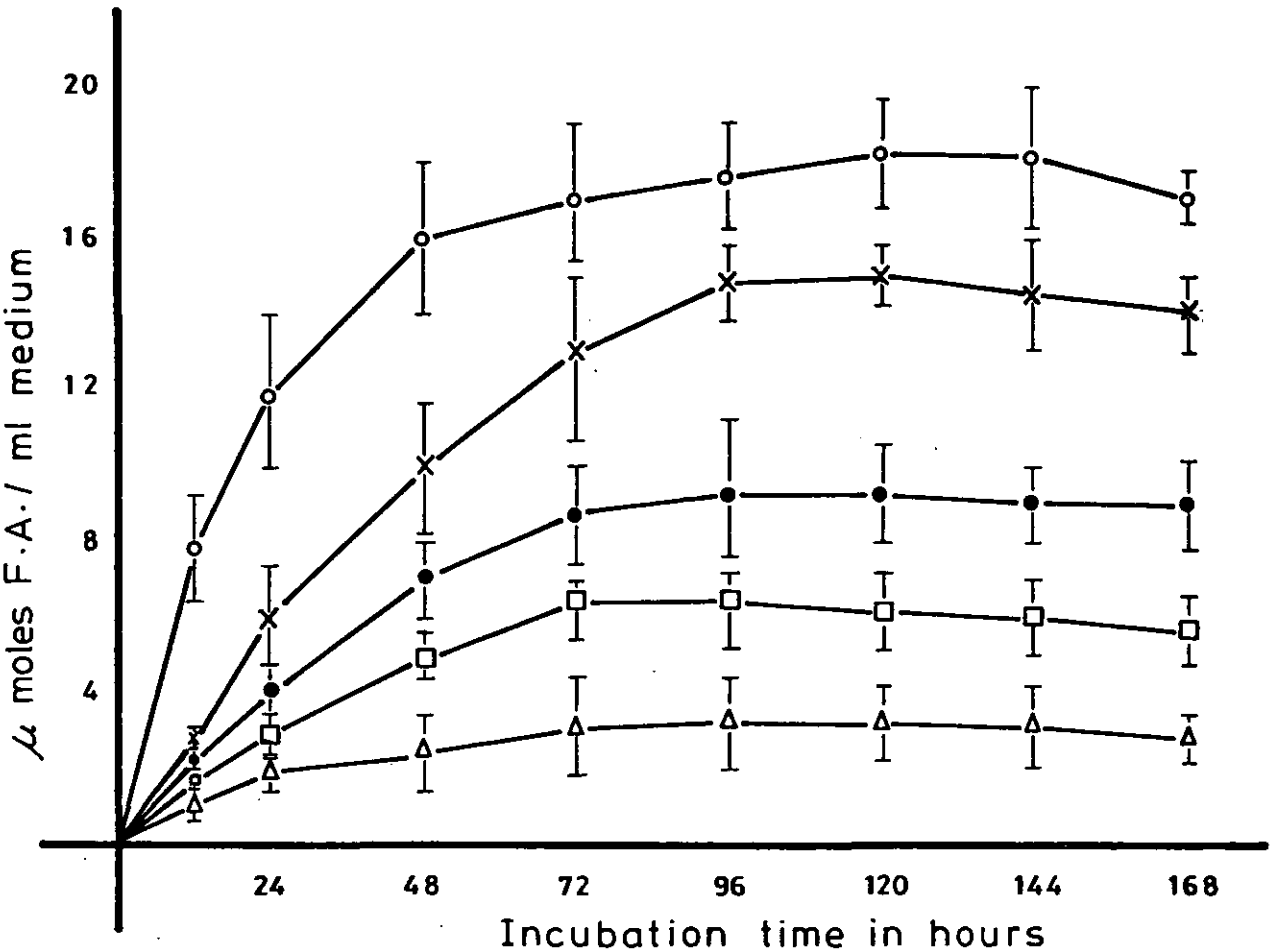
□——□ Butyric acid (B)

Δ——Δ Isobutyric acid (IB)

Fig. 17(b): Production of n-VFAs by B.asaccharolyticus (B536) in CMG medium over 7 days of anaerobic incubation at 37°C.

■——■ Succinic acid (S)

◊——◊ Lactic acid (L)


20
16
12
8
4
μ moles F.A. / ml medium
24
48
72
96
120
144
168
Incubation time in hours

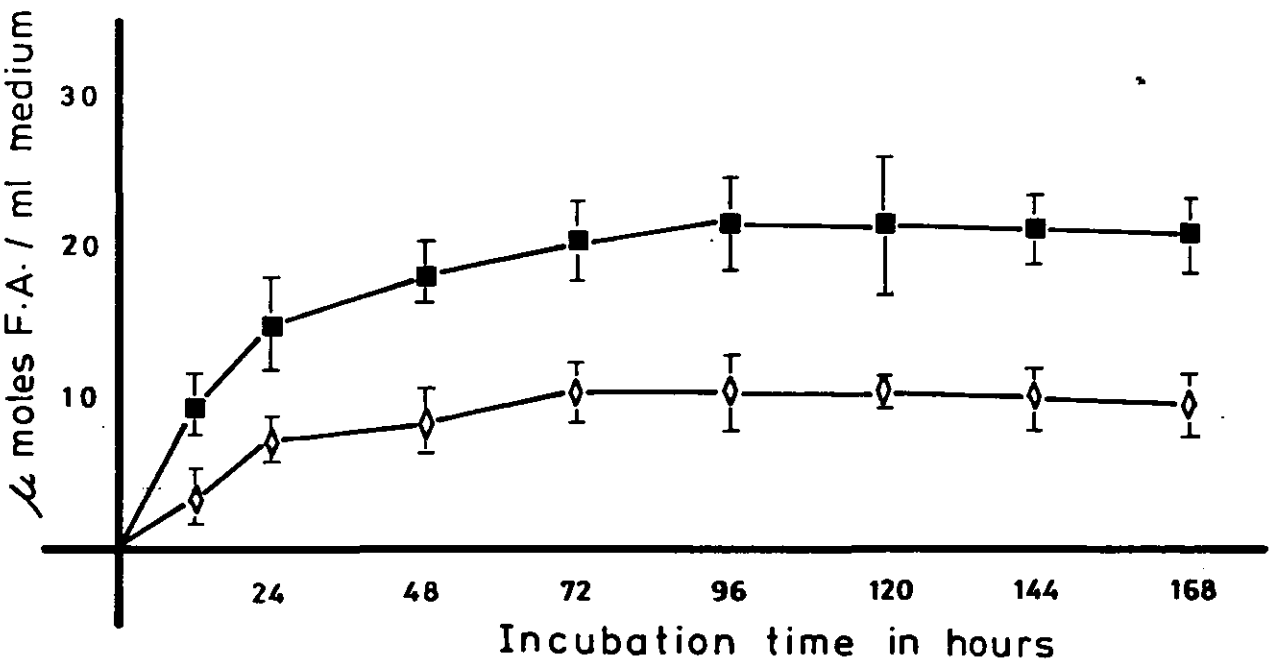

30
20
10
μ moles F.A. / ml medium
24
48
72
96
120
144
168
Incubation time in hours

Fig. 18(a): Production of VFAs by B.asaccharolyticus (B537) in CMG medium over 7 days of anaerobic incubation at 37°C.

o——o Propionic acid (P)

x——x Acetic acid (A)

●——● Isovaleric acid (IV)

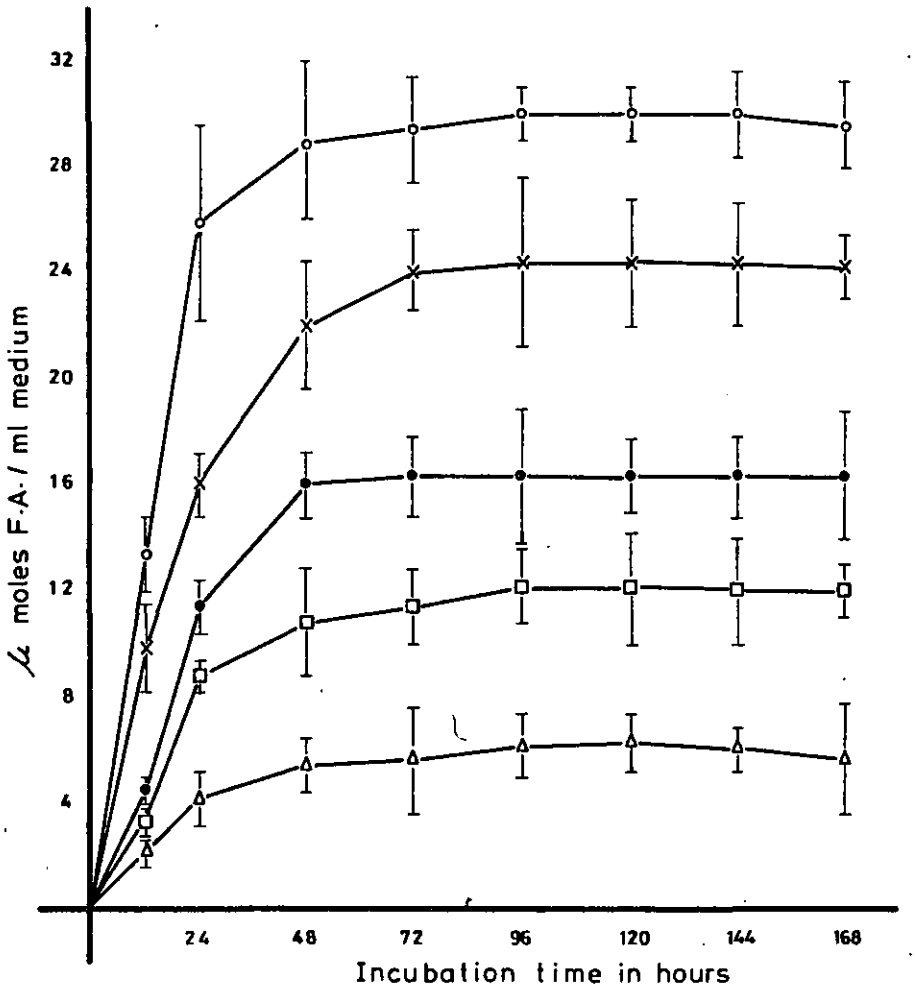
□——□ Butyric acid (B)

Δ——Δ Isobutyric acid (IB

Fig. 18 (b): Production of n-VFAs by B.asaccharolyticus (B537) in CMG medium over 7 days of anaerobic incubation at 37°C.

■——■ Succinic acid (S)

◊——◊ Lactic acid (L)


32
28
24
20
16
12
8
4
μ moles F.A. / ml medium
24
48
72
96
120
144
168
Incubation time in hours

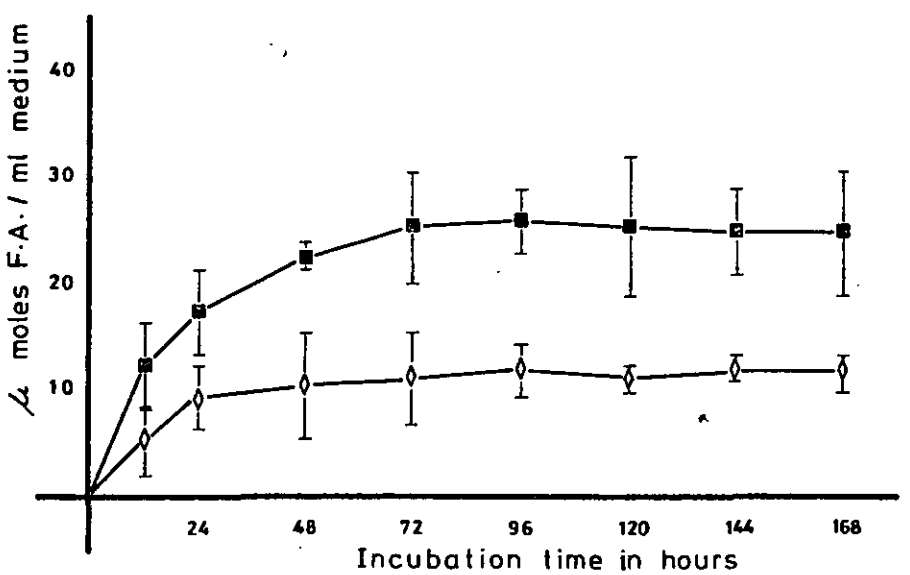

40
30
20
10
μ moles F.A. / ml medium
24
48
72
96
120
144
168
Incubation time in hours

Fig. 19(a): Production of VFAs by B.asaccharolyticus (W50) in CMG medium over 7 days of anaerobic incubation at 37°C.

o——o Propionic acid (P)

x——x Acetic acid (A)

●——● Isovaleric acid (IV)

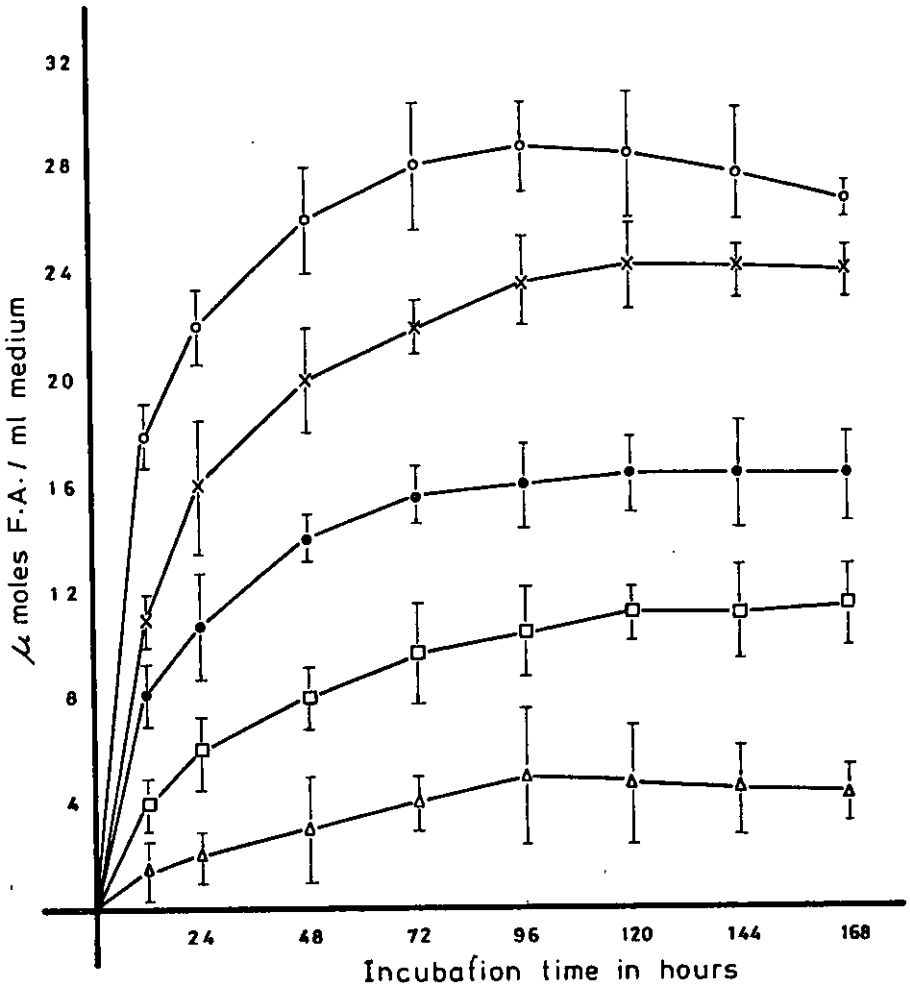
□——□ Butyric acid (B)

Δ——Δ Isobutyric acid (IB)

Fig. 19(b): Production of n-VFAs by B.asaccharolyticus (W50) in CMG medium over 7 days of anaerobic incubation at 37°C.

■——■ Succinic acid (S)

◊——◊ Lactic acid (L)


32
28
24
20
16
12
8
4
μ moles F.A. / ml medium
24
48
72
96
120
144
168
Incubation time in hours

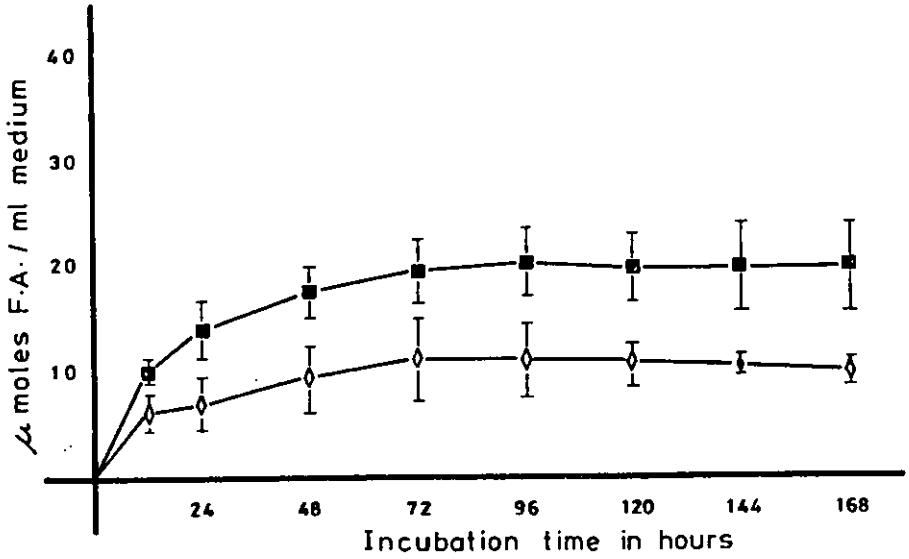

40
30
20
10
μ moles F.A. / ml medium
24
48
72
96
120
144
168
Incubation time in hours

Fig. 20(a): Production of VFAs by B.fragilis (NCTC 8560) in PYG medium over 7 days of anaerobic incubation at 37°C.

x——x Acetic acid (A)

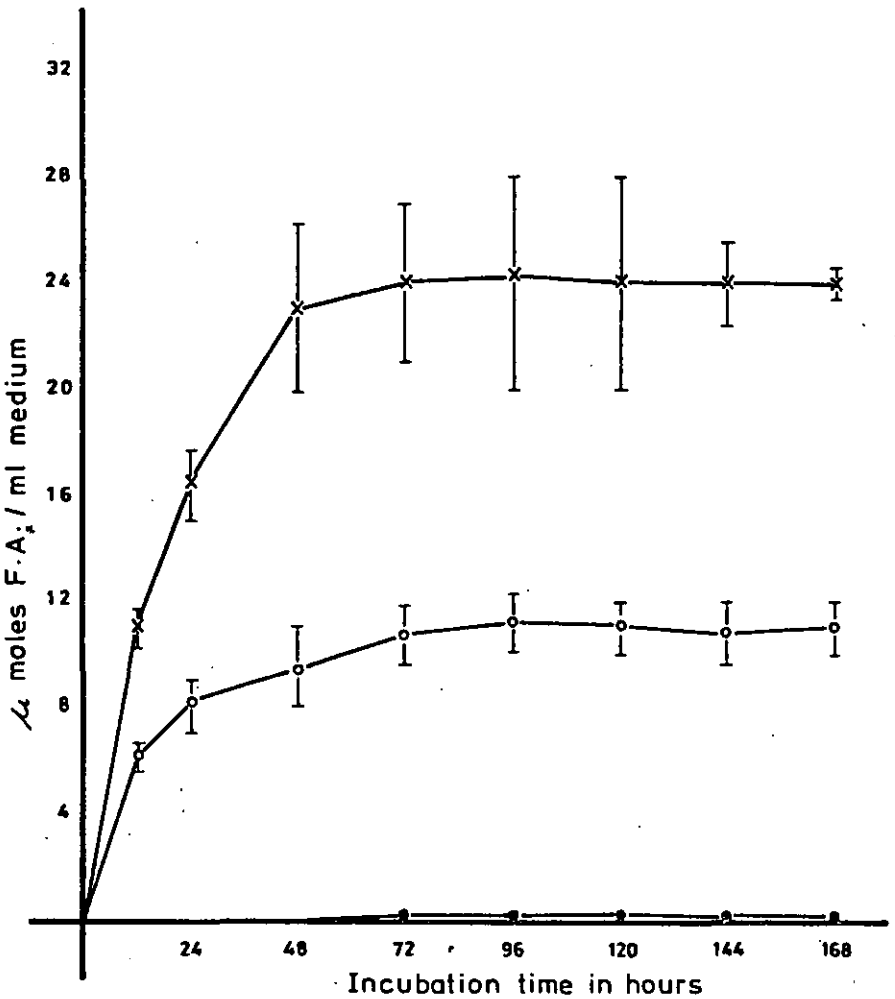
o——o Propionic acid (P)

●——● Isovaleric acid (IV)

Fig. 20(b): Production of n-VFAs by B.fragilis (NCT 8560) in PYG medium over 7 days of anaerobic incubation at 37°C.

■——■ Succinic acid (S)

◊——◊ Lactic acid (L)


32
28
24
20
16
12
8
4
μ moles F·A·/ ml medium
24
48
72
96
120
144
168
Incubation time in hours

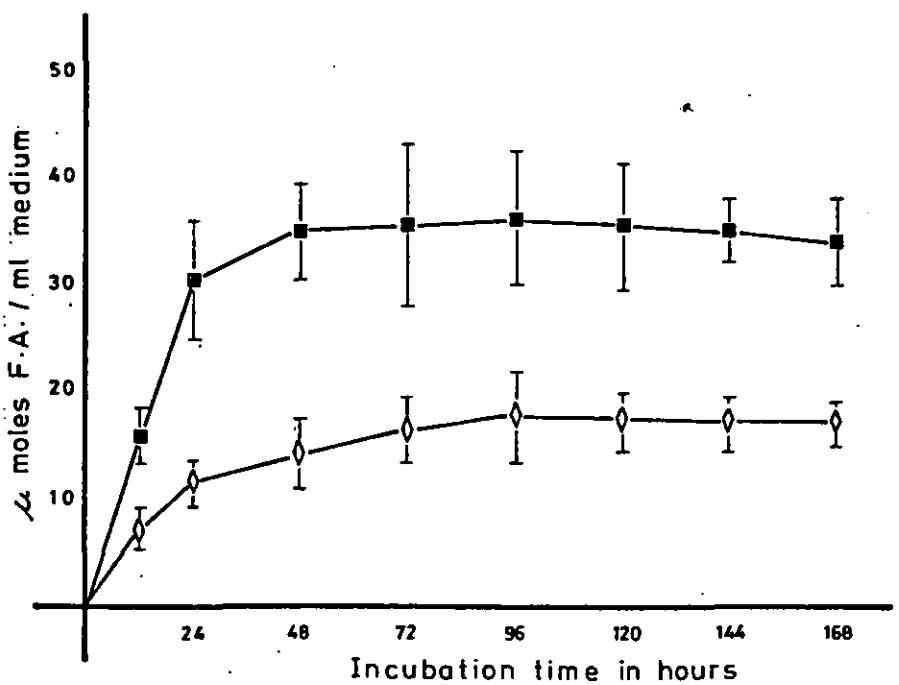

50
40
30
20
10
μ moles F·A·/ ml medium
24
48
72
96
120
144
168
Incubation time in hours

Fig. 21(a): Production of VFAs by B.fragilis (NCTC 9343) in PYG medium over 7 days of anaerobic incubation at 37°C.

x——x Acetic acid (A)

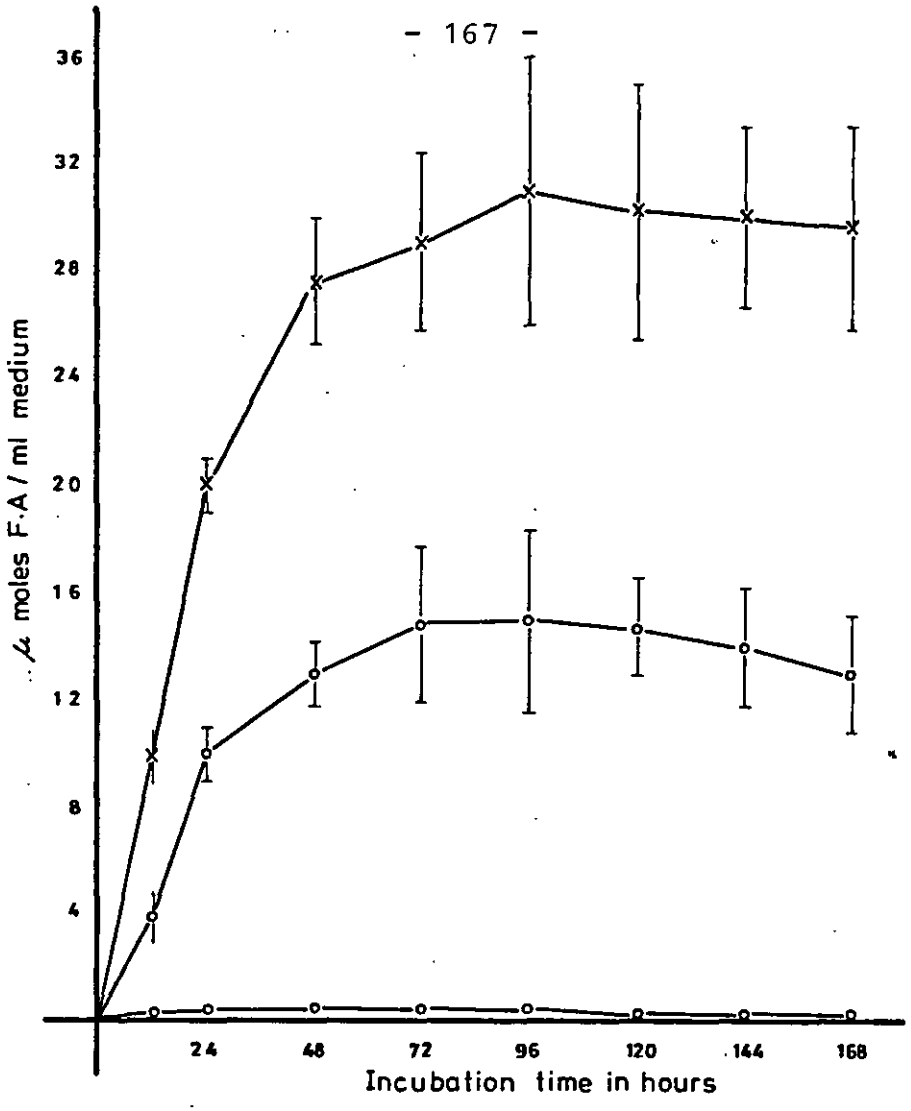
o——o Propionic acid (P)

●——● Isovaleric acid (IV)

Fig. 21(b): Production of n-VFAs by B.fragilis (NCTC 9343) in PYG medium over 7 days of anaerobic incubation at 37°C.

■——■ Succinic acid (S)

◊——◊ Lactic acid (L)


36
32
28
24
20
16
12
8
4
μ moles F.A / ml medium
24
48
72
96
120
144
168
Incubation time in hours

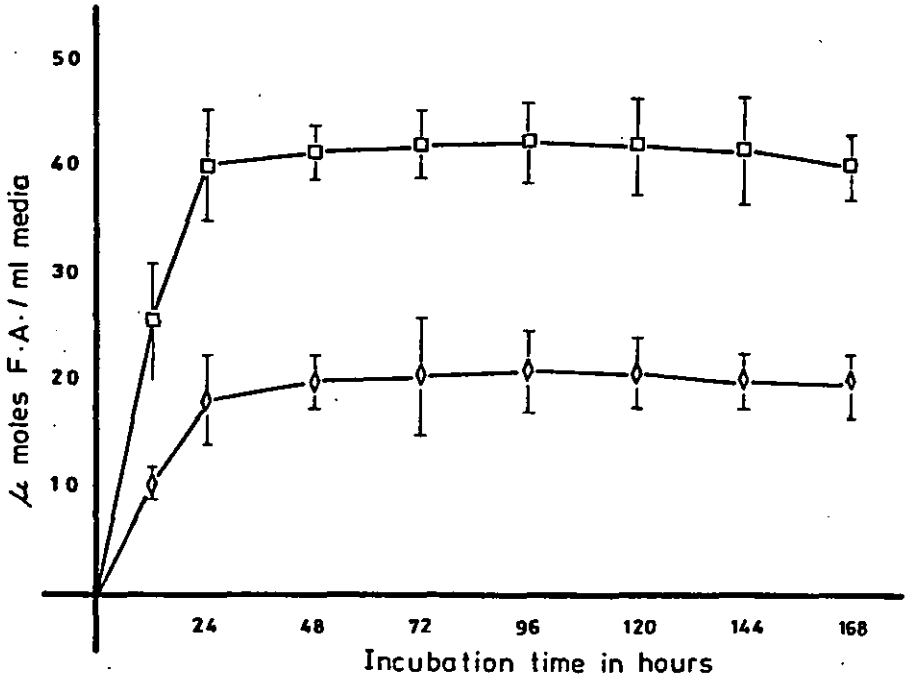

50
40
30
20
10
μ moles F.A. / ml media
24
48
72
96
120
144
168
Incubation time in hours

Fig. 22(a): Production of VFAs by B.asaccharolyticus (B536) in PYG medium over 7 days of anaerobic incubation at 37°C.

o—o Propionic acid (P)

x—x Acetic acid (A)

●—● Isovaleric acid (IV)

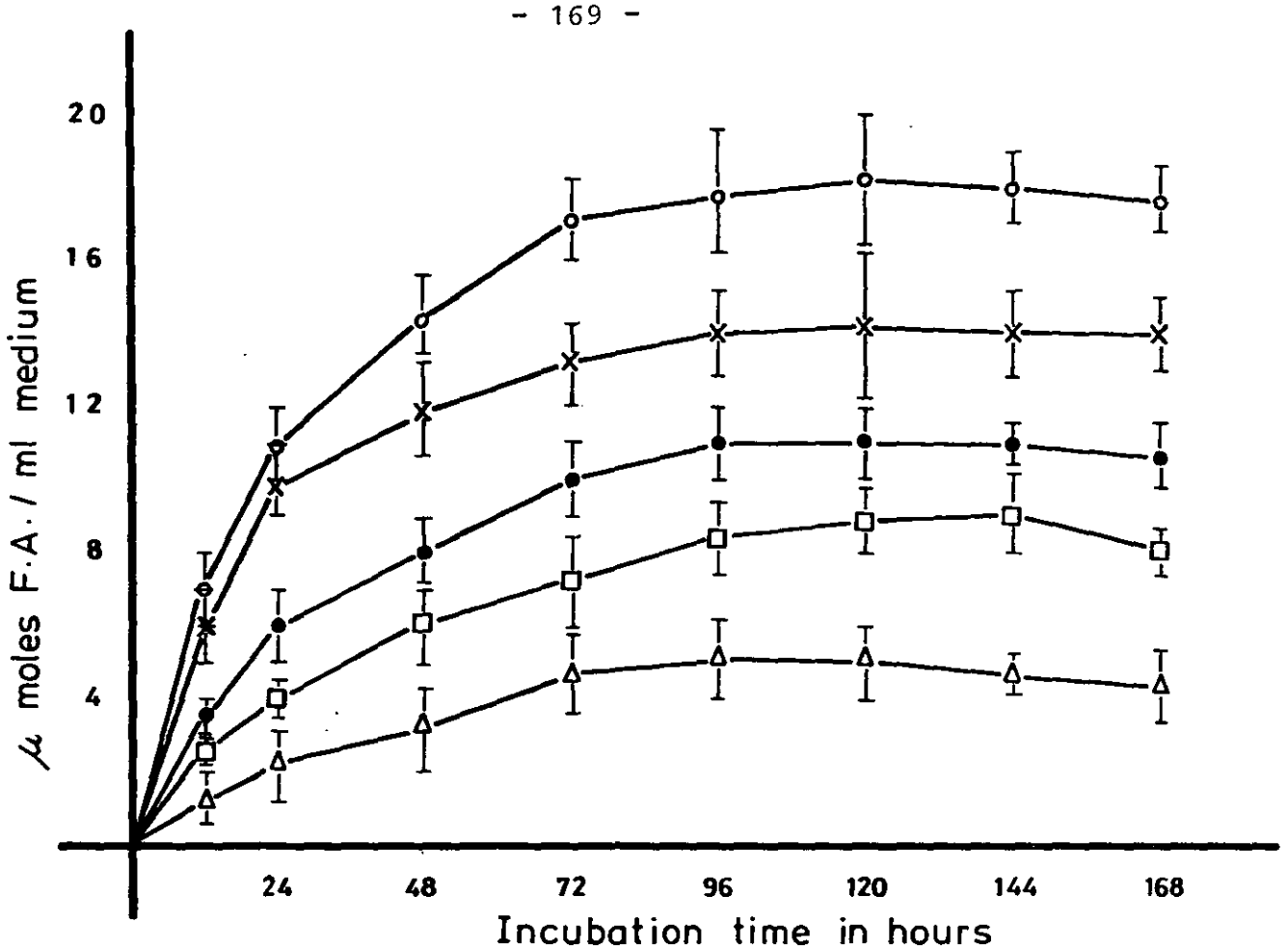
□—□ Butyric acid (B)

Δ—Δ Isobutyric acid (IB)

Fig.22(b): Production of n-VFAs by B.asaccharolyticus (B536) in PYG medium over 7 days of anaerobic incubation at 37°C.

■—■ Succinic acid (S)

◊—◊ Lactic acid (L)


20
16
12
8
4
μ moles F.A. / ml medium
24
48
72
96
120
144
168
Incubation time in hours

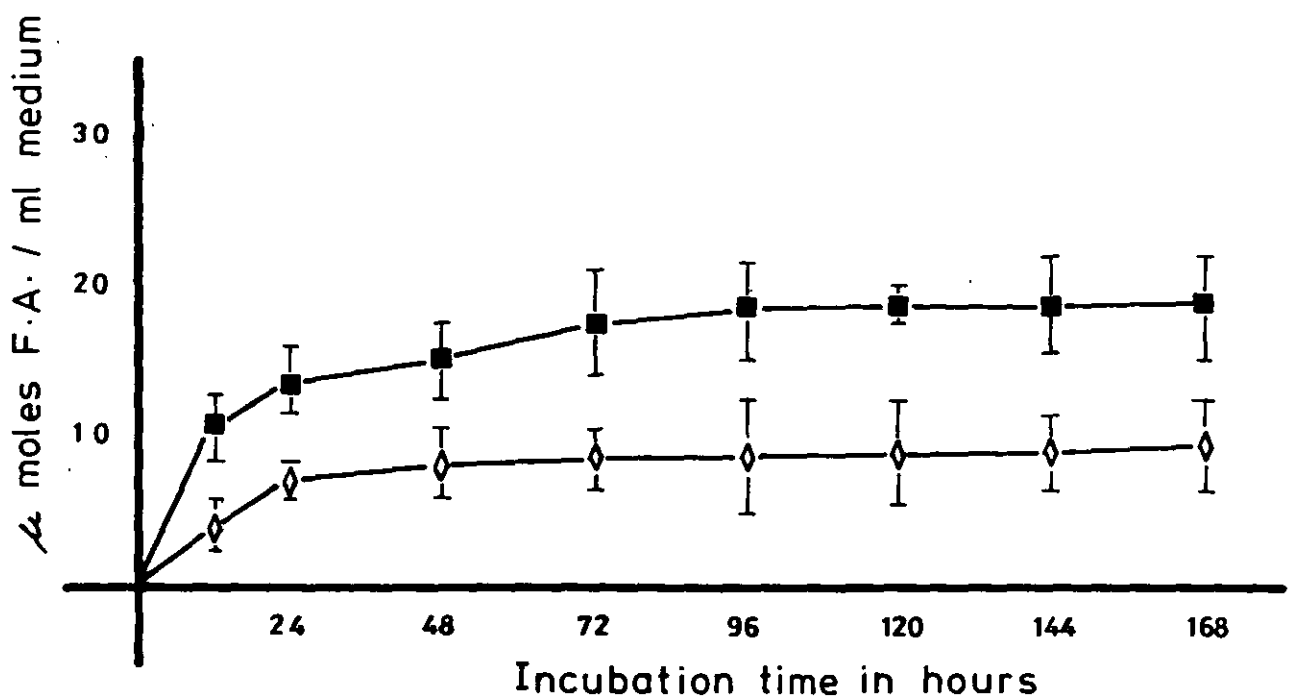

30
20
10
μ moles F.A. / ml medium
24
48
72
96
120
144
168
Incubation time in hours

Fig. 23(a): Production of VFAs by B.asaccharolyticus (W50) in PYG medium over 7 days of anaerobic incubation at 37°C.

o——o Propionic acid (P)

x——x Acetic acid (A)

●——● Isovaleric acid (IV)

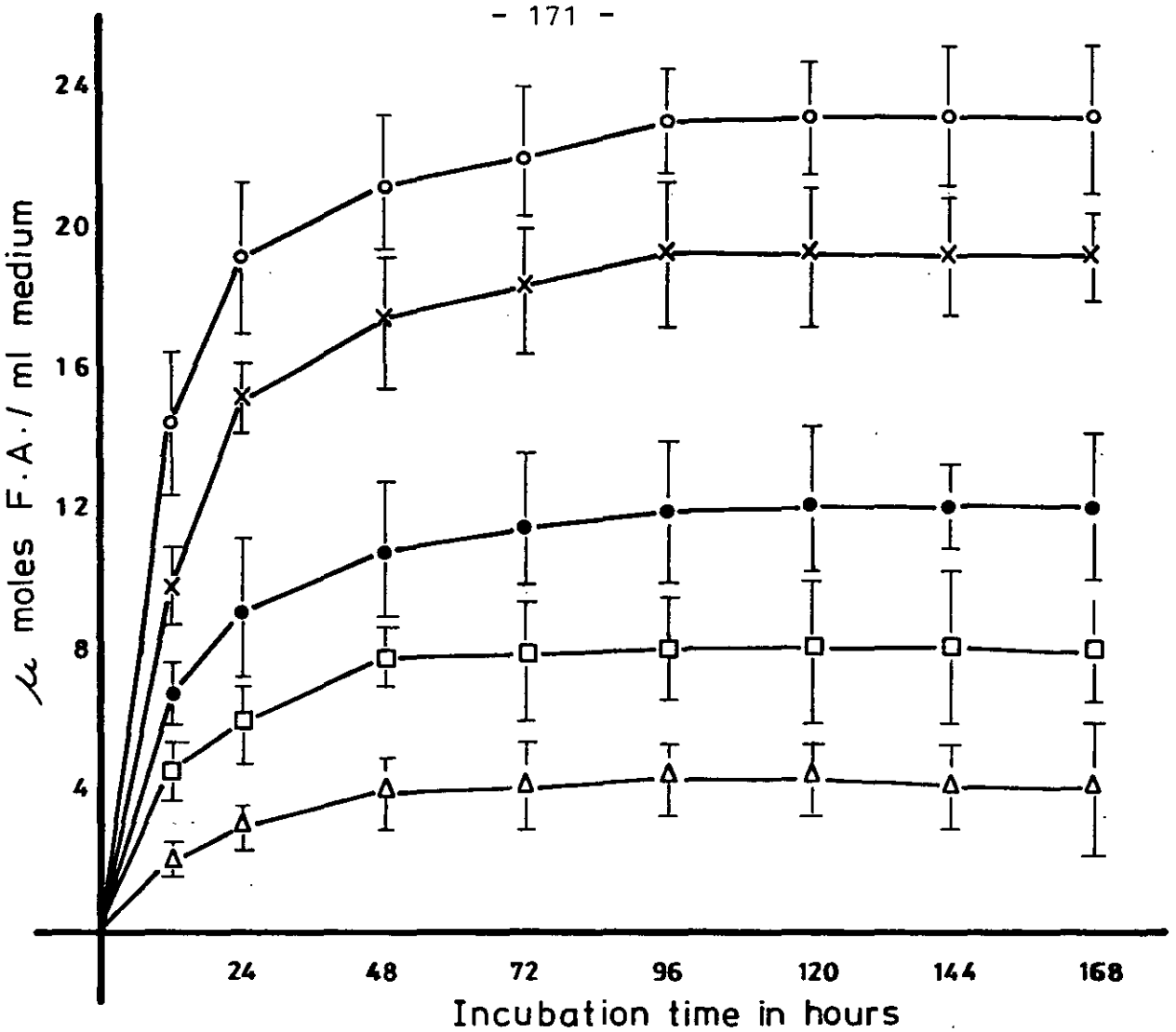
□——□ Butyric acid (B)

Δ——Δ Isobutyric acid (IB)

Fig. 23(b): Production of n-VFAs by B.asaccharolyticus (W50) in PYG medium over 7 days of anaerobic incubation at 37°C.

■——■ Succinic acid (S)

◊——◊ Lactic acid (L)


24
20
16
12
8
4
μ moles F.A. / ml medium
24
48
72
96
120
144
168
Incubation time in hours

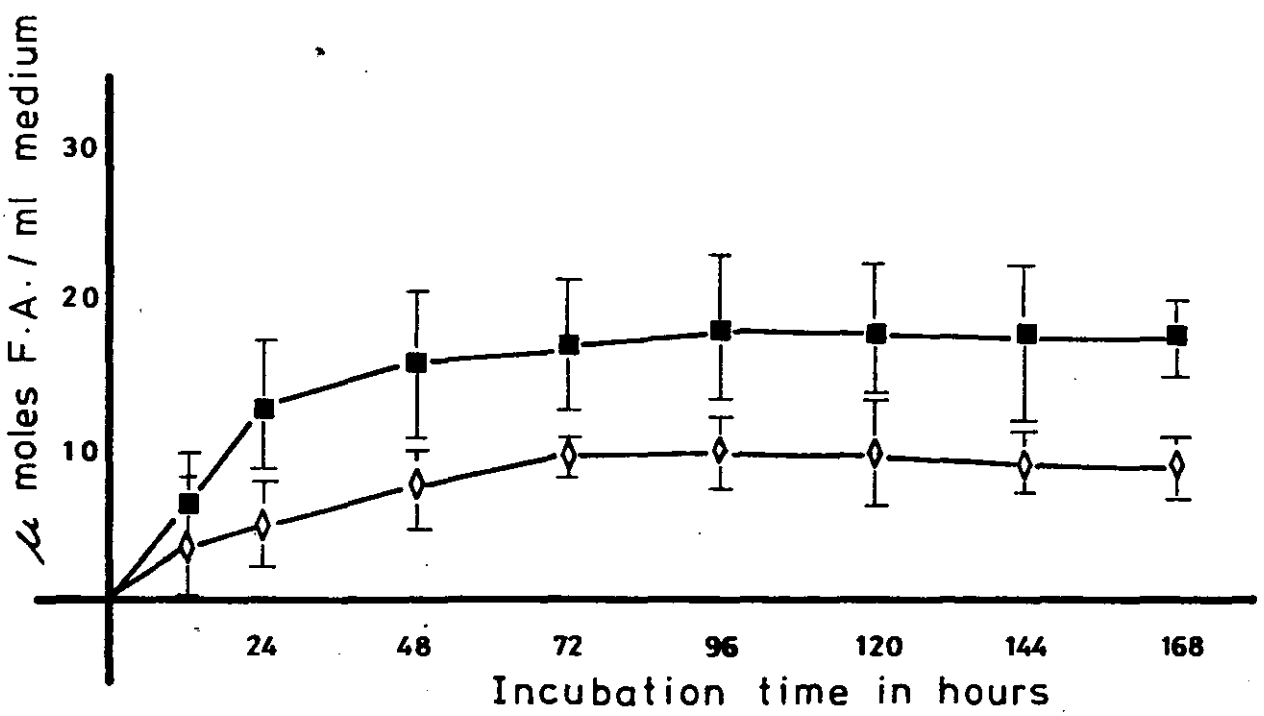

30
20
10
μ moles F.A. / ml medium
24
48
72
96
120
144
168
Incubation time in hours

Fig. 24(a): Production of VFAs by B.melaninogenicus ss. macacea (ATCC 33141) in PYG medium over 7 days of anaerobic incubation at 37°C.

o——o Propionic acid (P)

x——x Acetic acid (A)

●——● Isovaleric acid (IV)

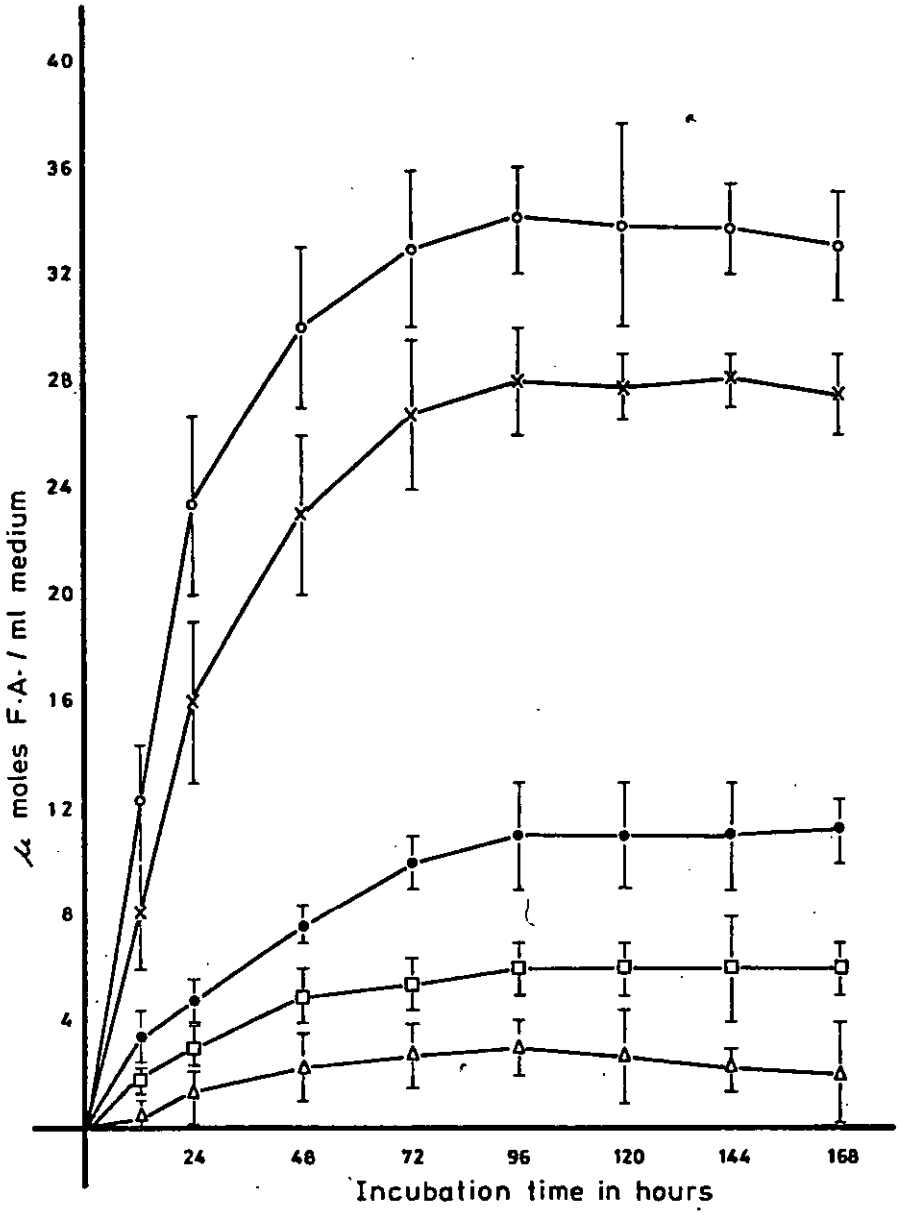
□——□ Butyric acid (B)

Δ——Δ Isobutyric acid (IB)

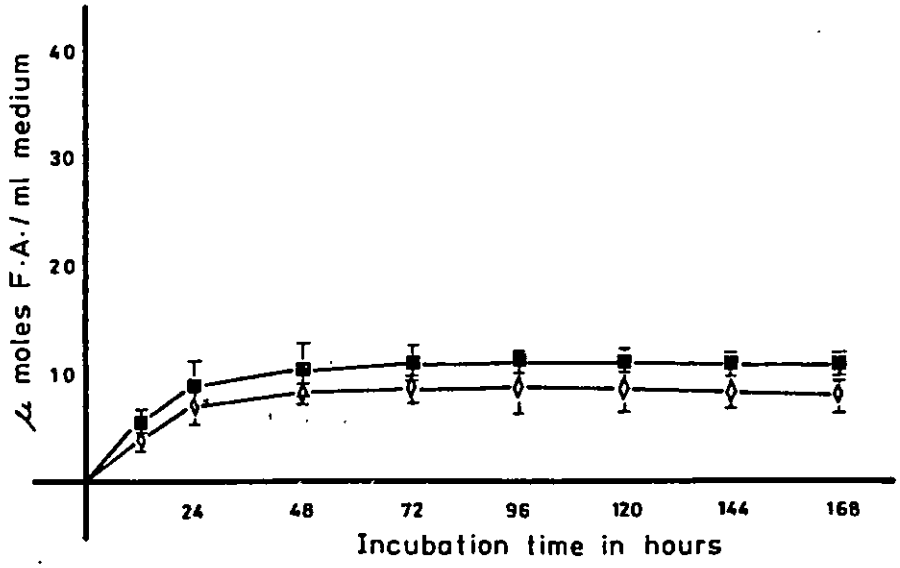
Fig. 24(b): Production of n-VFAs by B.melaninogenicus ss. macacea (ATCC 33141) in PYG medium over 7 days of anaerobic incubation at 37°C.

■——■ Succinic acid (S)

◊——◊ Lactic acid (L)


40
36
32
28
24
20
16
12
8
4
μ moles F·A· / ml medium
24
48
72
96
120
144
168
Incubation time in hours

24b


40
30
20
10
μ moles F·A·/ ml medium
24
48
72
96
120
144
168
Incubation time in hours

(3) Total Acid Production in CMG for Bacteroides Species

The total acid produced for 12 Bacteroides species in CMG and at 24, 96 and 168 h of incubation is shown in Fig. 25.

The results show the maximum total acid production by all strains was reached at 96 h and remained at a more or less steady state until the end of the incubation. The pattern obtained seems to divide the strains into three types. Type (a) include the highly productive strains such as, B.melaninogenicus SS. intermedius, B.distasonis, B.vulgatus, B.ovatus, B.thetaiotaomicron. All of these strains showed a similar pattern of behaviour except for B.distasonis which showed a lower SCFA level at 24 h than usual which seemed to increase at 96 h and until the end of the incubation time.

Type (b) include the moderately productive strains such as B.melaninogenicus SS.macacae, B.asaccharolyticus and B.fragilis (NCTC 9343).

Type (c) is the least productive strain and which include B.fragilis (NCTC 8560), B.ruminicola SS. brevis and B.melaninogenicus ss. melaninogenicus.

Fig.25 shows the black pigmented Bacteroides with their different species and subspecies have different rates of total acid production where B.melaninogenicus SS. intermedius has the highest rate of production, B.melaninogenicus SS. melaninogenicus has the lowest

production rate and B.melaninogenicus ss. macacae and B.asaccharolyticus occupying an intermediate position. Within the non-pigmented Bacteroides, B.fragilis seems to be differentiated by having a lower level of total acid production.

Fig. 25: Total acid production in CMG medium over 7 days of anaerobic incubation at 37°C for 12 Bacteroides species.

▲—▲ B.melaninogenicus ss. intermedius (T588)

■—■ B.distasonis (ATCC 8503)

●—● B.vulgatus (WBM137)

♦—♦ B.ovatus (ATCC 8483)

◊—◊ B.thetaiotaomicron (NCTC 10582)

x—x B.melaninogenicus ss.macacae (ATCC 33141)

▲—▲ B.asaccharolyticus (B537)

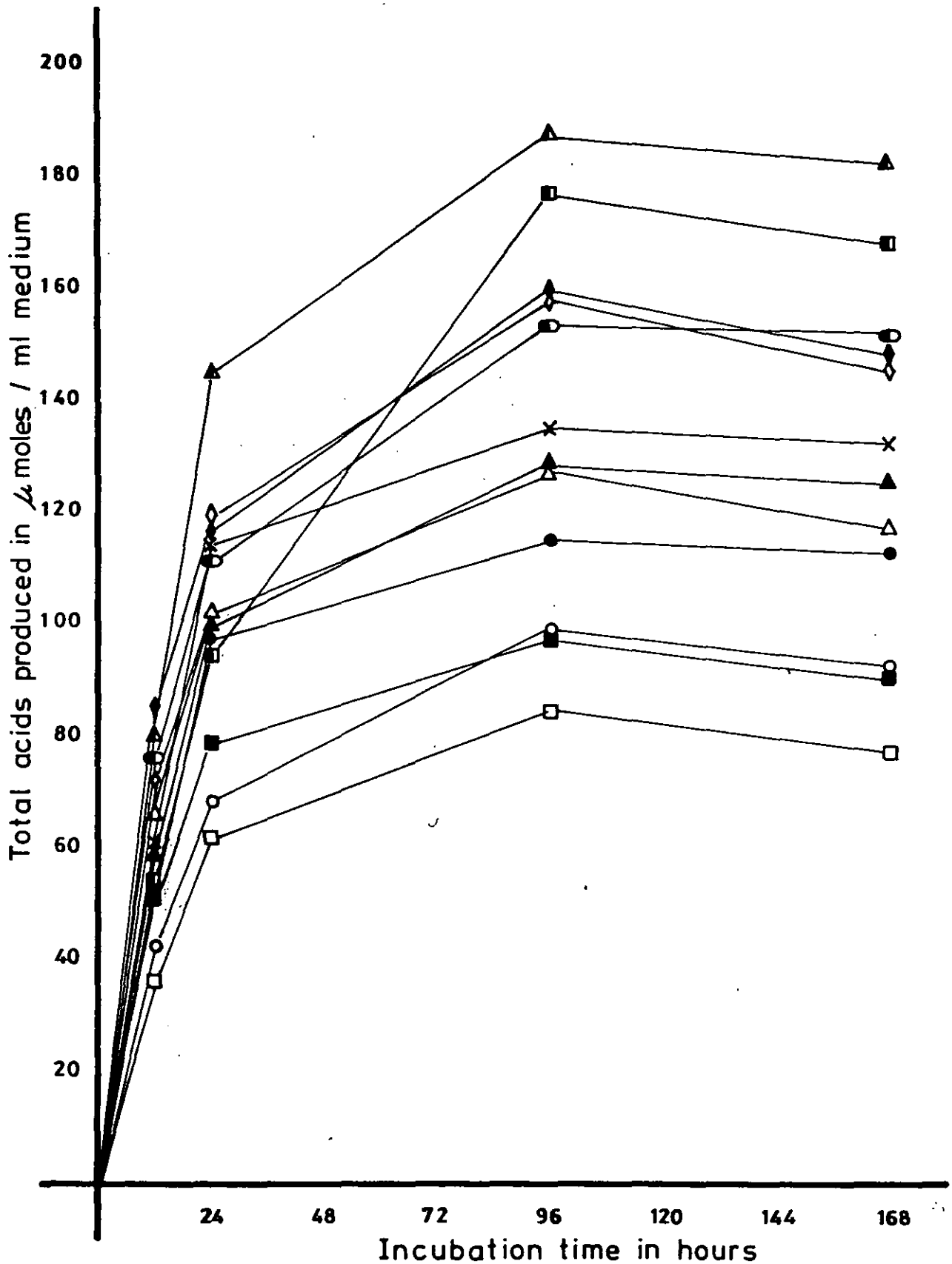
△—△ B.fragilis (NCTC 9343)

●—● B.asaccharolyticus (W50)

○—○ B.fragilis (NCTC 8560)

■—■ B.ruminicola ss. brevis (NP333)

□—□ B.melaninogenicus ss.melaninogenicus (VPI 9085)


Total acids produced in μ moles / ml medium
200
180
160
140
120
100
80
60
40
20
24
48
72
96
120
144
168
Incubation time in hours

(4) Comparative Study on Total Acid Production in CMG and PYG

The total acid production for 5 Bacteroides strains was compared in two different media CMG and PYG and the results is shown in Fig.26 .

The results show that CMG in general encourages a higher rate of production of SCFA than PYG. The largest difference was noted with B.melaninogenicus SS. macacae and the least with B.asaccharolyticus (B536)

The two strains of B.asaccharolyticus W50 and B536 , however, show different patterns with W50 giving a higher total acid production in CMG and PYG compared with B536 A similar observation was noted for the two B.fragilis strains where NCTC 9343 showed a much higher rate of total acid production in CMG and PYG compared with NCTC 8560.

All strains studied showed a much higher rate of total acid production in CMG than PYG with the degree of differences related to the species used.

Fig. 26: Comparison of total acid production by 5 Bacteroides species in CMG and PYG over 7 days of anaerobic incubation at 37°C.

●—● B.melaninogenicus ss. macacae (ATCC 33141) in CMG

○—○ " " " " (" ") in PYG

▲—▲ B.fragilis (NCTC 9343) in CMG

△—△ " " " in PYG

♦—♦ B.asaccharolyticus (W50) in CMG

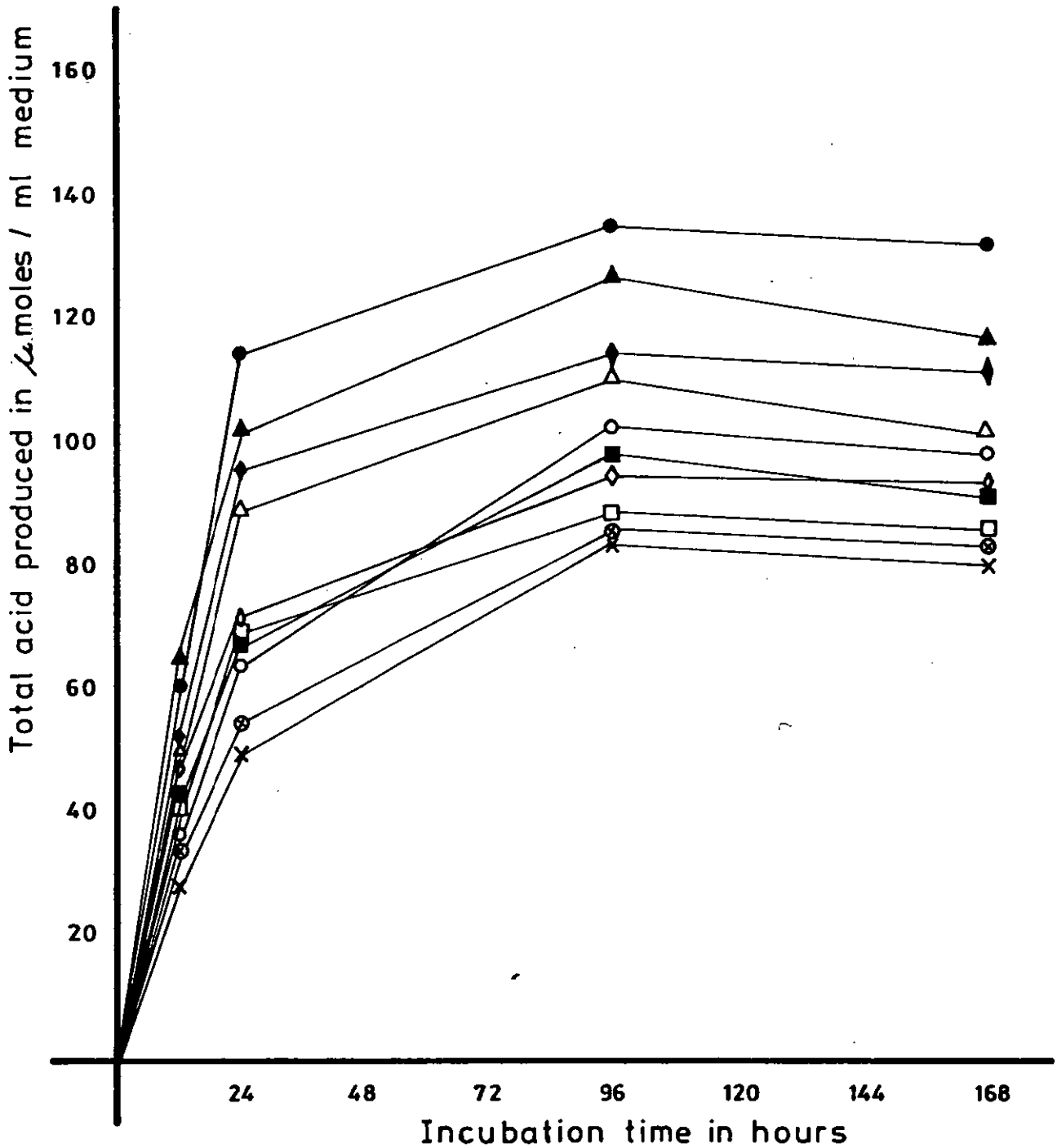
◊—◊ " " " in PYG

■—■ B.fragilis (NCTC 8560) in CMG

□—□ " " " in PYG

⊗—⊗ B.asaccharolyticus (B536) in CMG

✕—✕ " " " in PYG


Total acid produced in µ moles / ml medium
160
140
120
100
80
60
40
20
24
48
72
96
120
144
168
Incubation time in hours

(5) Comparative Study of S:A ratio in CMG Medium

The ratios of S:A for 8 different Bacteroides species were examined in CMG at 12 and 24 h of incubation with the results presented in Table 7.

The results shows that all the strains studied have a higher ratio of S:A at 12 h of incubation which decrease at 24 h. The ratio of S:A in B.ovatus was shown to be reduced by half at 24 h. B.melaninogenicus ss. intermedius showed a slight reduction in the ratio of these acids at the two different incubation time, and B.ruminicola ss brevis showed a slightly higher ratio at 12 h than 24 h.

The results shows CMG to favour higher production of S in comparison to A at 12 h of incubation, which seem to moderate at 24 h by increase in A production.

Table 7

Ratio of S:A in CMG medium at different incubation time for Bacteroides species

Ratio	Incubation Time (hour)	B.fragilis NCTC 9343	B.ovatus ATCC 8483	B.vulgatus WBM 137	B.distasonis ATCC 8503	B.thetaiotaomicron NCTC 10582	B.ruminicola SS.brevis NP 333	B.melaninogenicus SS.melaninogenicus VPI 9085	B.melaninogenicus SS.intermedius T 588
S:A	12	2.61	5.16	6.74	3.60	5.02	4.79	3.62	4.85
S:A	24	1.68	2.95	5.30	2.31	3.81	5.09	2.95	4.56

S = succinic acid a = acetic acid

(6) Determination of Types and Amount SCFA Present in CMG and PYG media

The type of SCFA detected in both media prior to inoculation were A, L and S and their amounts in $\mu mole.ml^{-1}$ is listed in Table 8.

Table 8

Types and amounts of SCFA ($\mu mole.ml^{-1}$) present in uninoculated CMG and PYG media

Acids Detected	Amount of SCFA ($\mu mole.ml^{-1}$	
	CMG	PYG
A	4.19 ± 1.01	3.11 ± 1.12
L	2.50 ± 1.3	1.99 ± 0.8
S	2.27 ± 0.42	1.10 ± 0.16

A = acetic acid, L = lactic acid S = sussinic acid

3.5 Growth and pH Curves

Growth and change in the medium pH were examined in association with SCFA analyses in PYG medium for two strains i.e. B.fragilis (NCTC 8560) and B.asaccharolyticus (W50) The results are shown in Fig (27).

B.fragilis grew exponentially for the first 48 h and this was associated with a fall in medium pH from 7.0 - 5.2 within the same period. The stationary phase occurred from 48 h - 96 h and the decline phase was observed from this period to the end of the incubation time. The pH of the medium remained unchanged at 5.2 throughout the further incubation.

In the case of B.asaccharolyticus the exponential phase continued until 72 h and was followed by the stationary phase until 120 h. The decline phase was observed from this period and until the end of the incubation time. B.asaccharolyticus showed slightly lower growth rate than that shown by B.fragilis. The drop in medium pH was very slight, i.e. 0.4 units, within the first 48 h after which it remained unchanged until the end of the incubation time.

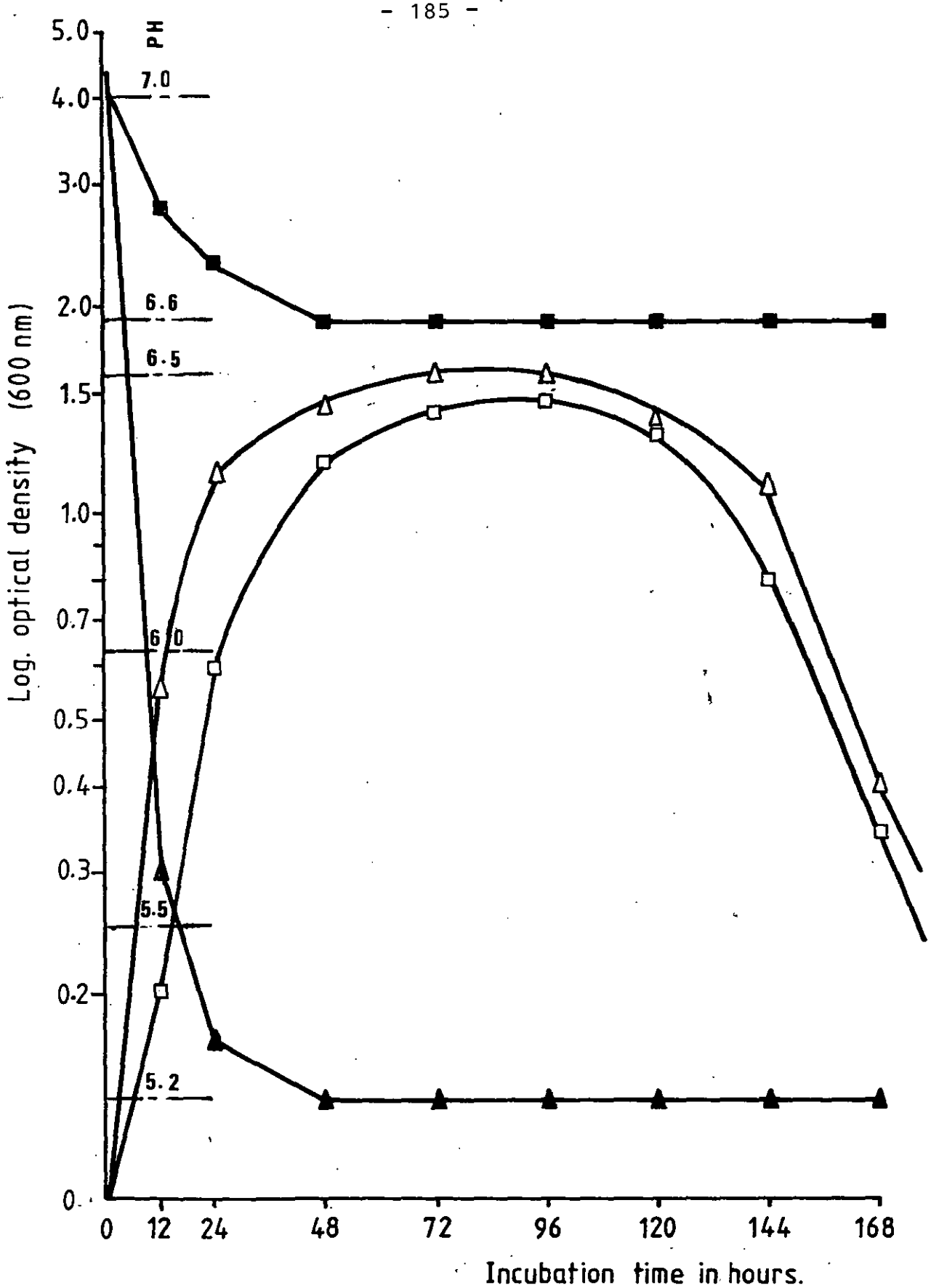
Fig. 27: B.fragilis (NCTC 8560) and B.asaccharolyticus (W50) growth and pH curves in PYG medium over 7 days of anaerobic incubation at 37°C.

△——△ B.fragilis (NCTC 8560) growth curve

▲——▲ " " " pH curve

□——□ B.asaccharolyticus (W50) growth curve

■——■ " " " pH curve


PH
5.0
4.0
3.0
2.0
1.5
1.0
0.7
0.5
0.4
0.3
0.2
0
7.0
6.6
6.5
6.0
5.5
5.2
Log. optical density (600 nm)
0
12
24
48
72
96
120
144
168
Incubation time in hours.

3.6 Metabolism of ^{14}C-acetate

The results of the radioactive assays with ^{14}C sodium acetate showed that both species tested B.asaccharolyticus, W50 and B.distasonis, ATCC 8503, were capable of using labelled acetic acid in the form of 1-^{14}C sodium acetate with the release of high number of labelled CO_2 into the culture atmosphere which was absorbed by the hyamine. The results also show little radioactivity was detected when 2-^{14}C sodium acetate was used (Table 9).

Table 9

Uptake of ^{14}C acetate by two representative Bacteroides species at 72 h of incubation

Species/Strains No.	Incubation Time (hour)	Absorbed $^{14}CO_2$ (DPM)	
		2-^{14}C	1-^{14}C
B.distasonis ATCC 8503	72 h	766	83549
B.asaccharolyticus W50	72 h	912	83885

3.7 Pyrolysis Gas Chromatography

The results of this part of the work were divided into two sections:

Section I: deals with pyrolysis data of 11 species (12 strains) of the genus Bacteroides.

Section II: deals with pyrolysis data resulting from direct analysis of pus material from infections caused by (i) the pure strains of B.fragilis and B.asaccharolyticus and (ii) a mixed anaerobic infection using E.coli, Streptococcus faecalis, Clostridium sporogenes and B.asaccharolyticus.

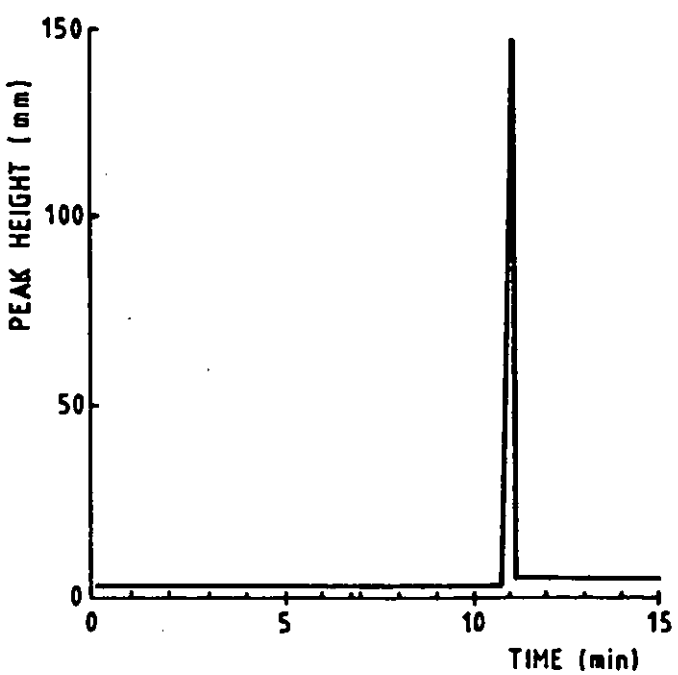
Section I: 5 representative pyrochromatograms from the 11 different bacteroides species are shown in Fig. 29 - 33 representing B.fragilis (NCTC 8560), B.vulgatus (WBM137) , B.melaninogenicus SS. melaninogenicus (VPI 9085), B.ruminicola ss. brevis (NP333) and B.melaninogenicus SS. macacae (ATCC 33141)respectively. Peak 8 in all

pyrograms represent the internal standard (polyhydroxy proline, (Fig. 28) added for more accurate identification of the peaks, where the relative retention time was used to identify the peaks. This peak was also found to be produced by all Bacteroides species studied. The peak heights for each pyrogram were normalized in relation to peak 13 which was found to be extremely reproducible in its amount and retention time.

The relative heights, their range, mean and standard of deviation were calculated and are presented in tables A.1 - A.12 (Appendix I).

Visual comparison of the Bacteroides strains revealed some characteristic peaks (finger print area), these were peaks 16; 17; 23; 24; 25; 26; 35; 36; 37; 38; and 39. The presence of these peaks within different heights or their absence gave valuable information in

Fig. 28: Pyrogram of internal standard DL, Polyhydroxy Proline (2 µl)



the differentiation of these species (Table 10).

Table 10: Finger-print area for differentiation of Bacteroides species

Species/Strain No.	Peak Number										
	16	17	23	24	25	26	35	36	37	38	39
B.vulgatus WBM137	+	+	+++	++	-	+++	-	-	++	-	++
B.fragilis NCTC 8560	+	+	+++	++	-	+++	-	++	++	++	++
B.ruminicola SS.brevis NP333	+++	++	+++	+++	++	+++	++	-	++	+++	++
B.mel.SS macacae ATCC 33141	++	++	+++	+++	+	+++	+++	++	++	++	++
B.mel.SS. melanino-genicus VPI 9085	+	+	+++	+++	+	+++	++	+	+++	+++	++

+++ present in large amount

++ " " moderate amount

+ " " small amount

- absent

Table 10 represents the qualitative and semiquantitative differences existing between the representative pyrograms (Figs. 29-33) of five different Bacteroides species.

Although these characteristic peaks help differentiate the species studied, visual comparison tends to be complicated by the differences in relative sample weight. Thus the need for a more quantitative and objective method for the assessment of pyrograms was needed and consequently canonical variate analysis was employed. Canonical variate analysis of 39 normalized peak heights for the 12 strains and representing 95% of the total generalized variation between the groups is shown in Fig. 34 & 35. The results shows PGLC capable of differentiating the 12 Bacteroides strains. Fig.34 shows the strains to be divided into three sets, the first set includes B.asaccharolyticus (9 and 10); B.melaninogenicus SS. intermedius (7) and B.melaninogenicus ss. macacae (11) which seem to be quite well differentiated from the members of the same set. Strains B536 and W50 which belong to species B.asaccharolyticus show some differences from each other which necessitate their separation (9 and 10 in Fig. 34). The second set includes B.vulgatus (6) and B.ruminicola SS. brevis (12) which has taken an intermediate position between the black pigmented and non-pigmented Bacteroides. The third set includes B.thetaiotaomicron (1); B.fragilis (4 and 5); B.distasonis (3); B.ovatus (2) and B.melaninogenicus SS. melaninogenicus (8). The latter species seem to be quite differentiated from the member of the last set.

All strains show sufficient variation to be separated into different groups except B.distasonis (3) and B.fragilis (4) which are not distinguished from each other.

Fig. 34 also shows the ability of PGLC to differentiate heterogenous strains of the same species as the case with strain numbers 9 and 10 which both belong to species

B.asaccharolyticus and strains number 4 and 5 which are B.fragilis strains.

Consideration of more than the first two canonical variate did not improve the differentiation. Fig.35 shows the third conanical variate vs. the first with the result showing the three previous sets of group quite well differentiated from each other. Sets one and three show clustering of their strains which make differentiation difficult. Strains numbers 6, 7 and 12 seems to be very well differentiated from each other whether the first or the third canonical variate was applied.

Canonical variate analysis of 8 Bacteroides species (Fig.36a & b) shows a different arrangement to that of fig.34 but still shows a well differentiated species with species number 2 and 3 undifferentiated. The third canonical variate vs. the first (Fig.36b) does not offer more differentiation of the species but to the contrary shows three species to be undifferentiated which is 2, 3 and 4 with the rest of the species well differentiated.

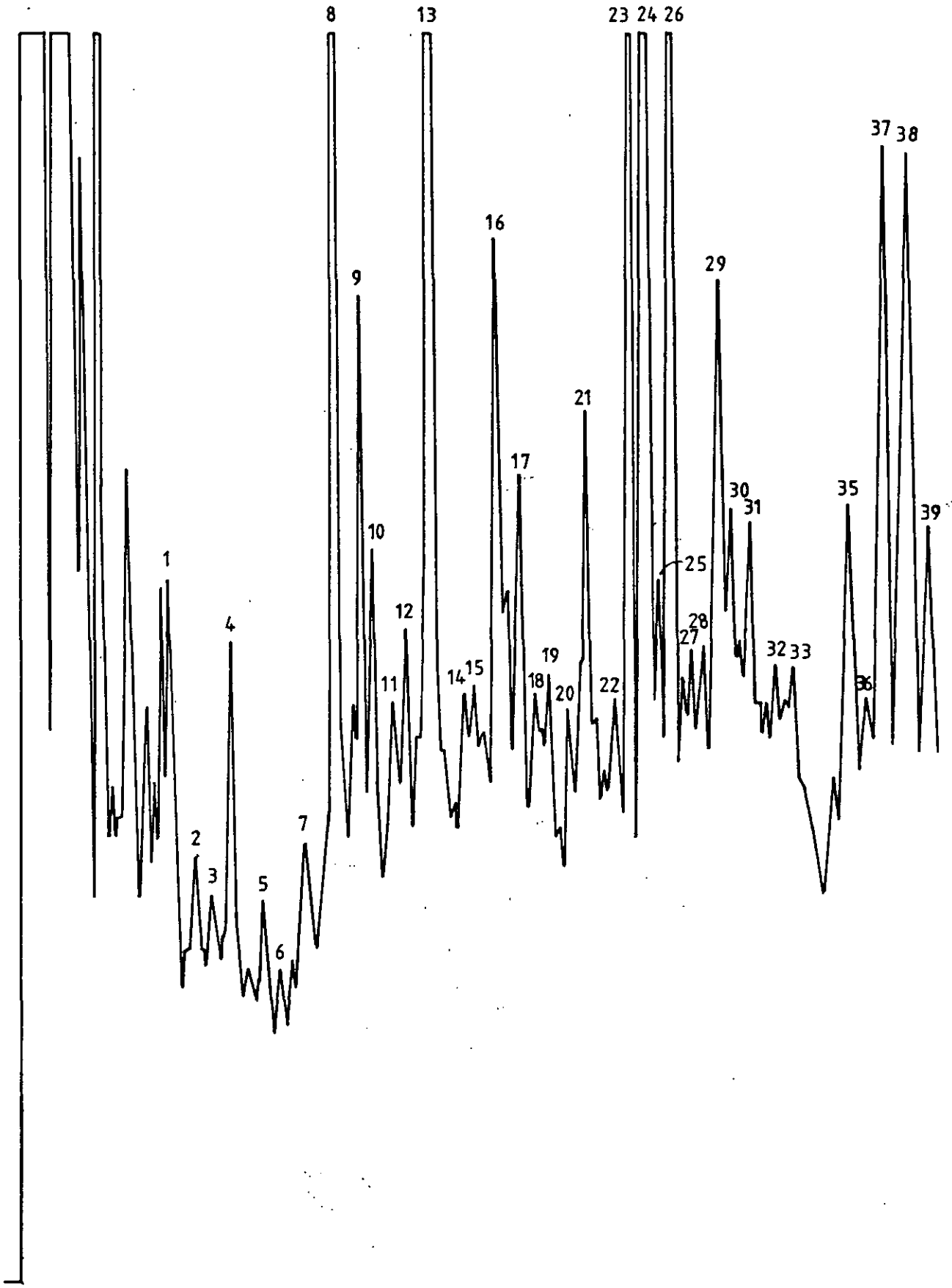
Section II: Representive pyrochromatograms obtained from the direct analysis of abscess material is shown in Fig. 37 and 38 representing infections caused by a pure strain of B.fragilis (NCTC 8560) and mixed anaerobic bacteria respectively.

Visual comparison of Fig. 29 with Fig. 37 shows a great similarity in both pyrograms, although very successful when dealing with pure strains such as B.fragilis and B.asaccharolyticus induced experimental

infection, it was not useful when dealing with an experimental infection resulting from a mixture of anaerobic organisms (Fig. 38). In this case the resulting pyrogram is produced from components contributed by the 4 species involved in the infection. For the purpose of applying canonical variate analysis to the infection work, pure strains of E.coli, Streptococcus faecalis and Clostridium sporogenes were pyrolysed in three successive weeks (Tables A.13 - A.18, Appendix I). The canonical variate analysis was also found to require data from sterile abscess. An attempt to produce such an abscess by injecting a suspension of 1%w/v kaolin was not successful. Unfortunately this resulted in no definite conclusion being drawn from the pyrolysis of pus from a mixed infection.

Fig. 29

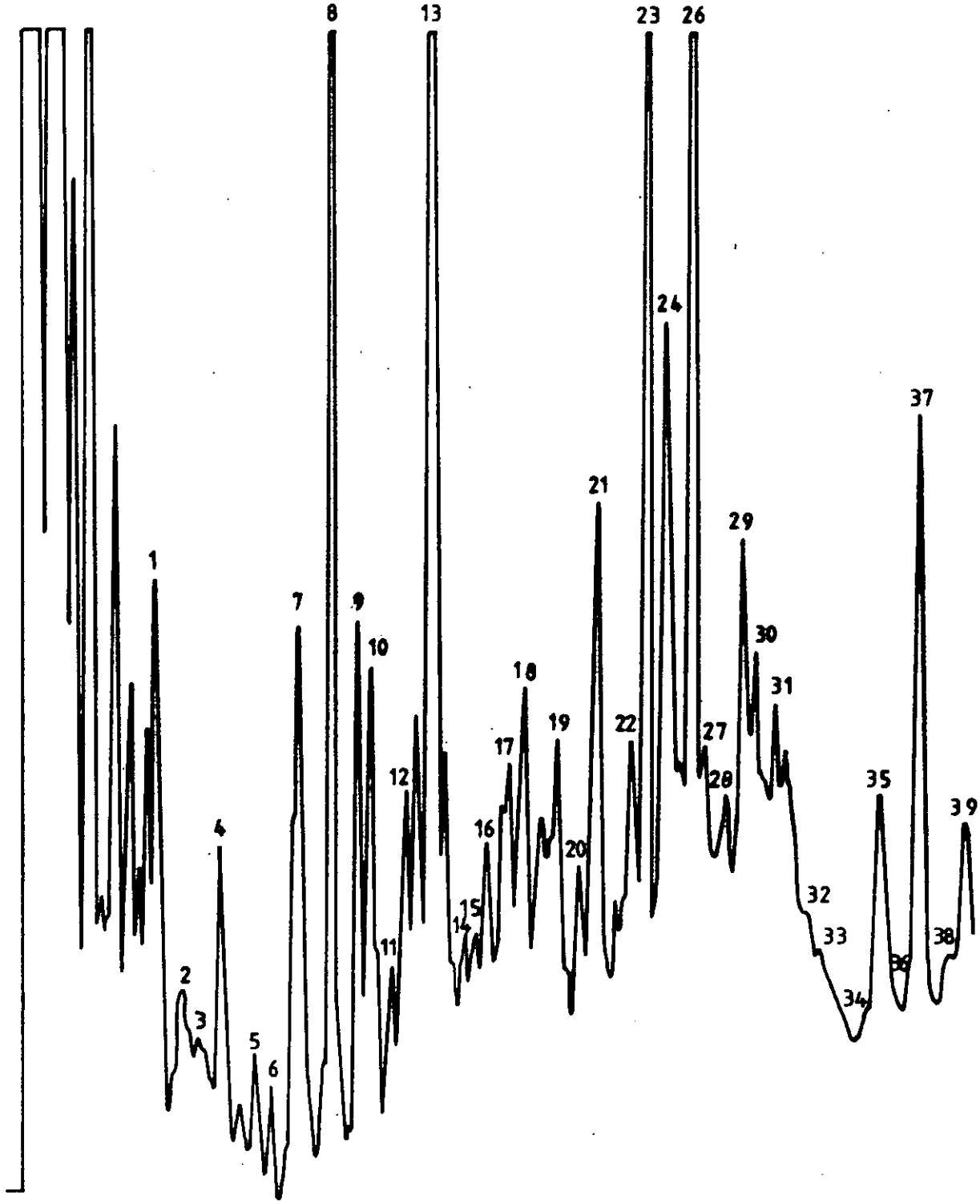
Pyrochromatogram of B.fragilis (NCTC 8560)



B. fragilis. (NCTC 8560)

Fig. 30

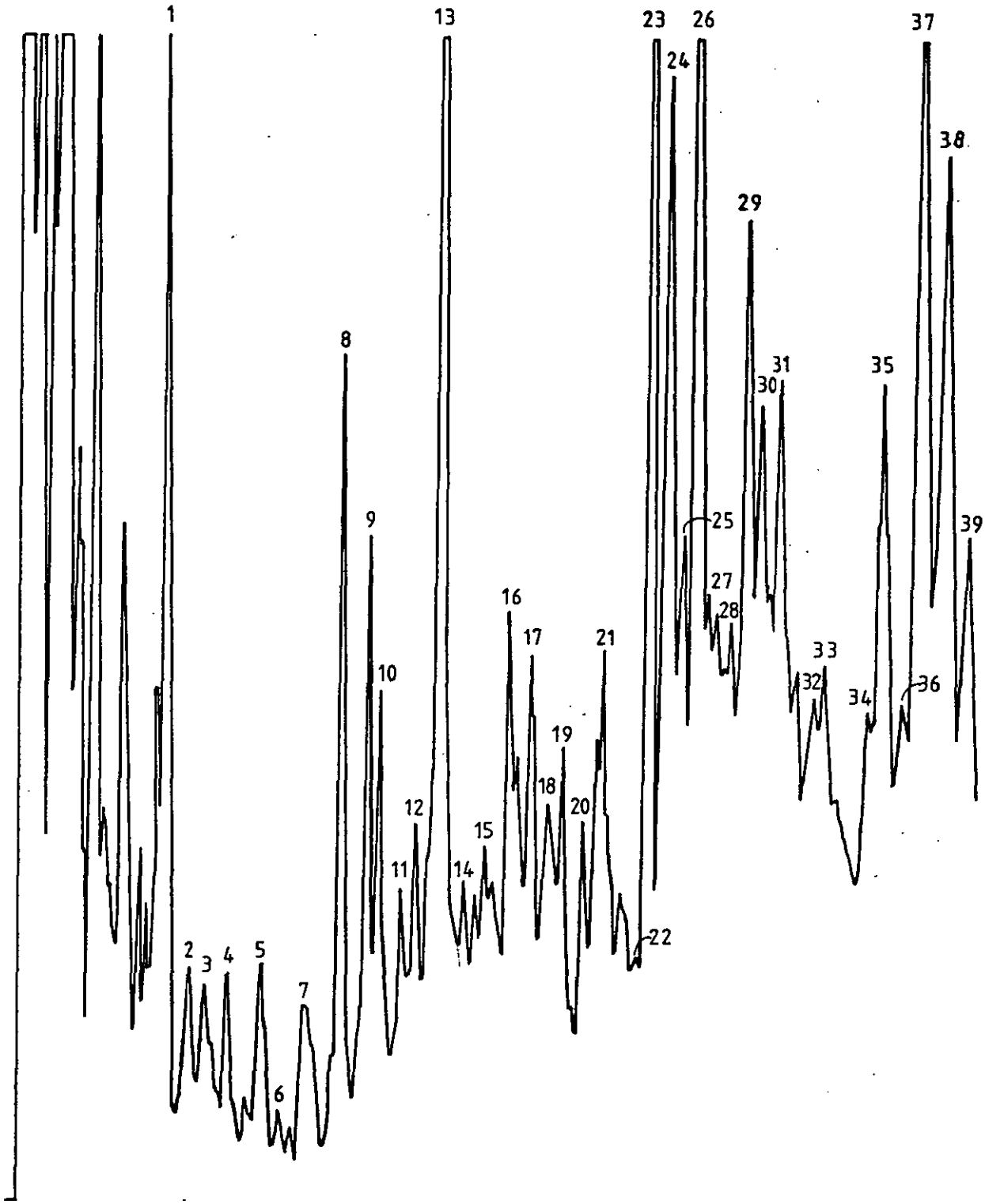
Pyrochromatogram of B.vulgatus (WBM137)



B. vulgatus (WBM137)

Fig. 31

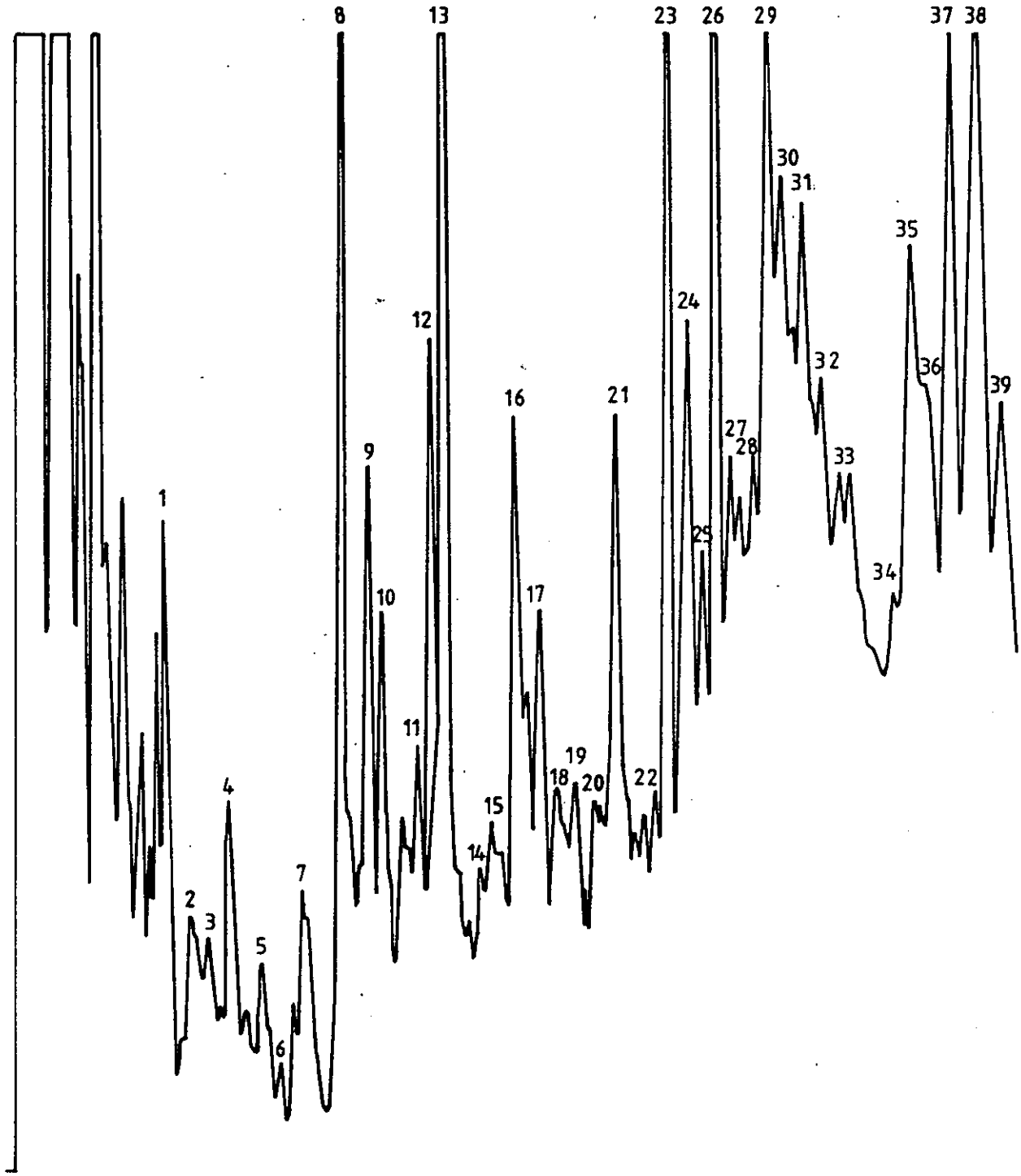
Pyrochromatogram of B.melaninogenicus ss. melaninogenicus (VPI 9085)



B. melaninogenicus SS. melaninogenicus (VPI 9085)

Fig. 32

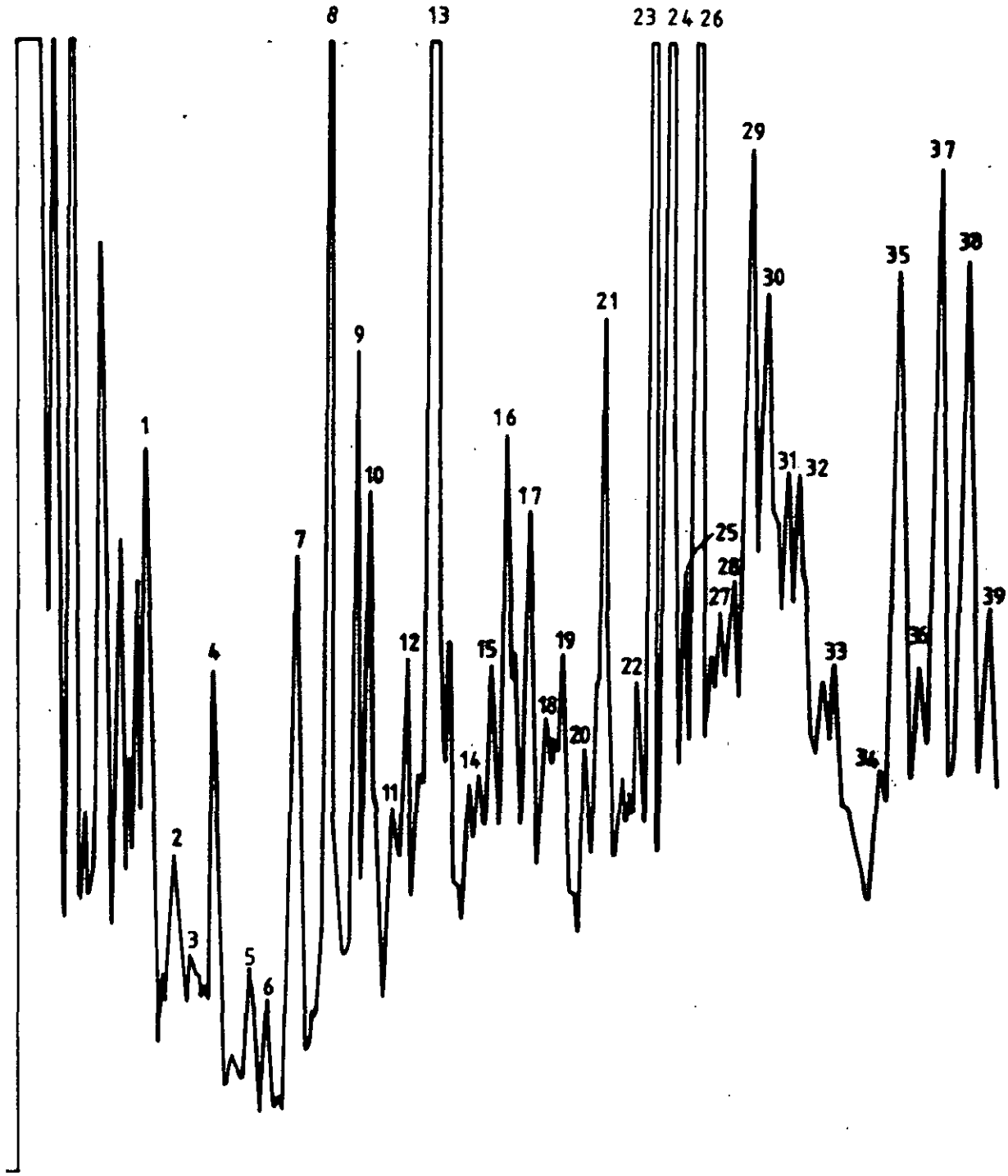
Pyrochromatogram of B.ruminicola ss. brevis (NP333)



B. ruminicola S S. brevis (NP 333)

Fig. 33

Pyrochromatogram of B.melaninogenicus ss. macacae (ATCC 33141)



B. melaninogenicus SS. macacae (ATCC 33141)

(1) B.thetaiotaomicron

(2) B.ovatus

(3) B.distasonis

(4) B.fragilis (NCTC 8560)

(5) B.fragilis (NCTC 9343)

(6) B.vulgatus

(7) B.melaninogenicus ss. intermedius

(8) B.melaninogenicus ss. melaninogenicus

(9) B.asaccharolyticus (B536)

(10) B.asaccharolyticus (W50)

(11) B.melaninogenicus ss. macacae

(12) B.ruminicola ss. brevis

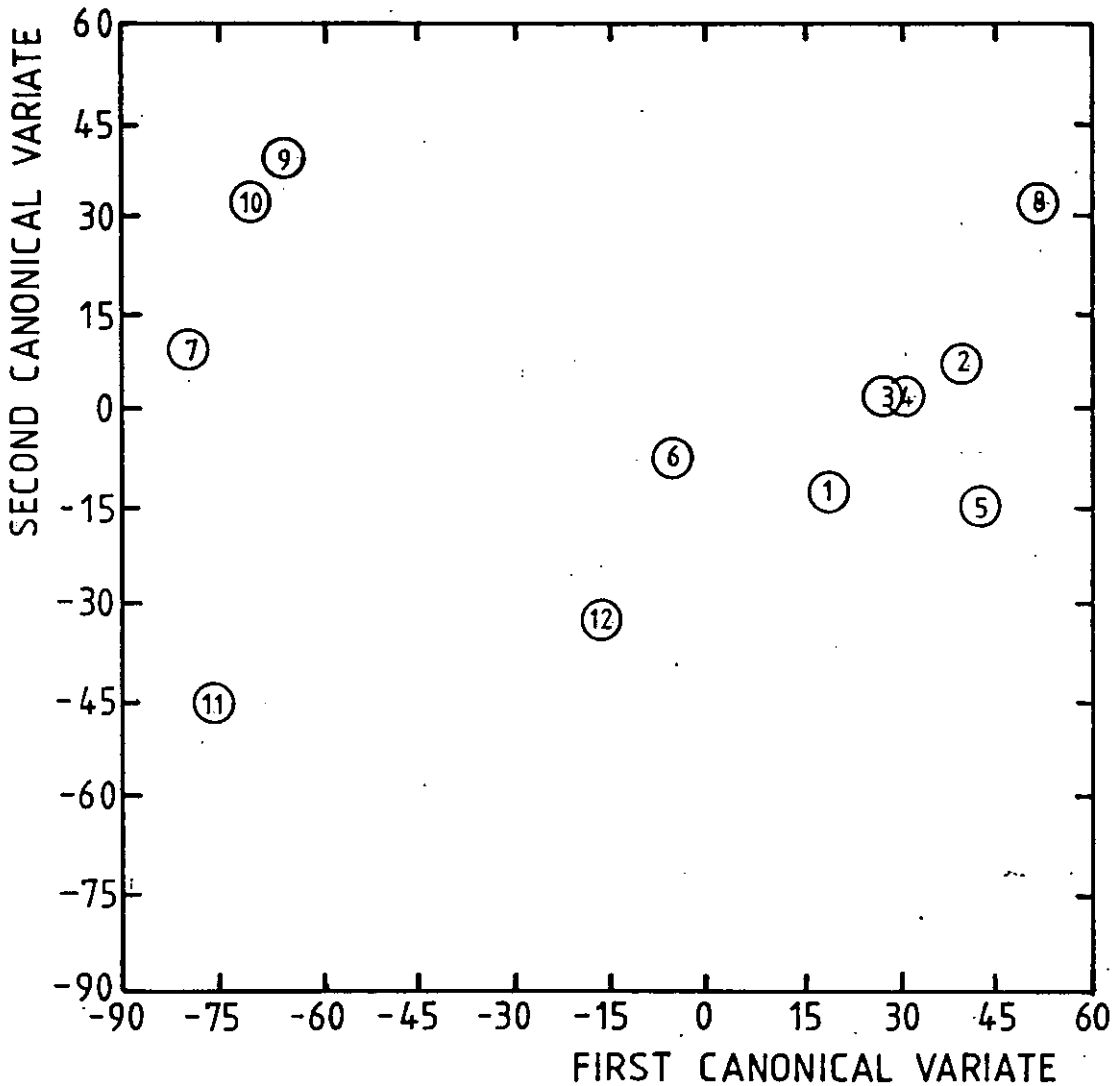


Fig. 34

Canonical variate analysis for 12 Bacteroides strains (2nd vs. 1st)

(1) B.thetaiotaomicron

(2) B.ovatus

(3) B.distasonis

(4) B.fragilis (NCTC 8560)

(5) B.fragilis (NCTC 9343)

(6) B.vulgatus

(7) B.melaninogenicus ss. intermedius

(8) B.melaninogenicus ss. melaninogenicus

(9) B.asaccharolyticus (B536)

(10) B.asaccharolyticus (W50)

(11) B.melaninogenicus ss. macacae

(12) B.ruminicola ss. brevis

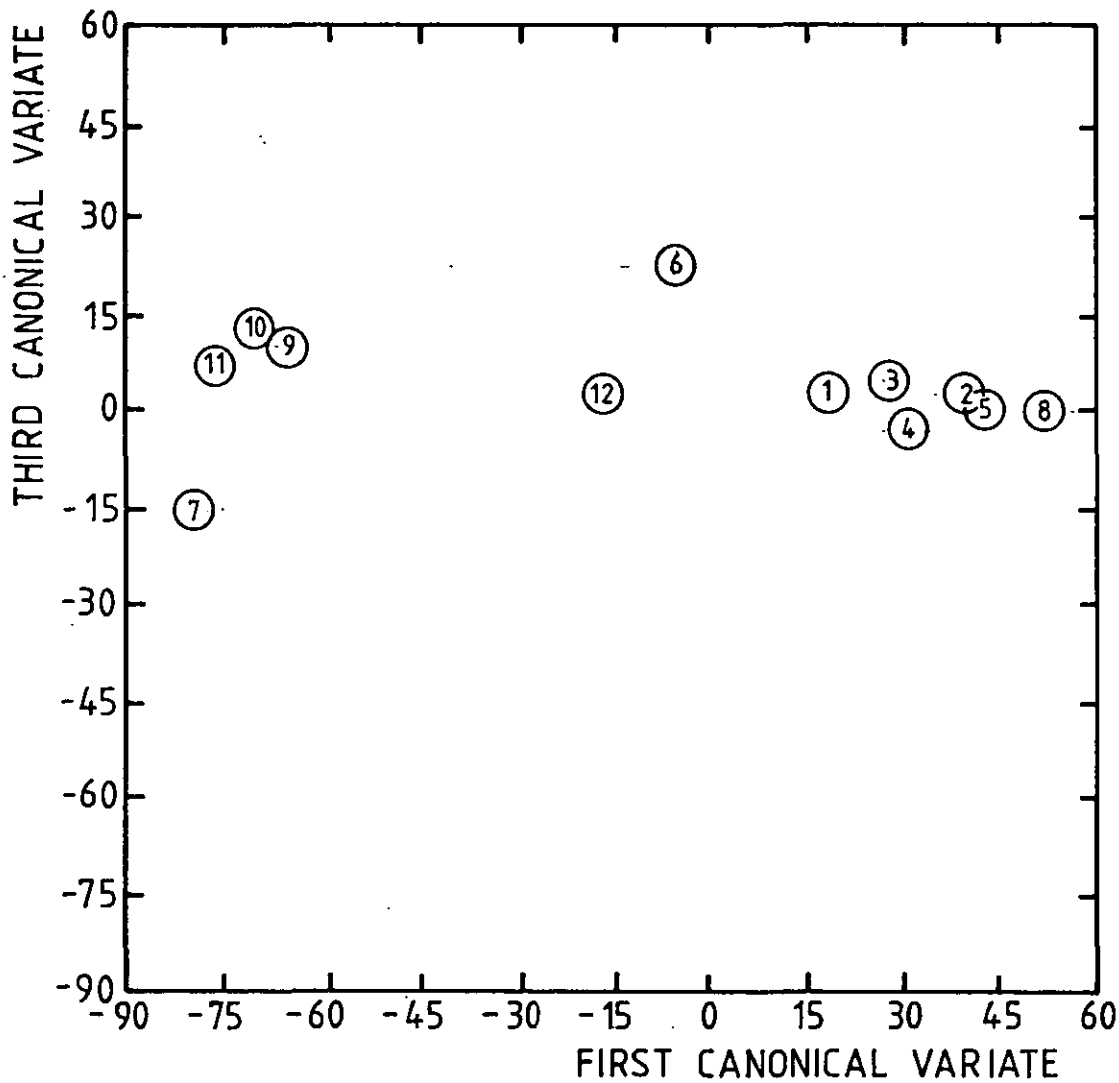


Fig. 35

Canonical variate analysis for 12 Bacteroides strains (3rd vs. ist)

Fig. 36a

Canonical variate analysis for 8 bacteroides strains (3rd vs. ist)

Fig. 36b

Canonical variate analysis for 8 Bacteroides strains (2nd vs. ist)

(1) B.thetaiotaomicron

(2) B.ovatus

(3) B.distasonis

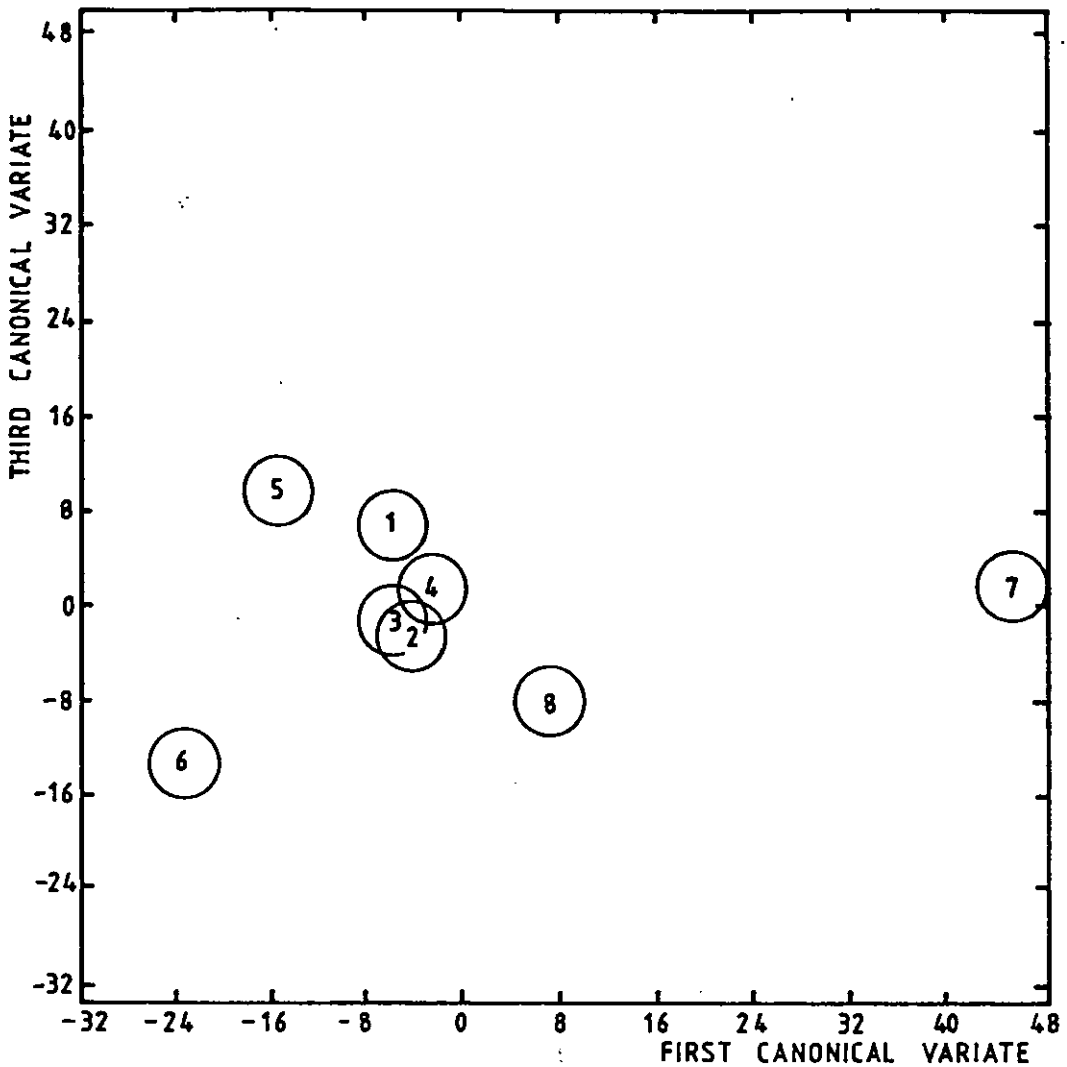
(4) B.fragilis (NCTC 8560)

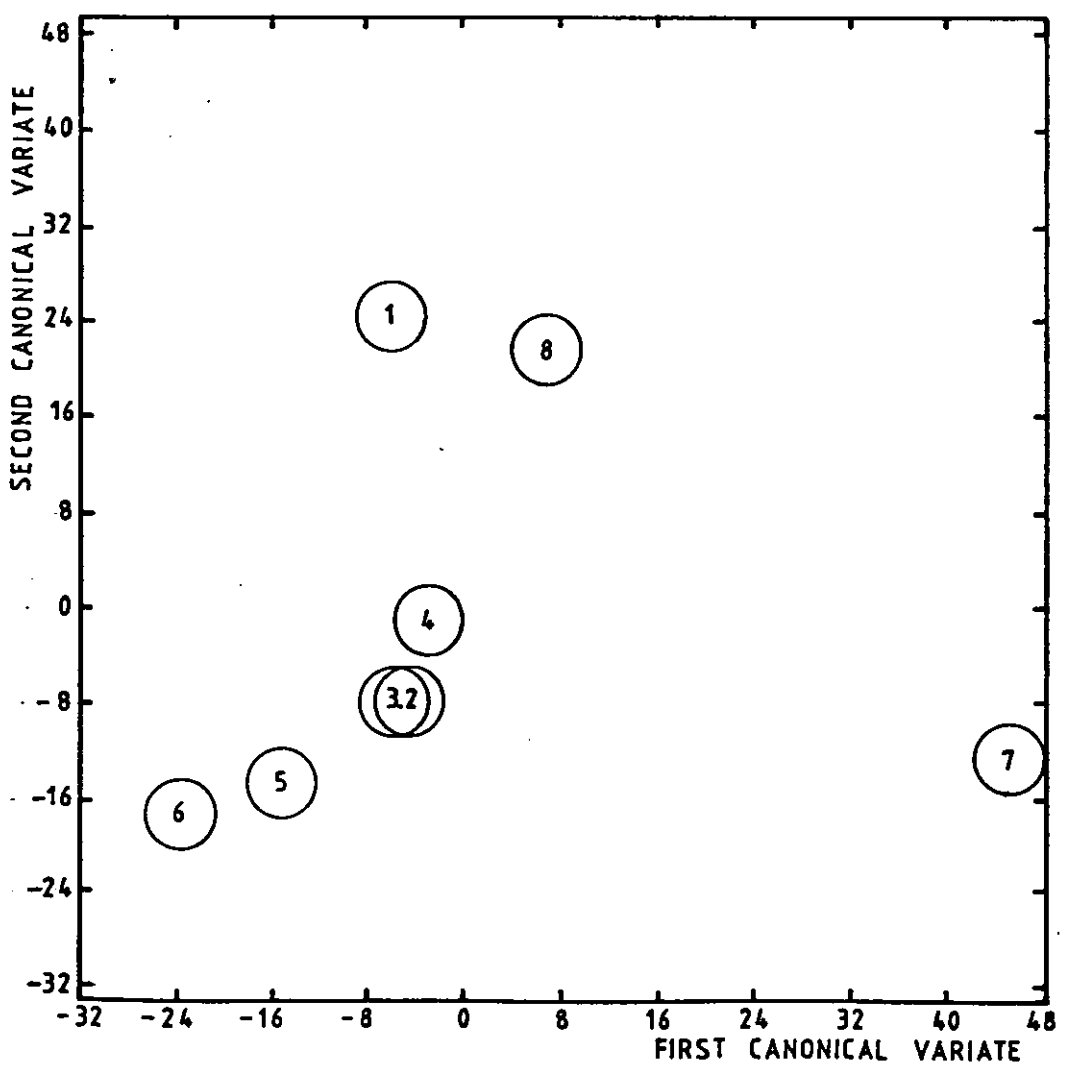
(5) B.fragilis (NCTC 9343)

(6) B.vulgatus

(7) B.melaninogenicus ss. intermedius

(8) B.melaninogenicus ss. melaninogenicus


THIRD CANONICAL VARIATE
FIRST CANONICAL VARIATE
48
40
32
24
16
8
0
-8
-16
-24
-32
-32
-24
-16
-8
0
8
16
24
32
40
48
1
2
3
4
5
6
7
8


SECOND CANONICAL VARIATE
FIRST CANONICAL VARIATE
48
40
32
24
16
8
0
-8
-16
-24
-32
-32
-24
-16
-8
0
8
16
24
32
40
48
1
2
3
4
5
6
7
8

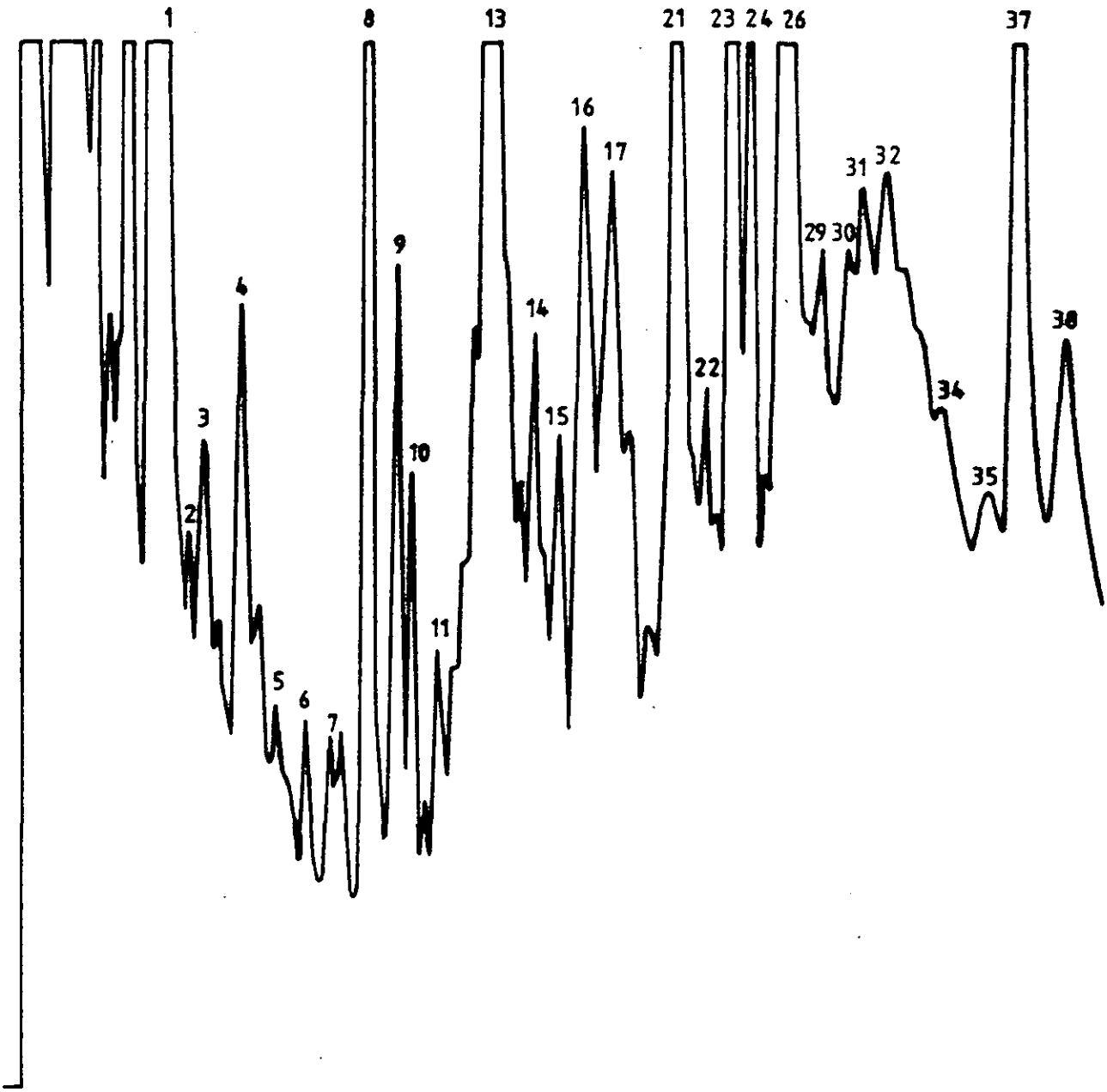
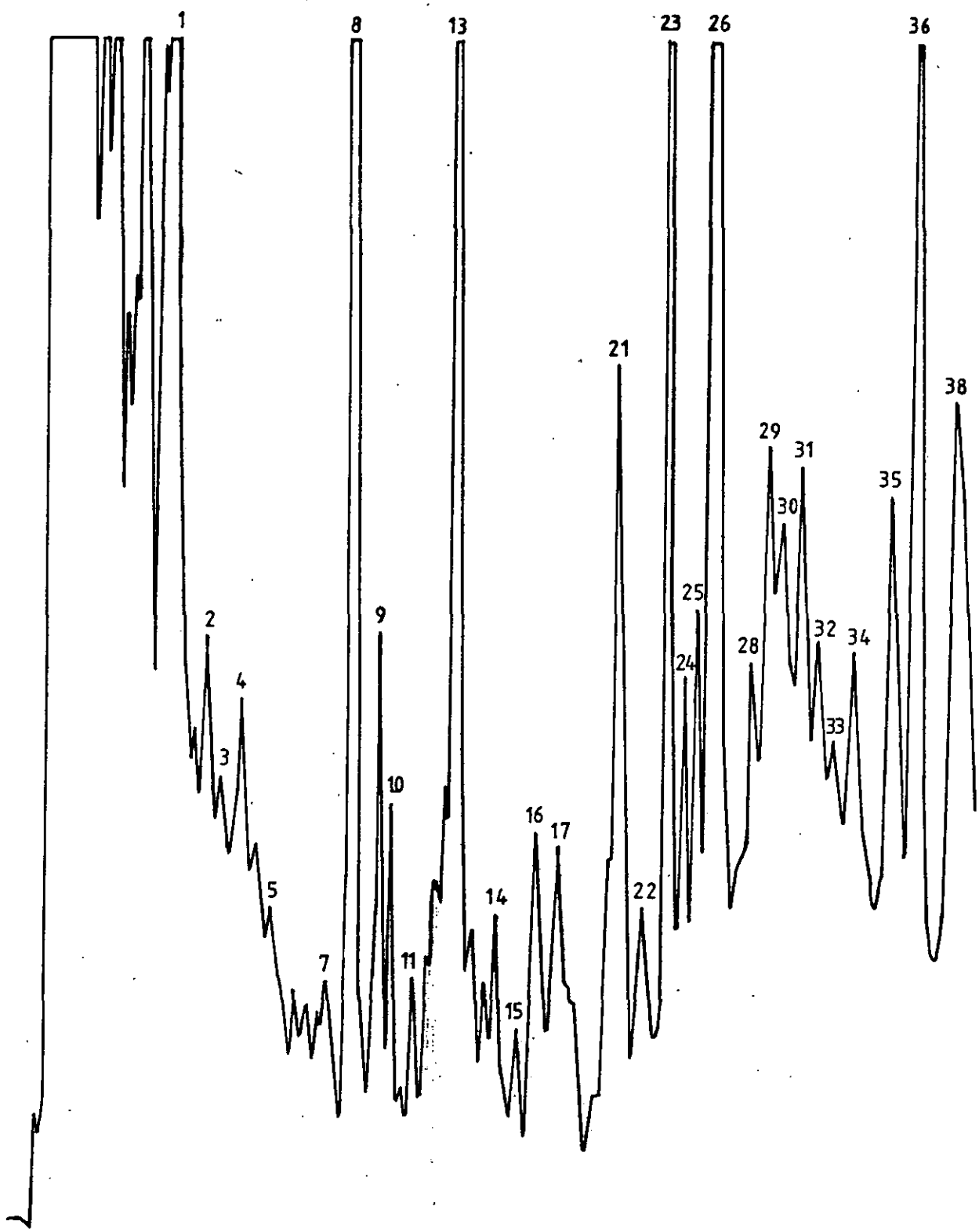


Fig. 37: Pyrochromatogram from direct analysis of pus from experimental animal infection by pure culture of B.fragilis (NCTC 8560)

Fig. 38

Pyrochromatogram from direct analysis of pus from experimental animal infection by mixed cultures (*E.coli*, *Cl.sporogenes*, *St.faecalis* and *B.asaccharolyticus* (W50)


1
8
13
23
26
36
21
38
29
31
35
30
25
2
9
32
34
28
24
4
33
3
10
16
17
5
14
22
7
11
15

3.8 Experimental Infection

(i) Pathogenic capability

The results obtained show clearly that B.fragilis, B.melaninogenicus ss. macacea and B.asaccharolyticus were capable of producing a localized abscess at a concentration of 10^8 cells.ml^{-1} B.fragilis injected subcutaneously (s.c) caused the formation of abscesses which varied from 16 - 22 mm in length at the site of injection (Fig. 39a). Intramuscular (i.m.) injection caused a haematoma without the formation of an abscess.

B.melaninogenicus SS. macacea injected subcutaneously (S.C.) caused the formation of abscesses which varied from 10 - 18 mm in length at the site of injection (Fig. 39c).

The abscesses were firmly attached to the mucosa of the skin and attempts to separate the abscesses from the skin were not successful. Therefore, for the purpose of identification and histology, the abscesses were removed intact and still attached to the skin. Also observed was the haematoma of the adjacent muscles by the abscesses. The i.m. injection caused a haematoma to the muscle without the abscess formation.

B.asaccharolyticus injected subcutaneously caused the formation of abscesses which varied from 18 - 25 mm in length at the site of injection (Fig. 39b).

The abscesses were firmly attached to the skin. The intramuscular injection caused the formation of abscesses which varied from 0 - 5 mm in length and which were surrounded by haematoma of the proximal muscles.

In all cases, the pus obtained from the abscesses were white-pale yellow in colour and creamy in texture.

All animals throughout the incubation period of 7 days showed no signs of discomfort except when examining the site of injection. No organisms were isolated from the heart, liver and spleen.

The histopathological examination of B.fragilis abscess (Fig.40a & b) showed a moderate infiltration by neutrophils to the surrounding tissue. It also shows normal muscle cells as well as many vacuoles. Control animals showed no abscess formation with both heat killed suspension and 1% kaolin suspension.

(ii) Effect of variation in inoculum size:

(I) The result of this part can be seen in Figs. 41a, b & c . There was no apparent effect when 10^2 cells.ml^{-1} were injected both subcutaneously and intramuscularly. Autopsy showed no abscess formation at the site of injection or elsewhere.

The abscesses caused by injection of 10^5 cells.ml^{-1} were 5-10 mm in length and located at the site of injection (Fig. 41a), whereas the injection of 10^8 cells.ml^{-1} gave bigger

abscesses 18 - 25 mm in length (Fig. 41b).

The abscesses caused by injection of 10^{10} cells.ml^{-1} caused an intensive necrosis to the muscles at the site of injection. The pattern of infection was similar to that caused by a mixed infection (Fig. 42c). The abscesses were firmly attached to the mucosa of the skin and the infection was characterized; by accumulation of black foul smelling pus in the area extended from the upper thigh region to the tail region; which caused the rabbits to be in a continually suppurating state. The effect of i.m. injection of 10^{10} cell.ml^{-1} can be seen in Fig. 41c which shows slight necrosis of the muscles. Control animals showed no abscess formation with both heat killed suspension or 1% sterile kaolin suspension.

The pus obtained at cell concentration of 10^5 and 10^8 cell.ml^{-1} were white pale-yellow and creamy in texture, whereas at 10^{10} cells.ml^{-1}, the pus was black, foul smelling and in liquid form.

Animals which received 10^2 cells.ml^{-1} were normal and healthy throughout the 7 days incubation period, those who received 10^5 and 10^8 cells.ml^{-1} showed no signs of discomfort except when examining the site of injection, their eating and drinking were normal.

At 10^{10} cells.ml^{-1} the animals were distressed and would not take food and drink after 24 h of injection. The animals' health deteriorated

rapidly and at 6 days the hind part of the rabbits was semiparalysed. The adjacent area contained much pus, which was enclosed by the skin. It was also observed that the tail became detached from the body and this was accompanied by a discharge of foul smelling pus,

The damage caused by s.c. injection at this inoculum level was much greater than that of the i.m. injection. No organisms however were isolated from heart, liver or spleen.

The histopathological examination showed the pattern to be similar to that caused by mixed infection (Fig.45a, b, & c) where there was a heavy infiltration of the skin and muscles with neutrophils. Higher magnification of the same area shows (Fig. 45b) a massive infiltration of the tissues with neutrophils. This section also shows some remains of necrotised muscle cell.

(II) The effect of high inoculum of B.fragilis and B.asaccharolyticus injection into the same rabbit can be seen in Fig.42a & b).

The infection caused gangrenous spreading lesions of the deep layer of the skin with the result that death occurred after 60 - 72 h of injection. After 24 h of injection the animals stopped eating, drinking and the rear legs showed impeded movement. After 48 h the rabbits

hind part seemed to be completely paralysed. Death always followed shortly after this stage.

The pus, was black, foul smelling and it was found to accumulate in the abdominal and tail region. Throughout the incubation, small amounts of pus were discharged from the tail region. The tail seemed to have become detached from the body.

The histopathological examination showed a massive invasion by neutrophils to the deep layer of the skin (Fig. 43a & b). Higher magnification (X 400) showed the lesions to be inflammatory, and heavily necrotic. Due to the severity of the infection differential histology of the surrounding tissue was not possible.

At autopsy all internal organs were examined for any changes. Only the liver exhibited any changes. There appeared to be damage and necrosis to the peripheral area of the liver. However histological examination revealed a normal liver.

B.fragilis was the only organism to be isolated from the heart and liver. Histological examination of the heart (Fig. 44a) showed slight damage to the muscles immediately adjacent to the heart chambers, though damage was not widespread and resembled an early necrosis. Examination of the adipose tissue surrounding

the heart showed a normal lymphnode (Fig. 44b) but the adipose tissue immediately adjacent showed a very heavy cellular infiltration (Fig. 44c). Lesions were very necrotic but some remaining cells were neutrophils and lymphocytes.

Control animals showed no abscess formation with both s.c. and i.m. injections.

(iii) Mixed infection

The effect of 10^5 cells.ml^{-1} injection of mixed culture of E.coli, Streptococcus faecalis, Clostridium sporogenes and B.asaccharolyticus can be seen in (Fig. 42c).

The pattern of infection was similar to that produced by 10^{10} cells.ml^{-1} of B.asaccharolyticus. The infection had spread from the upper thigh region to the tail region with accumulation of black, foul smelling pus, which was enclosed by the skin. The effect of s.c. injection was much greater than that of i.m., as in the latter the infection did not seem to have been well established even after 7 days of incubation in the rabbit. The pattern of infection following i.m. injection was similar to that of Fig.41c).

After 48 h of injection, the animals were normal in their eating and drinking behaviour, the rabbit showed discomfort on examination of the site of injection. Beyond 48 h however the

infection seem to progress rapidly with the animals rear legs showing signs of impeded movement; the rabbits at this stage, were eating and drinking less and showing sluggish movement.

The histopathological examination of the abscess (Fig. 45a, b & c) shows a normal epidermis with hair follicle and sebaceous gland. It also shows a zone of heavy cellular infiltration to the inner skin layer (Fig. 45a). At X 400 (Fig. 45b) the section shows heavy accumulation of neutrophils, it also show the disintegration of the muscle.cells caused by the infection. A Gram strained X 1000 section (Fig. 45c) of the area shows the presence of Gram positive rod which is probably Clostridium sporogenes. Streptococcus faecalis cells were seen in other sections but none of the Gram-negative bacteria were seen in any sections.

No organisms were isolated from the internal organs.

Direct analysis of the pus gave excellent results when pyrolysis was the method applied for re-identification of the Bacteroides species. The pyrogram obtained from direct pyrolysis of the pus material showed a great visual similarity to the pyrogram obtained from pure cultures. Direct GLC on the other hand gave variable results. The (VFA) extracted from

B.fragilis abscesses gave a typical profile in which acetic and propionic acids were the major acids detected, whereas the (VFA) extracted from B.asaccharolyticus abscesses gave a typical but weak profile which was always detected. Methylation of the pus suspension gave good, reproducible results from B.fragilis but gave negative results when working with B.asaccharolyticus. The results obtained when using API 20A system were similar to those obtained by pure culture.

3.9 Qualitative Assay for Enzyme Activity

The results of the assay for hyaluronidase, chondroitin sulphatase, collagenase and other enzymes, using two strains, B.fragilis, NCTC 8560 and B.asaccharolyticus, W50, are shown in Table (11). The results showed B.fragilis to be capable of hydrolysing all the substrates tested. The extent of hydrolysis varied from substrate to substrate. The organism showed a strong tendency to hydrolyse fibrin, chondroitin sulphate and collagen readily and to a lesser extent hyaluronic acid and hide powder. B.asaccharolyticus was also capable of hydrolysing all of the substrate like fibrin, hide powder and chondriotin sulphate rapidly and to lesser extent hyaluronic acid and collagen (Table 11). Hide and fibrin powder when hydrolysed released the blue colour into the broth and a blue halo was formed at the top of positive tubes. Whereas the control tube contained the blue

substrates, with the dye still bound, deposited at the bottom leaving a clear broth at the top.

Chondroitin sulphate, collagen and hyaluronic acid formed an opaque halo at the top of positive tubes with the complete digestion of the substrate. Whereas the control tubes showed a clear broth with a whitish substrate deposited at the bottom.

Table 11

Qualitative assessment of enzymes produced by two selected Bacteroides species

Species/ Strains No.	Substrates				
	Fibrin	Hide Powder	Chond-roitin sulphate	Hyaluronic acid	Collagen
B.fragilis NCTC 8560	+++	++	+++	++	+++
B.asacch-arolyti-cus W 50	+++	+++	+++	++	++

+ Weak positive reaction

++ Positive reaction

+++ Strong positive reaction

Fig. 39

Induced experimental infections in rabbits by three different Bacteroides species at an inoculum level of 10^8 cells.ml^{-1}

(a) B. fragilis (NCTC 8560)

(b) B.asaccharolyticus (W50)

(c) B.melaninogenicus ss. macacae (ATCC 33141)

Fig. 40

Cross section into B.fragilis subcutaneous abscess

(a) showing heavy cellular infiltration of muscles and the presence of vacuoles which is an indication to the gas produced by the infecting organisms. H & E stain (X10).

(b) Gram-stained section (X 1000) showing muscle necrosis and accumulation of neutrophils with no infecting organisms present.

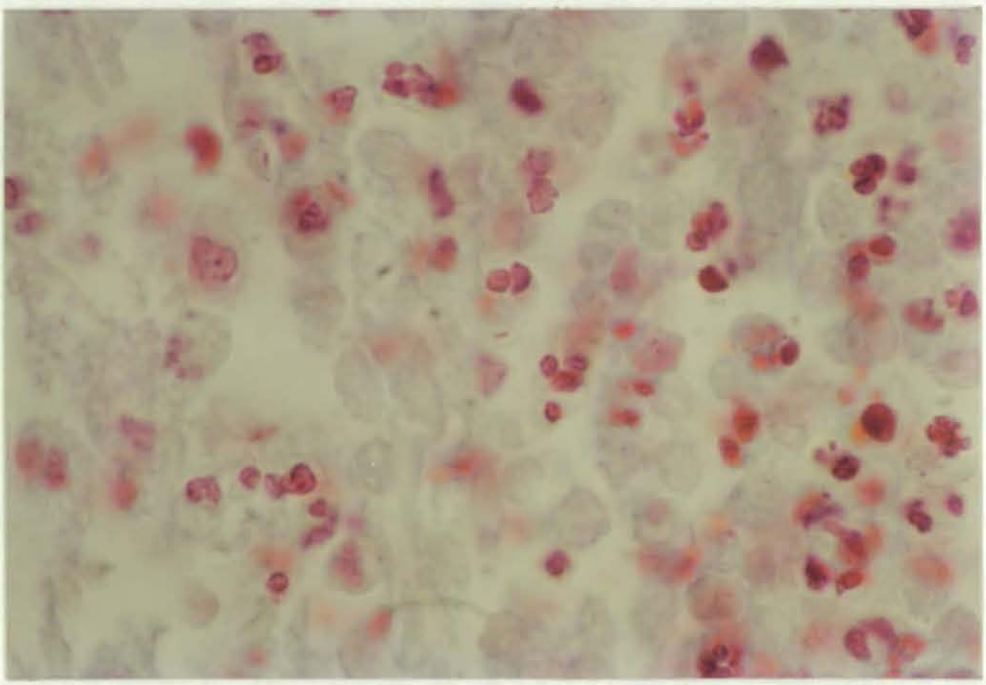


Fig. 41

Effect of increase in inoculum size on the abscess size in induced experimental infection in rabbit using B.asaccharolyticus (W50)

(a) 10^5 cells.ml^{-1}

(b) 10^8 cells.ml^{-1}

(c) 10^{10} cells.ml^{-1}

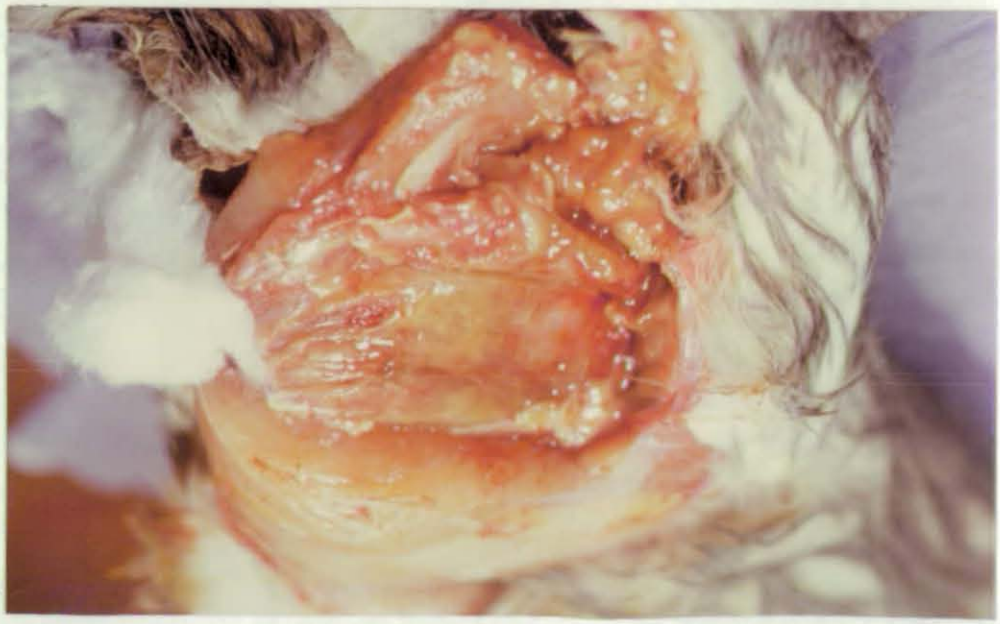


Fig. 42 a & b

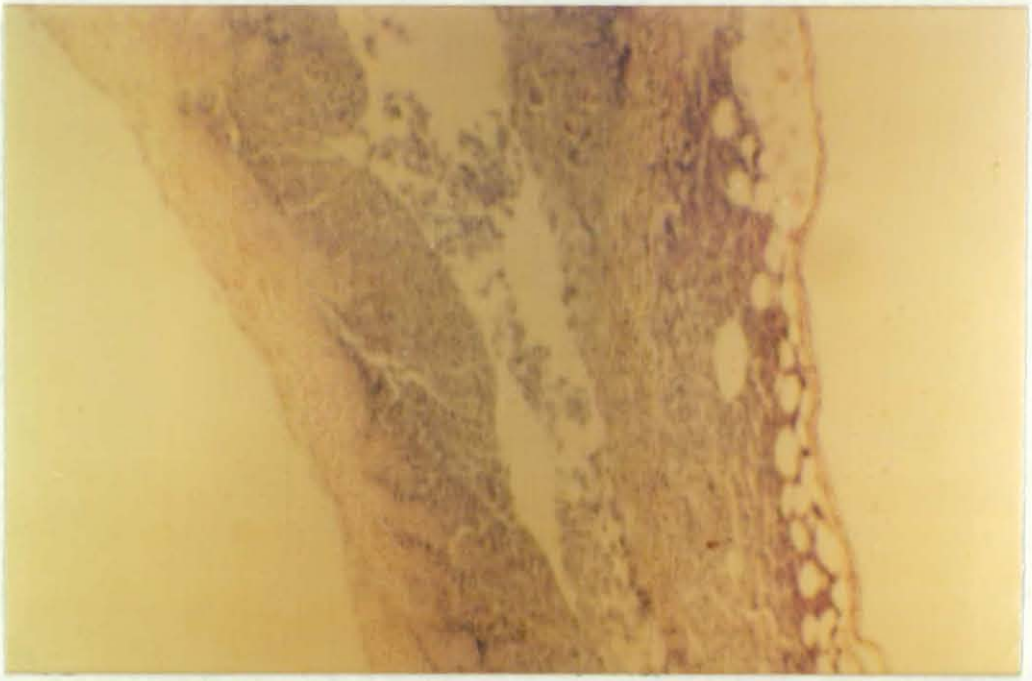
Fatal effect of high doses of B.fragilis (NCTC 8560) and B.asaccharolyticus (W50) injected separately into the same rabbit (10^{10} cells.ml^{-1})

Fig. 42c

Mixed anaerobic infection caused by 10^5 cells.ml^{-1} of E.coli, St.faecalis, Cl.sporogenes and B.asaccharolyticus (W50)

Fig. 43a: Massive necrosis and disintegration of muscles fibres by B.fragilis and B.asaccharolyticus infection. H & F stain (X10)

Fig. 43b: Deposition of stained granules unknown in origin in all heavily necrotic areas of the abscess. H & E stain (X400)



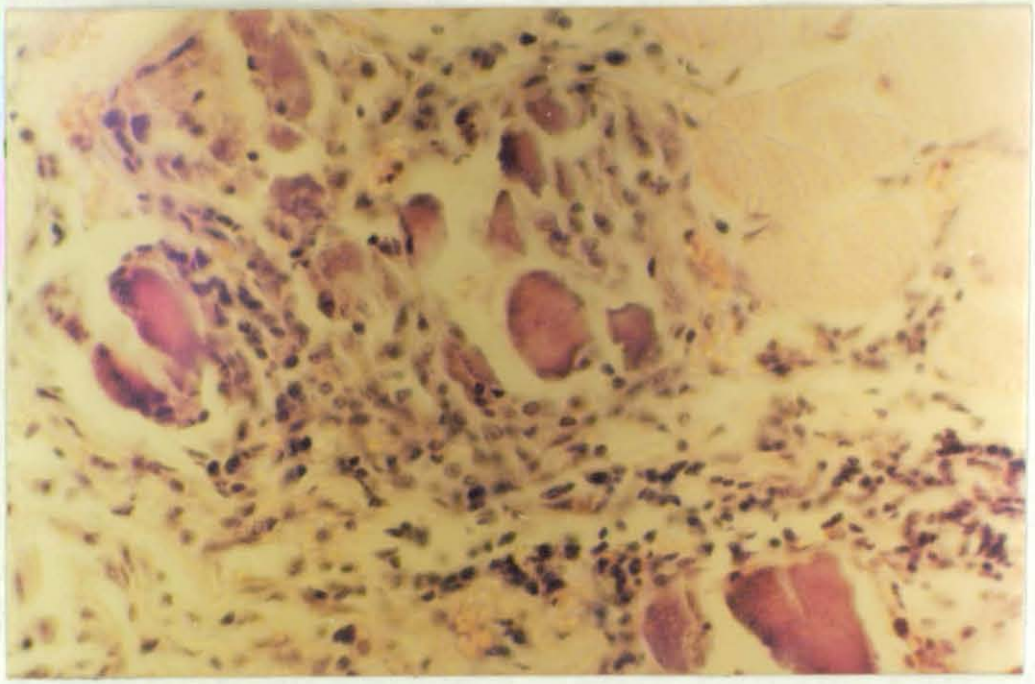
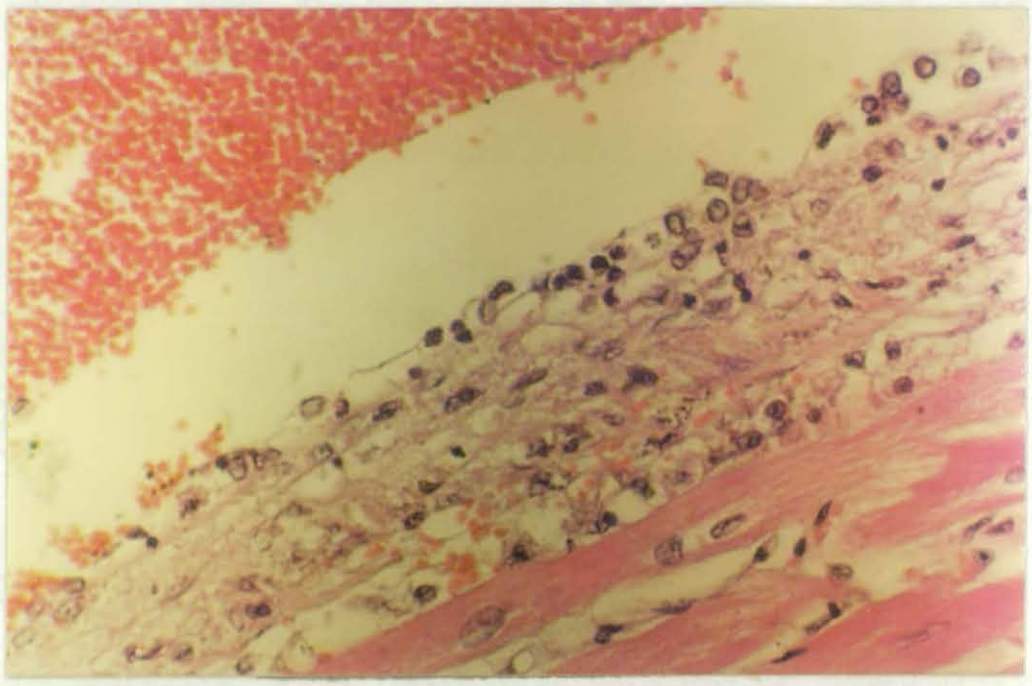
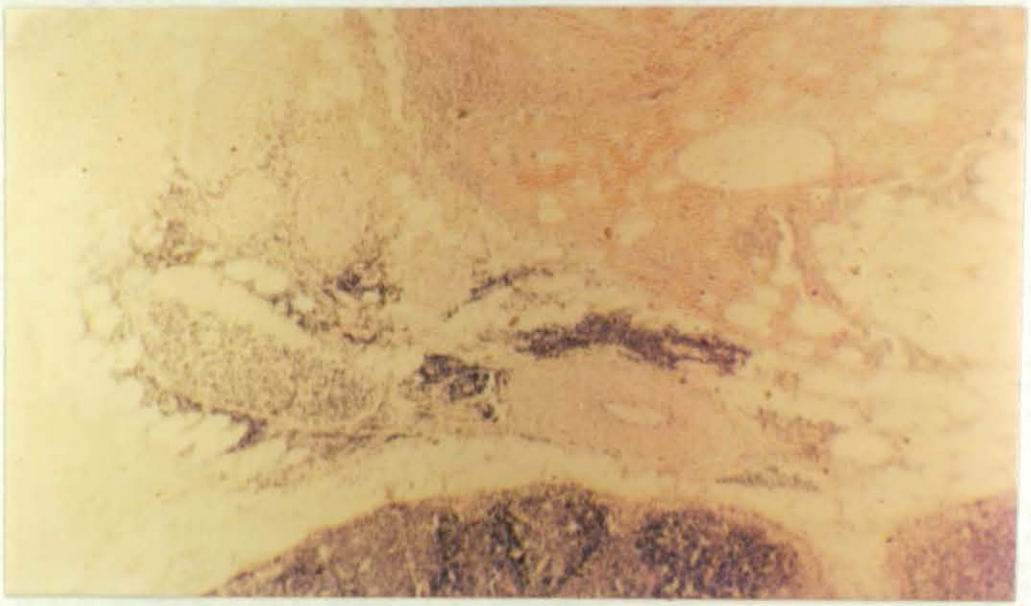


Fig. 44a: Cross section into the heart showing early stages of necrosis of the heart chamber epithelial layer. H & E stain (X400)

Fig. 44b: Cross section into the heart and its surrounding tissues showing no pathological changes to the area and the presence of normal lymphnode

Fig. 44c: Heavy cellular infilteration of the adipose tissue immediately adjacent to the heart. H & E stain (X400)





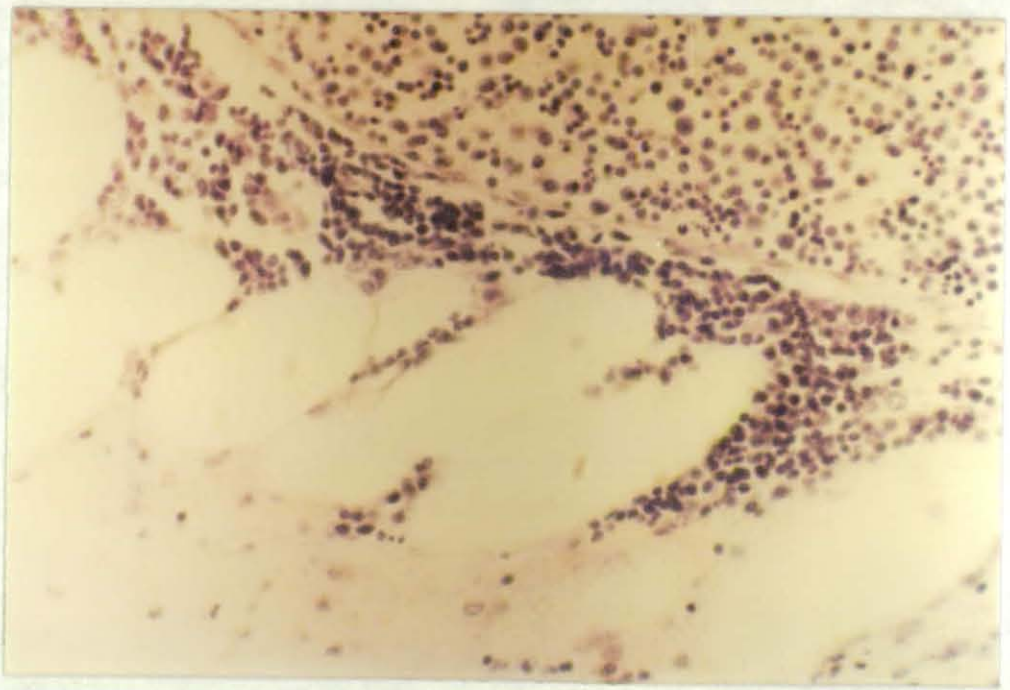
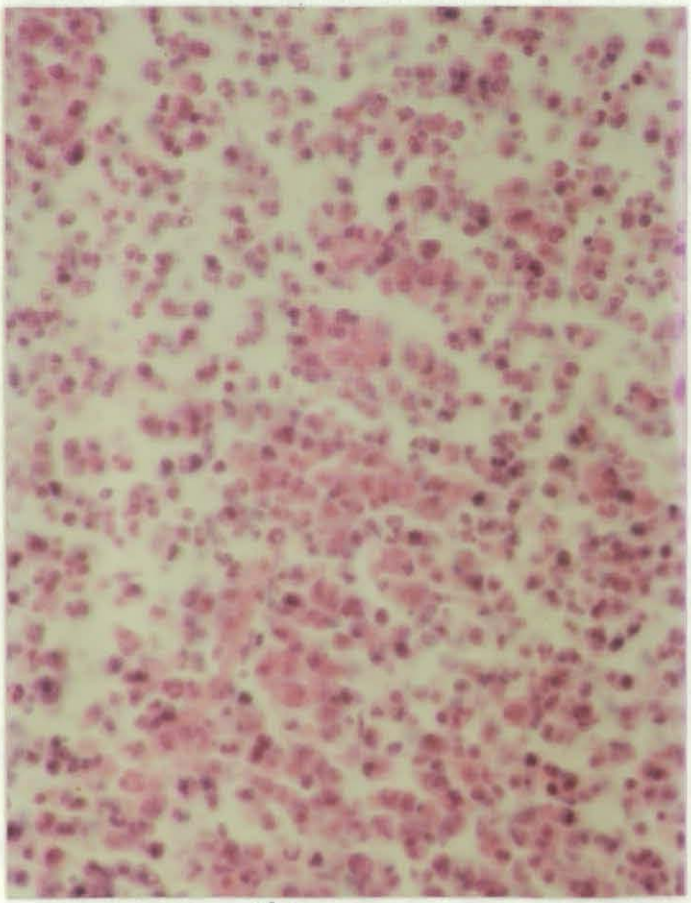


Fig. 45a: Cross-section into an abscess caused by mixed anaerobic infection showing normal epidermis layer and heavily infilterated dermis layers. H & E stains (X10)

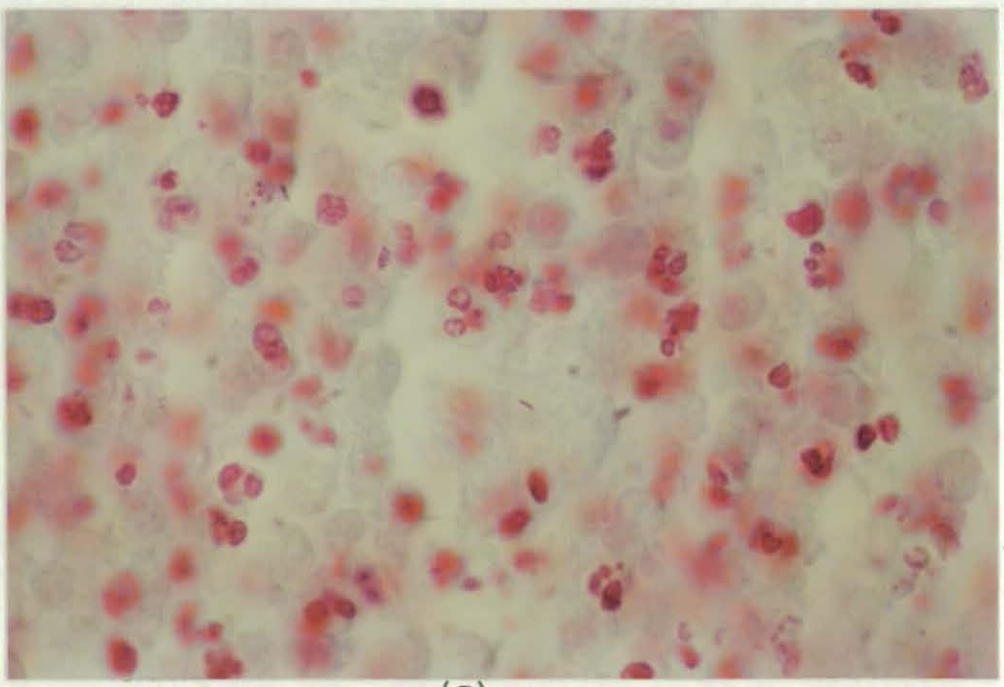
Fig. 45b: Higher magnification (X400) of the dermis showing masses of neutrophils with disintegrated muscle fibres.

Fig. 45c: Gram-stained section showing the presence of Gram-positive bacilli along with normal muscle cells and lymphocyte (X1000).



(b)

(a)



(c)

CHAPTER 4

DISCUSSION

Anaerobic bacteria enjoy a commensal existence with their host in areas such as gastrointestinal tract, oropharynx, vagina and skin. To propagate, these micro-organisms must abstain atmospheric oxygen, paradoxically the very element that is so necessary for the survival of their host.

As a working definition, Mcbee, et.al., (1955) suggested that "An anaerobe is a bacterium that requires a reduced oxygen tension for growth and fails to grow on the surface of solid media in 10% CO_2 in air (18% O_2)". Among these bacteria there is, however, a spectrum of oxygen sensitivity ranging from those for which oxygen is bactericidal at very low concentration, such as Methane bacteria, Spirochetes and certain strains of Clostridium, to those that tolerate exposure to air and for which atmospheric oxygen is bacteriostatic such as Bacteroides species (Loesche, 1969; Rosenblatt, et.al., 1973; Morris, 1975 and Tally, et.al., 1975). As a general rule, anaerobes associated with infective processes are relatively aerotolerant (Tally, et.al., 1975).

Various mechanisms have been suggested for the lethal effect of molecular oxygen (Haugaard, 1968; McCord, et.al., 1971; Morris & O'Brein, 1971 and Morris, 1975) and it seems likely that the toxicity of oxygen is multifactoral including the self-killing of the organisms by its formation of peroxides, by-products of the bacterial metabolism of oxygen, and the effect of atmospheric oxygen on the

oxidation-reduction potential (Eh) of the microenvironment of anaerobes.

The increasing recognition of Bacteroides as human pathogens has led to marked improvements in their identification. Identification may be defined as "The assignment of unidentified organisms to a particular class in a previously made classification" (Sneath, 1978a). Identification methods employed vary considerably ranging from physiological tests such as antibiotic resistance (Sutter, et.al., 1972), Serology (Lambe, 1974; Lambe & Jerris, 1976), tolerance to dyes and bile salts (Holbrook & Duerden, 1974; Duerden et.al., 1976) to chemotaxonomic methods, which include chromatography (Holdeman, et.al., 1977)(8),(9), electrophoresis (Swindlehurst, et.al., 1977)*(1),(10),(11), cell wall analysis (Hofstad & Kristoffersen, 1971a; Kasper, 1976), lipid analysis (Shah & Collins, 1980)(8) and DNA base composition (Shah, et.al., 1976 and Johnson, 1973),(1),(2),(4),(5),(6),(7).

The identification of Bacteroides using conventional methods requires several biochemical tests (Table 2 and 3) including some of primary importance such as pigment production, growth in 20% bile, catalase and carbohydrate fermentation tests. The latter are of discriminatory value between B.fragilis groups (group I) and the black pigmented group (group II). Conventional methods were found to be time consuming, required considerable preparation and, in addition, some inconsistencies were encountered in carbohydrate fermentation tests. This may be partially explained by the presence of peptone and yeast extract in the test medium which under anaerobic conditions, may support a variety of reactions; thus the end - products

often become a balance between acid production from the breakdown of carbohydrates and alkali production from the breakdown of protein constituents (Schreckenberger & Blazevic, 1976). In a rapid test system, such as ApI 20A, multiple reactions were not likely to occur, since the system was devoid of nutrients except for the single carbohydrate being tested.

Rapid testing of anaerobic bacteria has been reported as far back as 1941 by Reed & Orr. ApI 20A is a standardised system which allows the rapid and reliable performance of a large series of biochemical tests coupled with computer assisted identification. The results obtained with ApI 20A are more consistent on repeated testing of the strains, rapid and easily performed. As a result it was preferable to the conventional methods.

Table 6 summarizes the biochemical tests and their results that were found to be of discriminatory value in the identification of Bacteroides. The results show the genus Bacteroides to have species with a wide range of behaviour towards carbohydrates: B.fragilis groups and B.ruminicola with high affinity for carbohydrates, B.asaccharolyticus with its inability to utilize carbohydrates and B.melaninogenicus having a relatively intermediate position. Several authors have linked this behaviour with the environment in which these organisms normally live. B.fragilis and B.ruminicola occupy a niche in the large intestine or the rumen where carbohydrates are readily available (Bryant & Robinson, 1962 and 1963; Hungate, 1966). On the other hand B.melaninogenicus and B.asaccharolyticus predominate in

the periodontal pocket where mainly amino acids and peptides are available (Brill, 1959; Makinen, 1966 and Newman, 1979).

A few carbohydrate tests in Table 5 seem to be of limited use for presumptive identification, due to the variation encountered on repeated testing of the same strain. These were cellobiose, trehalose and arabinose for B.melaninogenicus ss. melaninogenicus, arabinose for B.runinicola ss.brevis, cellobiose and aesculin for B.distasonis and trehalose for B.melaninogenicus ss. intermedius.

The identification and classification of Bacteroides has been greatly facilitated by the use of gas chromatography. Among the numerous application of gas chromatography in biological sciences, the analysis of volatile and non-volatile fatty acids produced during the growth of anaerobic bacteria represents an essential method in the present system of classification for anaerobes (Holdeman, et.al., 1977). The impact of the method, however, varies according to the anaerobic taxon involved. Only a few species, e.g. Peptostreptococcus anaerobius, Eubacterium limosum and Megasphaera elsdenii can be unmistakably identified by their acidic product alone. Closely related species such as B.fragilis, B.thetaiotaomicron and the other taxa in the saccharolytic bacteroides group may show the same fermentation pattern. As the fermentation pattern of anaerobic bacteria may be assumed to be more stable than the utilization of single carbohydrates (which

might be changed by the mutation of a single enzyme), analysis of metabolic end products by GLC can generally be considered a taxonomic character of prime importance. On the other hand, an anaerobic bacterial strain cannot be properly identified without due regard to other taxonomic criteria such as biochemical, physiological and morphological properties.

The SCFA produced by large range of anaerobic bacteria have been studied extensively by the VPI group (Holdeman, et.al., 1977). Their studies used the chromatographic analysis of these acids in discriminating between wide groups of anaerobic bacteria. The VPI manual showed and stressed the importance of SCFA profiles (qualitative) in distinguishing between different genera and species of anaerobic bacteria. All the work that followed its publication concentrated on the qualitative aspects of chromatographic analysis of these acids with little attention to its quantitative potential. Most of these authors used only a semiquantitative method (as suggested by the VPI manual) to calculate the amounts of these acids produced by anaerobes. The use of an internal standard is more accurate and can account for a lot of variations occurring throughout the extraction and chromatographic analysis.

The one aspect of this study was to concentrate on the quantitative analysis and to assess whether this approach can contribute any information towards differentiation of heterogeneous strains (such as, B.fragilis, NCTC 8560, 9343 and B.asaccharolyticus, W50 and B536; Shah & Collins, 1980), or the closely related Bacteroides strains (B.fragilis, B.ovatus, B.vulgatus, B.thetaiotao-

micron and B.distasonis) which had previously been established as being unidentifiable from their fatty acid profile (Cato & Johnson, 1976). Additionally it was intended to assess the reliability and repeatability of the method as repeatability though stated previously to be excellent from culture to culture within strains (Moore, 1970), this had not been supported with figures. For this purpose strictly standardised cultural and chromatographic conditions were employed with the use of internal standards for more accurate estimation of these acids.

The B.fragilis group produces A, P, L, and S as the main products of fermentation. Qualitatively they all have a similar pattern but this pattern can differentiate them from the black pigmented Bacteroides where, B.asaccharolyticus and B.melaninogenicus ss. macacea produce A, P, IB, B, IV, L and S as the main fermentation products and B.melaninogenicus ss. intermedius which produces A, IV, L and S. B.fragilis also differs from B.ruminicola ss. brevis from the SCFA profiles since the latter subspecies produces A,L, and S as the main products. This profile is similar to B.melaninogenicus ss. melaninogenicus, but B.ruminicola can be differentiated from B.melaninogenicus by its failure to produce black pigment on blood agar and by biochemical tests.

The types and relative amounts of these acids are similar from strain to strain (whether fresh, isolated or reference strain) and they are produced in the medium in distinctive molar proportions (Moore, 1970 and Bohannan, et.al., 1978). The types and relative proportion of these acids have been shown to be sensitive to many factors such

as medium composition and to length of incubation (Mayhew, et.al., 1975 and Bohannan, et.al., 1978), also the amount of L acid produced in combination with other acids may be sensitive to haemin content of the medium. In addition the total amount of non-volatile fatty acids is significantly greater in medium containing haemin and Vit.K_1 (Holdeman, et.al., 1977). The presence of monosaccharide was also found to affect the production of SCFA by inducing not only a large quantity of end products and at a faster rate, but also favour increased S production in relation to A (Mayhew, et.al., 1975).

Table 12 shows the qualitative and quantitative differences between the thirteen Bacteroides strains tested in CMG medium. The effect of incubation time on the ratio of four acids (A, P. L and S) was noted at 24, 96 and 168 h. All ratios were obtained in relation to A acid. The ratios of L and S to A show a slight decrease with increase in incubation time, with all strains showing a similar pattern. Though the ratio of P to A was variable, the overall variation was slight so it is possible to consider the ratio constant over the different incubation times. Table 12 also shows some qualitative differences between the strains such as, the production trace amount of B and IV by both strains of B.fragilis which was not detected in B.ovatus and B.vulgatus, B.thetaiotaomicron. Similarly B.ruminicola ss. brevis produced a trace amount of IV. As for B.melaninogenicus ss.intermedius, P and IB were produced in trace amounts. The black pigmented species seem to be well differentiated qualitatively and quantitatively from

Table 12: The ratios and types of SCFA produced by thirteen strains of Bacteroides at different incubation time

Organisms/ Strain No.	Incubation Time (hr)	Relative Proportion of Fatty Acid Detected						
		A	P	IB	B	IV	L	S
B.fragilis NCTC 8560	24	1	0.56	-	Tr.	Tr.	0.73	1.37
	96	1	0.50	-	Tr.	Tr.	0.71	1.39
	168	1	0.42	-	Tr.	Tr.	0.66	1.35
B.fragilis NCTC 9343	24	1	0.43	-	Tr.	Tr.	0.82	1.68
	96	1	0.49	-	Tr.	Tr.	0.74	1.47
	168	1	0.48	-	Tr.	Tr.	0.73	1.39
B.ovatus, ATCC 8483	24	1	0.38	-	-	-	0.61	2.95
	96	1	0.58	-	-	-	0.51	2.37
	168	1	0.53	-	-	-	0.51	2.48
B.vulgatus, WBM137	24	1	0.63	-	-	-	0.72	5.30
	96	1	0171	-	-	-	0.54	3.12
	168	1	0.72	-	-	-	0.48	2.99
B.distasonis, ATCC 8503	24	1	0.49	-	-	Tr.	0.32	2.31
	96	1	0.45	-	-	Tr.	0.32	2.43
	168	1	0.37	-	-	Tr.	0.30	2.48
B.thetiaotao-micron, NCTC 10582	24	1	0.64	-	-	Tr.	1.04	3.81
	96	1	0.86	-	-	Tr.	0.85	3.17
	168	1	0.83	-	-	Tr.	0.80	3.08
B.ruminicola ss. brevis,NP333	24	1	-	-	-	Tr.	1.44	5.09
	96	1	-	-	-	Tr.	1.12	3.74
	168	1	-	-	-	Tr.	1.06	3.71

Table 12, continued

B.melanino-genicus ss. melanino-genicus, VPI9085	24 96 168	1 1 1	- - -	- - -	- - -	- - -	1.42 1.16 1.07	2.95 2.60 2.55
B.melanino-genicus ss. intermedius, T588	24 96 168	1 1 1	Tr. Tr. Tr.	Tr. Tr. Tr.	- - -	0.67 0.74 0.74	0.60 0.49 0.49	4.56 4.15 4.15
B.melanino-genicus ss. macacae, ATCC33141	24 96 168	1 1 1	1.33 1.27 1.22	0.13 0.16 0.15	02.6 0.30 0.29	0.40 0.46 0.44	0.32 0.40 0.37	0.36 0.53 0.55
B.asaccha-ralyticus (a) W50	24 96 168	1 1 1	1.36 1.20 1.10	0.12 0.20 0.17	0.38 0.43 0.46	0.67 0.68 0.69	0.43 0.46 0.41	0.86 0.84 0.81
(b) B537	24 96 168	1 1 1	1.60 1.22 1.20	0.27 0.25 0.23	0.55 0.50 0.49	0.99 0.67 0.67	0.57 0.47 0.48	1.08 1.07 1.02
(c) B536	24 96 168	1 1 1	1.08 1.23 1.25	0.22 0.36 0.31	0.39 0.59 0.57	0.60 0.78 0.75	0.74 0.64 0.67	1.38 1.30 1.31

A = acetic acid, P = Propionic acid, IB = isobutyric acid,
B = butyric acid, IV = isovaleric acid, L = lactic acid,
S = succinic acid

each other, as they vary in their pattern and ratio of SCFA production where B.melaninogenicus ss.melaninogenicus produce A, L and S, whereas B.melaninogenicus ss.intermedius produces A, IV, L and S. A more complex pattern is produced by B.melaninogenicus ss.macacae and B.asaccharolyticus where they share a similar profile but different ratios. The ratios of P and IB in subspecies macacae are similar to that of B.asaccharolyticus whereas IV and S are produced at a lower rate. The representative strains of B.asaccharolyticus studied showed W50 to be less productive then B536 and B537 (which have a relatively similar ratio of production).

All strains listed in Table 12 showed a quantitative difference from each other. The most important difference is the ratio of S which when considered with that of L and P provides a mean of differentiation of the closely related B.fragilis species. For further information on this point, the ratios obtained at different incubation times (12 h - 168 h) for each species were averaged and listed in Table 13, part A. The results shows B.fragilis, B.ovatus, B.distasonis, B.thetaiotaomicron and B.vulgatus to have different ratios although they have a similar SCFA profile. The ratio of these acids are sufficiently different with different species to facilitate their identification. Table 13, part B, show a comparison of the ratio (average) of acids produced in two different media CMG and PYG, for four species. The figures clearly show a similar ratio of production in the two media; it also shows a slightly higher production rate for B.fragilis, NCTC 9343 compared with NCTC 8560. However, the ratios for the same species

Table 13: Average ratio of S.C.F.A. for some Bacteroides species

Part A: Average ratio in CMG

Organisms/Strain No.	Fatty acids detected			
	A	P	L	S
B.ovatus,ATCC 8483	1	0.48	0.53	2.81
B.Vulgatus, WBM 137	1	0.67	0.59	3.98
B.distasonis, ATCC 8503	1	0.44	0.32	2.55
B.thetaiotaomicron, NCTC 10583	1	0.77	0.96	3.56
B.ruminicola ss.brevis, NP 333	1	-	1.19	4.20
B.melaninogenicus ss.mel. VPI 9085	1	-	1.27	2.80
B.melaninogenicus ss. intermedius T 588	1	-	0.55	4.27
B.asaccharolyticus, B 537	1	1.29	0.49	1.07

Part B: Comparison of the average ratio in CMG & PYG

Organisms/Strain No.	Media	Fatty Acids Detected			
		A	P	L	S
B.fragilis, NCTC 8560	CMG	1	0.52	0.72	1.42
" " " "	PYG	1	0.46	0.68	1.53
B.fragilis, NCTC 9343	CMG	1	0.47	0.80	1.59
" " " "	PYG	1	0.47	0.75	1.62
B.melaninogenicus ss. macacae ATCC 33141	CMG	1	1.28	0.37	0.49
" " " "	PYG	1	1.28	0.35	0.45
B.asaccharolyticus, W50	CMG	1	1.26	0.46	0.84
" " " "	PYG	1	1.23	0.46	0.85

A = acetic acid, P = propionic acid, L = lactic acid

S = succinic acid

in different media are the same.

The SCFA were surveyed in two growth media, CMG and PYG. CM is a traditional liquid medium with meat particles that has been used for growth of anaerobic bacteria since the 19th century. However, it was not until 1916 that Robertson formulated a medium based on bullock's heart that formed the basis of the modern cooked meat medium. Lepper & Martin (1930) investigated the characteristic of CM medium and found that the meat particles absorb oxygen due to auto-oxidation of unsaturated fatty acids and was catalysed by haemin. In addition to the removal of oxygen from the medium, a glutathione system in the meat lowers the redox potential.. All strains used in the present study (except B536) showed excellent growth in this medium and as the amount of growth may effect the total amount of SCFA produced (Moore, 1970), the use of CMG was preferred to pre-reduced PYG in which growth of the strains tested was good to moderate but which was not as consistent as that achieved using CMG.

The total acid production ($\mu mole.ml^{-1}$) of twelve strains of Bacteroides in CMG was plotted against different incubation time (12, 24, 96 and 168 h) and is shown in Fig (25). The results divided the strains into three groups. Group one comprises the highly productive strains (range 140 - 190 $\mu moles.ml^{-1}$) which include: B.melaninogenicus ss. intermedius (T588) B.distasonis (ATCC 8503), B.vulgatus (WBM 137) B.ovatus (ATCC 8483) and B.thetaiotaomicron (NCTC 10582). This high rate of production was caused by high amount of S produced (Table 12). Group two includes the moderately productive strains (range 110 - 135

μmoles.ml^{-1} namely B.melaninogenicus ss. macacae (ATCC 33141), B.asaccharolyticus (W50 and B536) and B.fragilis(NCTC 9343). The third group includes those strains which are least productive (range 75 - 100 μmoles.ml^{-1}) namely B.fragilis (NCTC 8560), B.ruminicola ss.brevis (NP333) and B.melaninogenicus ss. melaninogenicus (VPI 9085). The results also show B.fragilis to be well differentiated from its closely related species. In addition B.fragilis, NCTC 9343, seems to produce a higher level of acids than NCTC 8560. A similar situation can be seen in Fig (25) where heterogenous strains of B.asaccharolyticus, B 537 and W50 show different total acid production with B 537 producing higher rate of SCFA than W50.

A comparison carried out on the effect of medium composition on the total acid production of five Bacteroides strains (Fig. 26) showed all strains to have a higher total acid production in CMG than in PYG. This could be partly attributed to the excellent growth of most strains in CMG. The largest difference was produced by B.melaninogenicus ss. macacae (ATCC 33141), and the smallest difference was shown by B.asaccharolyticus B536. By examining the amounts of fatty acid produced by different strains (Fig. 7 - 24) and looking at the SD of each mean value, it is apparent that the absolute value fluctuates by ± 20%. Similar results have been reported by other workers in this field, it is therefore better to express the results as acid ratios rather than absolute amounts. For this purpose acetic acid was used as a marker due to its production by all strains in moderate amounts.

It is of interest to note that after 12 h of incuba

tion in CMG medium a few Bacteroides species show a significant shift in their SCFA production in favour of S production which was found to moderate at 24 h of incubation possibly due to an increase in A production to that of S (Table 7). The situation might be explained by the view of Mayhew, et.al., (1975) who suggested that the presence of glucose in the medium causes a faster rate of production of SCFA and S production is favoured in relation to A.

In this system,the amount of A produced is of vital importance so care was taken to detect the amount initially present in both media (CMG and PYG). The acids detected were A, L and S acids and their amounts are listed in Table 8. These values were used to set the zero level for all strains in CMG and PYG.

During the present study, the levels of SCFA detected by some species were very low, the first case arose when dealing with B.distasonis, ATCC 8503, in CMG in which the amount of acids detected at 12 and 24 h of incubation was lower than that present in the broth medium, though the production increased to well above the basic level after 48 h. It seems possible that this species uses these SCFA present in the medium as a substrate, so these results were initially discarded. A similar result was, however, obtained with B.asaccharolyticus, B536, in CMG and in PYG media. The amount of A produced by B536 in CMG at 12 h was 2.53 $\mu moles.ml^{-1}$ compared with the amount of A present in CMG broth which was 4.19 $\pm$ 1.01 $\mu moles.ml^{-1}$. The production increased to 6.16 $\mu moles.ml^{-1}$ at 24 h which is still very low when compared with the amount of A present

in the broth. The level of A increased gradually from 48 h and upwards till the end of incubation period. These figures tend to support the view that this strain used the SCFA present in the medium. 'A' acid was not the only one affected and B_{536} produced all acids at a much lower rate than the other strain (B537) which showed greater and more consistent pattern of production.

The amount of A produced by all strains is of great significance in the system of ratios used. Consequently, the result for B_{536} in CMG and PYG could not be used for comparison purposes. These results may be attributed to a reduction in total acid production due to poor growth (Moore, 1970), whereas if one acid had been affected it could have been for various reasons other than growth such as inefficient extraction procedure or to column contamination.

The ability of these species to use the acids present in the medium was evaluated using C-14 labelled sodium acetate. Two representative species, B.distasonis, ATCC 8503, and B.asaccharolyticus, W50, were chosen. Preliminary work with 2-^{14}C sodium acetate showed a very small amount of labelled CO_2 detected by these two species (Table 9). The situation was improved greatly with the use of 1-^{14}C sodium acetate where high level of labelled CO_2 was detected at 24 h and remained the same up to 72 h of incubation (Table 9). It was of interest to note that the high level of labelled CO_2 detected using 1-^{14}C suggest that C_1 and not c_2 goes directly into the formation of CO_2 by the test strain, indicating that both species were capable of using acetic acid present or added to the medium.

It is apparent that the first 24 h of Bacteroides growth cycle shows maximum growth rate (Fig. 27), maxium SCFA production (Figs. 7 - 24) and highest rate of uptake of 1-^{14}C, (Table 9) with the formation of high levels of labelled CO_2. These results are in agreement with those of Mayhew, et.al., (1975). Preliminary work with Bacteroides species presented problems related to their growth which was overcome by time and experience. However, a few factors were noted which could affect their growth, these include:

(1) The use of moderately freshly prepared additives (filter sterilized Vit.K and haemin solutions were discarded if not used within 4 weeks of preparation).

(2) The use of fresh whole blood which was found to enhance growth especially in the absence of other additives. The use of oxidated blood or chocolate agar did not support good growth and affected pigment formation.

(3) The type of anaerobic jar was found to affect growth: BBL plastic jars supported good growth of the non-pigmented species (except B.ruminicola) but a modification was necessary for the growth of the black pigmented Bacteroides and B.ruminicola. The modification involved the fitting of evacuation valves.

(4) The constant use of enriched media in routine practice may render the growth of these species dependent on the presence of these additives

(auxotrophy) in a readily available form.

(5) The use of faulty gas-pak generators envelopes: Ferguson, et.al., (1975) noted that sometimes these envelopes provided little or no CO_2 due to the fact that borohydride used to produce hydrogen causes a rise in pH which lowers the amount of CO_2 released by the action of the water on citrate bicarbonate in the mixture. This may present a problem as CO_2 has been shown to be an important growth factor for Bacteroides species (Watt, 1973).

The method followed in this thesis for the growth of Bacteroides species involved the use of freshly prepared blood agar with no additives and the use of an anaerobic jar employing, evacuation and replacement technique (Dowell & Hawkins, 1976) as well as the gas-pak system. This method gave an excellent recovery of all strains.

There have been few reports in the literature on factors that might affect the growth of these species (especially the black pigmented Bacteroides) which is surprising as the growth and maintenance of Bacteroides species must present a constant worry to many workers in this field.

In general the maximum production of SCFA for most strains seems to coincide with maximum growth which took place from 0 - 48 h as can be seen from the growth curve of two representative strains, B.asaccharolyticus, W50, and B.fragilis, NCTC 8560, in PYG (Fig. 27). CMG was not suitable for growth assessment due to the presence of

extraneous matters which made the OD reading extremely high and variable.

Many authors have described the effect of incubation time and media on the relative proportions and rates of production of SCFA (Mayhew, et.al., 1975; Bohannan, et.al., 1978 and Holdeman, et.al., 1980) when in fact they were describing the effect of these factors on the absolute value of SCFA detected. The results of this project show that the two statistics (absolute value and rate of production) vary considerably and as a result each one should be considered separately. When dealing with absolute values (Figs. 7 - 24) these results were in complete agreement with those of the above authors in that the growth medium and incubation time were found to affect the amounts of fatty acids detected. When results were expressed as a ratio to acetic acid, the ratios was almost unaffected by incubation time (Table 12) and when these ratios were averaged the results obtained showed the differentiating ability of this system between all strains (Table 13, part A).

For comparative purposes the data from this study and that derived from all the work available in the literature on the quantitative analysis of SCFA produced by Bacteroides species is listed in TAble 14. The purpose of this table is to provide an easy and quick method for comparison of all data and also includes some experimental informations.

The study to follow the VPI (Holdeman, et.al., 1974) qualitative work on Bacteroides was the quantitative study of Mayhew, et.al., (1975) which is the principal publication on the effect of time and growth medium on

SCFA produced by B.fragilis. Their data detected at 72 h of incubation and presented in Table (14) is the mean of five strains of B.fragilis (four B.fragilis ss.fragilis and one ss.vulgatus). The results of the comparison show a high rate of production of all acids using the present technique. The biggest difference found was in the amount of A, P and S respectively with a significant difference in the total acid production between the two studies. The repeatability (SD) of their technique was in the range of 5 - 17%.

Gas solid chromatography (GSC) had been preferred by many authors as it does not require sample preparation, permits immediate sample analysis and both VFA and n-VFA can be analysed using the same column. Lindner & Marcelis (1978) used this technique in the semiquantitative analysis to differentiate some clinical isolates belonging to the genus Bacteroides. They grew their strains on selective medium containing vancomycin. The results of the comparison shows a significantly higher rate of the individual acids as well as the total acid production from their recorded data.

The differences between the two studies could be partly attributed to the use of selective medium which does not support good growth as does CMG and partly to the semi-quantitative GSC which is not as sensitive as the quantitative GLC employed in this study.

A similar situation occurred when comparing the results of this study with those of Bohannan, et.al., (1978) who also used a semiquantitative GSC for analysis

of fermentation end products of anaerobic bacteria. The results of the comparison show quantitative GLC to be more sensitive and accurate in detecting SCFA of metabolic fermentation. Bohannan, et.al., (1978) consider the production of 3.1 $\mu moles.ml^{-1}$ by B.vulgatus to be a significant amount, yet this is small in comparison with the 77.14 $\mu moles.ml^{-1}$ detected in this study. They also suggested that the amount of acids detected in two different media (CM and PYG) were medium dependant (Table 14). Their reported difference, however, is small enough to be easily encountered on repeated testing of the same strain in the same medium; additionally the incubation procedure for CM and PYG were different making comparison difficult. Strains grown in CM were incubated aerobically whereas strains in PYG was incubated anaerobically.

Deacon, et.al., (1978) used GSC for identification of large number of Bacteroides species. There is some correlation between their findings and that derived from this study on certain points (Table 14). For example the production of A & S by B.fragilis, NCTC 8560, is similar, though differences are apparent in the level of P and L between the two sets of data. B.thetaiotaomicron, NCTC 10582, also showed a similar A level with their strain but significantly higher rates were noted for the other acids. As for the rest of the species studied by Deacon, et.al., (1978) a higher rate of production was recorded using this technique. Table 14 also shows the quantitative variations by three reference strains, B.ruminicola ss. brevis, NP333 (formerly identified as B.oralis), (1), (3), (4), (11), B.melaninogenicus ss. intermedius and B.melaninogenicus ss. melaninogenicus analysed by Deacon, et.al., (1978) on two

separate occasions. Their results show large differences between the two repeats for the same strain and in the same medium. This level of difference is often encountered and close results are often only possible within replicas but not for repeats. A similar argument was used earlier in discussing the results of Bohannan, et.al., (1978) in that variation between CM and PYG may be insignificant as much bigger differences may be encountered on repeated testing of the same strain in the same medium.

The variations encountered in this study (repeatability) was represented as SD value and listed with the actual value obtained.

B.melaninogenicus ss. macacae is a new species proposed by Slots & Genco (1980) to accommodate a strain of Gram-negative,catalase producing rods which produce black colonies on blood agar plates. For the identification and classification of these organisms, they employed biochemical tests, GLC analysis of metabolic end products and serology.

The results show a much higher rate of production of A and P, compared with the results of this study, with a small amount of IB, IV and S, with no L detected. It has been shown from previous results (Fig. 25) that B.melaninogenicus ss. macacae belong to the moderately productive strains so it is possible to consider the amount of A and P detected by Slots and Genco (1980) to be extremely high for 24 h of incubation. The highest amount detected throughout this study was 41.56 $\mu moles.ml^{-1}$ which was by B.distasonis, ATCC 8503 and after 96 h of incubation.

As for P the highest amount detected was 41.15 μmoles.ml^{-1} by B.melaninogenicus ss. macacae, ATCC 33141, after 96 h of incubation.

Another study based on the findings of Bohannan, et.al., (1978) is the recent publication of Turton, et.al., (1983) who analysed the effect of eight growth media on VFAs profile of ten anaerobic bacteria, namely, B.fragilis, B.melaninogenicus ss. intermedius, four Clostridium species, two Fusobacterium species and two Peptococcus species. The media examined were PYG, four CM media from different manufacturers, fastidious anaerobe broth (FAB), thiol broth and thioglycollate medium.

Comparison of the two datas (Table 14) showed the insensitivity of their technique in detecting the VFAs produced by the two Bacteroides species. It was also noted that the total amount of VFAs detected in their study for CMG and PYG broth were found to be 4.19 ± 1.01 μmoles. ml^{-1} and 3.11 ± 1.12 μmoles.ml^{-1} respectively (Table 14) thus it can be said that their B.fragilis strain in PYG and at 72 h of incubation has failed to produce any detectable VFAs and the same apply for their B.melaninogenicus ss. intermedius strain in CM (Difco)..

It was interesting to note that they have failed to grow the two strains in CM (Oxoid) which we found it to represent an excellent and quick method for the growth of all Bacteroides species.

The low level of VFA detected by Turton, et.al., (1983) could be attributed to their extraction procedure or to the storage of the samples.

Table 14: Comparative study of the data available on gas chromatography of Bacteroides species with the data from the present study

Author/Year	Species/ Subspecies	Strain No.	Media	Special Remarks	Incubation Time (h)	Fatty Acids Produced in µmoles.ml^{-1}							Total Acid Produced in µmoles. ml^{-1}
						A	P	IB	B	IV	L	S	
Mayhew, et.al 1975	B.fragilis		CMG	quantitative G.L.C.	72	15±2.6	2±0.6	0.1±0.05 0.03	-	0.8±0.4	2.2±0.87	46±2.44	66.1
			PYG	=	72	10±1.3	2±0	0.1±0.02	<0.1	0.1±0.05	-0.6±0.5	24.4±0	36.7
Present Study	B.fragilis	NCTC 8560	CMG	quantitative G.L.C.	72	24.78 ±4.8	13.31 ±3.10	-	0.079 ±0.02	0.871 ±0.39	17.59 ±2.25	36.35 ±4.5	92.68
		=	PYG	=	=	24.06 ±3.10	10.660 ±0.94	-	0.078 ±0.005	0.099 ±0.001	16.559 ±2.65	35.39 ±6.99	86.84
		NCTC 9343	CMG	=	=	34.16 ±6.49	16.49 ±4.50	-	0.409 ±0.09	0.576 ±0.11	24.99 ±4.10	48.17 ±5.69	124.79
		=	PYG	=	=	29.29 ±3.40	14.99 ±2.99	-	0.068 ±0.002	0.166 ±0.043	20.38 ±4.8	42.14 ±3.3	107.03
Lindner & Marcelis 1978	B.vulgatus	NCTC 10583	HVL	Semi-quantitative GSC	168	8.8	2.4	-	-	-	-	9.1	20.3
	B.fragilis	NCTC 9344	HVL	=	=	9.4	2.5	-	-	-	2.1	8.4	22.4
Present study	B.vulgatus	WBM137	CMG	quantitative G.L.C	168	28.89 ±2.09	20.87 ±3.12	-	-	-	14.083 ±1.15	86.55 ±9.16	150.39
	B.fragilis	NCTC 9343	CMG	=	=	32.23 ±2.75	15.52 ±1.21	-	0.289 ±0.1	0.308 ±0.09	23.54 ±4.4	44.97 ±7.6	116.85
Bohannan et.al 1978	B.vulgatus		CM	semi-quantitative G.S.C.	24	0.8	3.2	-	-	-	0.9	3.1	8
					48	7.0	16.2	-	-	-	1.6	3.1	27.9
			PYG	=	24	4	2.3	-	-	-	2	4.2	12.4
					48	8.8	2	-	-	-	2.8	5.4	19
Present Study	B.vulgatus	WBM137	CMG	quantitative G.L.C	24	14.48 ±2.67	9.209 ±1.96	-	-	-	10.51 ±1.91	77.14 ±12.5	111.33
					48	19.31 ±2.15	12.01 ±3.10	-	-	-	12.32 ±2.02	85.07 ±12.7	128.71
Deacon et.al 1978	B.fragilis	NCTC 8560	PPYSG	semi-quantitative G.S.C	48	22	1	-	-	<1	4	35	63
	B.thetaiotaomicron	NCTC 10585	=	=	=	21	<1	-	-	-	3	26	51
	B.ovatus	ATCC 8483	=	=	=	21	1	-	-	<1	3	33	58
	B.distasonis		=	=	=	4	4	<1	1	<1	7	22	39
	B.vulgatus		=	=	=	5	5	<1	-	<1	17	18	47

Table 14, continued.

Deacon et.al 1978 contd.	B.oralis	NP333	=	Study I	=	19	2	-	-	1	3	36	61
			=	Study II	=	10	1	-	-	-	-	22	33
	B.melaninogenius ss.intermedius		=	Study I	=	20	1	-	-	-	-	25	46
				Study II	=	14	-	-	-	-	-	27	41
	B.melaninogenicus ss.melaninogenicus		=	Study I	=	26	2	1	0	1	12	38	80
			=	Study II	=	20	1	1	-	1	6	39	68
Present study	B.fragilis	NCTC 8560	CMG	quantitative G.L.C.	48	22.70 ±3.99	12.99 ±0.19	-	0.079 ±0.009	0.381 ±0.09	16.15 ±3.8	34.74 ±3.6	87.04
	B.thetaiotaomicron	NCTC 10582	=	=	=	22.21 ±2.40	16.05 ±2.22	-	-	1.223 ±0.21	21.46 ±4.5	83.32 ±9.50	144.26
	B.ovatus	ATCC 8483	=	=	=	34.30 ±5.10	13.3 ±2.6	-	-	-	17.692 ±2.2	79.76 ±7.69	145.05
	B.distasonis	ATCC 8503	=	=	=	34.04 ±4.8	14.13 ±3.66	-	-	0.979 ±0.088	9.325 ±1.15	79.43 ±15.2	137.90
	B.vulgatus	WBM137	=	=	=	19.31 ±2.15	12.01 ±3.10	-	-	-	12.32 ±2.02	85.07 ±12.70	128.71
	B.ruminicola ss.brevis	NP333	=	=	=	12.45 ±2.16	-	-	-	0.137 ±0.88	17.54 ±2.10	59.6 ±11.9	89.72
	B.melaninogenicus ss.intermedius	T588	=	=	=	26.14 ±3.99	0.418 ±0.11	0.590 ±0.09	-	17.41 ±2.50	15.14 ±2.99	114.98 ±10.23	174.67
	B.melaninogenicus ss.melaninogenicus	VPI9085	=	=	=	14.65 ±1.99	-	-	-	-	19.64 ±3.6	39.94 ±5.6	74.23
Slots & Genco 1980	B.melaninogenicus ss.macacae	ATCC 33141	PYG	quantitative G.L.C.	24	60	61	3	5	6	-	5	140
Present study	B.melaninogenicus ss.macacae	ATCC 331 1	PYG	=	24	15.993 ±2.99	23.46 ±3.15	1.221 ±1.06	3.144 ±0.69	4.064 ±0.99	6.882 ±1.99	8.998 ±2.02	64.30
	=		CMG	=	=	29.962 ±4.9	39.88 ±3.99	3.925 ±1.35	7.948 ±1.95	12.097 ±2.10	9.87 ±1.7	10.826 ±0.72	114.50
Turton et.al 1983	B.fragilis	NCTC 9343	PYG	quantitative G.L.C.	72	0.3	0.6	-	0.1	-	-	-	1
			CM-(lab-M)	=	=	17.1	5.5	-	-	0.2	-	-	22.8
			CM (Mast)	=	=	9	0.1	0.1	0.1	0.2	-	-	9.5
			CM (Difco)	=	=	13.7	23.7	0.1	0.1	1.9	-	-	39.5
			CM (Oxoid)	=	=	NO GROWTH							
	B.melaninogenicus ss.intermedius	NCTC 9336	PYG	=	=	10	0.4	-	7.4	-	-	-	17.8
			CM (Lab-M)	=	=	5.6	12.5	-	-	0.1	-	-	18.2
			CM (Mast)	=	=	4.9	4	-	0.1	-	-	-	9
			CM (Difco)	=	=	3.7	0.2	-	0.1	-	-	-	4
			CM (Oxoid)	=	=	NO GROWTH							

Table 14, continued

Present study	B.fragilis	NCTC 9343	PYG	quantitative G.L.C.	72	29.29 ±3.40	14.99 ±2.99	-	0.068 ±0.002	0.166 ±0.043	20.38 ±4.8	42.14 ±3.3	107.03
			CMG (cmoid)	-	-	34.16 ±6.49	16.49 ±4.50	-	0.409 ±0.09	0.576 ±0.11	24.99 ±4.10	48.17 ±5.69	124.79
	B.melaninogenicus ss.intermedium	T588	CMG (cmoid)	-	-	27.52 ±3.50	0.566 ±0.15	0.703 ±0.07	-	21.65 ±1.56	15.19 ±2.50	117.59 ±14.39	183.21

*NP333 reclassified as B.ruminicola ss. brevis CMG = cooked meat glucose, PYG = Peptone yeast glucose

A, Acetic; P, propionic; IB, isobutyric; B, Butyric; iv, isovaleric; L, lactic; S, succinic

Turton, et.al., (1983) study like all previous studies was interested with the profile of VFAs for anaerobes rather than applying a rigid and strict procedures for maximum and efficient detection of these acids and we agree with their suggestion that fermentation profiles not only vary with growth medium but also the differences observed with a single strain can be shown statistically to be at least as great as differences observed between different strains separately grown in the same medium. They also recommended that laboratories that use VPI manual (Holdeman, et.al., 1977) should adhere to VPI medium and procedures, otherwise each laboratory should create their own library of fermentation profiles and carefully test each new batch of medium using standard anaerobic inocula.

It is reasonable to add that although big variations were encountered between different authors (Table 14) the use of the ratio system employed in this study might help more interlaboratory reproducibility under strict chromatographic and growth procedures which should be followed for maximum detection of these metabolites.

All authors listed in Table 14 measured the SCFA produced at different incubation times ranging from 24 - 168h. Bohannan, et.al., (1978) stated that the pattern of these acids only become evident after 24 h which disagrees with the finding of the present study which can be seen in Figs. (7 - 26) and Table 7 and shows that at 12 h of incubation SCFA pattern for all species is already established, which is extremely useful for differentiation purposes and diagnosis of the Bacteroides species.

The variations that are apparent in Table 14 between the results obtained in this study and other workers contributed to the use of a semiquantative technique for the detection of SCFA without the use of an internal standard which is very critical for more accurate method of estimation, as well as the chromatographic technique employed e.g. GSC or GLC.

By examining and comparing all data in Table (14) it is possible to conclude that GSC could well be reliable and rapid but not necessarily as sensitive as had been suggested. GSC provide a good method for qualitative work bearing in mind that some qualitative difference can help differentiate certain Bacteroides species. Shah, et.al., (1976) using GSC failed to detect P for B.melaninogenicus ss. intermedius (T588) which was found to be produced in small amounts at concentration of 0.418 $\mu moles.ml^{-1}$ for the same strain at 48 h in CMG. They also failed to detect L produced by. B.melaninogenicus ss. melaninogenicus (VPI 9085) which was found to be 19.64 $\mu moles.ml^{-1}$ for the same strain at 48 h in CMG and finally S acid was not detected by their technique for B.asaccharolyticus (B536) and was found to be 15.09 $\mu moles.ml^{-1}$ at the above conditions.

GSC inspite of its popularity with other workers in this field has some disadvantages, in particular, that related to column ageing, where the part of the column near the injector must be cleaned and refilled after several analysis. Secondly, S acid was found to be eluted very late (Hammann & Werner, 1980). In addition, due to the sample preparation procedure which only involve the centrifugation of the fermentation broth, Bohannan, et.al.,

(1978) found that syringes get clogged regularly when using CM medium due to the extraneous matter that stay in suspension even after centrifugation and as a result they preferred the use of PYG in which this problem did not occur.

The extraction procedures followed in this thesis were those of the VPI manual (Holdeman, et.al., 1977) with slight modification to the methylation procedure in which borontrifluoride methanol was used instead of methanol, and the heating in the water bath was carried on for longer period (45 min).

By sticking rigidly to these procedures it greatly increases the sensitivity of detection of these acids and in that respect GSC* are only detecting minor amount of SCFA (Table 14) due to the lack of these procedures. In addition these procedures help protect the column from contamination and thus prevent the necessary periodic replacement of the column top (which has been noted by all workers with GSC). This finding supports that of Mayhew, et.al., (1975) who stated that they performed thousand of chromatographic runs without substantial signs of column decomposition or alteration in its quantitative repeatability.

The variations encountered at any early stage mounted up to 50% between repeats as was the case with some data in Table 14, such as Deacon, et.al., (1978) the situation necessitated a strict standardization of all conditions involved in this study. The results now show the variation between runs or the repeatability of this

* techniques

technique (SD) to be in the range of 5 - 20% under the strict conditions employed. The major factor found to affect the estimation of SCFA was cellular growth. The optimization of growth especially the black pigmented species could help bring down the SD to a lower level. Other factors were related to sample preparation procedure as each procedure carries an attendant risk of error due to pipetting or incomplete partition, but it is recommended that the sample preparation procedure should follow a set routine of time and vigour of shaking. In addition, samples should be analysed immediately and should not be stored, especially the n-VFA in methylated form due to their extreme volatility.

A summary of all the parameters which need to be under strict control for GLC analysis were as follows:

(A) Microbiology

1. Nature of medium - type - pH, Eh .... etc.
2. Temperature of incubation
3. Time of incubation
4. Aerobic/anaerobic incubation
5. Sample preparation procedures

(B) Chromatography

1. Type of stationary and liquid phase
2. Column degradation
3. Flow rate of carrier gas
4. Nature of carrier gas
5. Flow rate of detector air and hydrogen supplies

6. Maintenance of flame ionization detector sensitivity
7. Recorder speed and sensitivity
8. Consistency of baseline fitting.

The high production of SCFA by Bacteroides species (Fig. 7 - 24) probably contributes to its virulence and the excessive production of A and S might be responsible for the development and persistance of inflammatory reactions in tissues, Takazoe, et.al., (1971).

The mechanism responsible for the pathogenicity of Bacteroides species is, however, not clear and various authors hold different views about how these species cause infection. Gorbach (1979) gives an excellent summary of the various factors, which he believes, may work collectively to cause an infection, these are: (a) Bacteroides spend the greater part of their natural existence as harmless commensals;)b) certain pathological conditions predispose to anaerobic infection (such as a compromised vascular supply, mechanical trauma, tissue destruction and infection due to other organisms); (c) the microenvironment of an anaerobic abscess tends to ensure its survival because of its low Eh $\simeq$-250 mv, with extremely low concentration of oxygen and the presence of a large concentration of organisms (approximately 10^8 - 10^9 cells ml^{-1}). Both these conditions would counteract the body defence mechanisms, (d) Bacteroides possess many virulence factors (Chapter 1, page 51).

Previous studies of the pathogenic potential of Bacteroides have demonstrated a lack of infectivity with

species injected in pure culture (Hill, et.al., 1974; Mayrand & Macbride, 1980) and without the use of special additives (Walker & Wilkins, 1976). Additives found effective are listed by Onderdonk, et.al., (1976) and include: hog gastric mucin and a combination of barium sulphate and sterile caecal contents.

The present study was partly concerned with the ability of pure Bacteroides species (without the interference of additives) to produce local or generalised infections. The results from the work using different species of infective organisms show that B.fragilis, B.melaninogenicus ss. macacae and B.asaccharolyticus are pathogenic at a concentration of 10^8 cell.ml^{-1} (Fig. 39 a, b, & c) which is similar to the level of organisms recorded for typical anaerobic abscess (Gorbach 1979). The subcutaneous infection located at the sight of injection within 7 days was similar for the three different species and ranged in length from 10 - 25 mm. The intramuscular injection showed a small abscess which varied in size and was distributed between the muscle strands.

Histopathological examination of these abscesses clearly demonstrated the gas production characteristic of these species. Gas is produced along tissue planes thus facilitating dissemination of these organisms into the dermis (Fig. 40a). The Figure also shows the infiltration of the layer with neutrophils. Higher magnification of the same area (Fig. 40b, X 1000) shows accumulation of white blood cells with lobular nuclei as well as normal and damaged muscle cells.

Walker & Wilkins (1976) found it necessary to use 0.5 ml of 10^9 cell.ml^{-1} in concentrated chopped meat carbohydrate broth to be able to cause a subcutaneous abscess using pure B.fragilis strain. Their frequency in causing abscess was found to be 55 - 80%. The frequency increased (90 - 100%) when the same size inoculum was used in chopped meat carbohydrate semisolid medium. The abscess size at the site of injection at 5 days of incubation was 5 mm in diameter and increased to 15 - 20 mm by the end of the eighth week.

The comparison of the results obtained in this study with that of Walker & Wilkins (1976) is made difficult by their use of the medium as the presence of the semisolid agar would have greatly increased their chances of abscess formation. It is therefore reasonable to assume that these additives could have contributed to the pathogenicity of B.fragilis thus introducing unknown factors which have to be taken into account when discussing pathogenicity. Walker & Wilkins (1976) recommended the use of semisolid agar for initation of B.fragilis infections in mice compared to the other additives. They found that semisolid agar could be easily prepared without significant variation between different batches and was thus preferable to mucin which was used by Pulaski, et.al., (1952); Hill, et.al., (1974); Renza, et.al., (1975).

It was found in this study that the three species tested were pathogenic at a level of 10^8 cells.ml^{-1} which is lower than that used by Walker & Wilkins (1976). Also, no special preparation was necessary and no additives were needed to potentiate the virulence of the three species.

It was also found that a 24 h culture of B.fragilis and 48 h culture of B.melaninogenicus ss. macacae and B.asaccharolyticus were virulent enough to produce abscesses at a frequency of 95% even in the abscence of additives. 24 h cultures of B.fragilis have been shown to be more virulent than 12 h cultures and this may be attributed to the oxygen sensitivity of younger cultures and capsule formation of the older culture (Walker & Wilkins, 1976).

Onderdonk, et.al., (1977) tested the frequency of abscess formation when either encapsulated or unencapsulated strains of B.fragilis were used and showed that the unencapsulated strains required the presence of a facultative organisms (enterococci) to produce an abscess. Encapsulated strains, however, produced abscesses without the presence of a second organism. Although B.fragilis and B.asaccharolyticus used in this study were not examined for capsular polysaccharide, it is apparent that this material is* found in [illegible] members of these species (Bartlett, et.al., 1978; Kasper, 1979; Mansheim & Coleman, 1980).

B.melaninogenicus ss. macacae is a recent clinical isolates from induced periodontal disease in monkeys and which has been isolated and classified by Slots & Genco (1980). The results (Fig. 39C) shows this strain to be as virulent as B.fragilis and is capable of causing a local subcutaneous infection at an inoculum level of 10^8 cell. ml^{-1} (0.5 ml).

B.asaccharolyticus has been strongly implicated as an important pathogen in actively destructive periodontal disease. Slots & Hausmann (1979) in their study on the

* commonly

relationship of microflora and bone loss have reported that B.asaccharolyticus species increased in proportion from a few per cent to 66% of the total isolates with the development of this disease in monkeys.

The only available publication on induced experimental infection using strains of B.asaccharolyticus is that of Mayrand, et.al., (1980). They studied the characterization of B.asaccharolyticus and B.melaninogenicus oral isolates and used infectivity assay as a means of identification. Their inoculum consisted of a mixture of B.asaccharolyticus with four anaerobic rods, two Gram-negative filamentous rods, one Gram-positive rod and one Gram-negative facultative rod.

It can be concluded that the present study offers a more accurate assessment of the pathogenic potential of the three Bacteroides species mentioned above and describes an easy and reproducible method for induced experimental infection using pure strains only.

The second part of the current study was concerned with the effect of cell concentration of inoculum on the abscess size using a pure strain of B.asaccharolyticus. The results show that the enhancement of the infection appeared to be dose related, as higher doses of organisms produced larger abscesses in all animals (Fig. 41 a, b and c). 10^2 cell.ml^{-1} of B.asaccharolyticus was found to be uninfective. However, at a concentration of 10^5 cell.ml^{-1} this strain started to manifest its pathogenic potential (Fig. 41a) and further increase in inoculum size caused an increase in the overall size of the abscess (Fig. 41b).

At a concentration of 10^{10} cell.ml^{-1} the infection

was severe with a large abscess spreading from one thigh to the other (the resulting infection was similar in appearance to that in Fig.(42c) and the skin covering the rabbit hind legs seemed to be detached from the underlying tissue forming a sac filled with black foul smelling pus which, upon subculturing, gave rise large numbers of viable cells (> 10^7 cells.ml^{-1}) of pure B.asaccharolyticus. The intramuscular injection of 10^{10} cell.ml^{-1} of this strain did not cause a localized abscess (Fig. 41C) but the infection seemed to involve all of the muscle layers right down to the femur with a surface coat of black foul smelling pus which upon aerobic and anaerobic subculturing revealed the presence of B.asaccharolyticus alone with no other species present.

The adopted procedure for this study was to inoculate one thigh with the recommended concentration s.c. and a similar amount would be injected i.m. into the other thigh. It was found repeatedly that at an inoculum level of 10^{10} cell.ml^{-1} of B.asaccharolyticus, the infection became generalised and involving both thighs within 7 days of inoculation. The explanation for this phenomena could be, in part, attributed to the possession by B.asaccharolyticus of various enzymes capable of destroying tissue (Table 11) such as collagenase, chondriotin sulphatase, hyaluronidase which favour invasiveness. The latter findings are in agreement with the result obtained by Mayrand, et.al., (1980). Indeed proteolytic activity may also be assumed within the range of enzymes produced since some of the substrates used for enzyme detection are also used in routine evaluation of proteolytic enzymes (Nelson, et.al.

1961).

Experimental animals receiving unusually large and separate doses of B.asaccharolyticus and B.fragilis died within 60 - 72 h from severe and rapidly spreading infection (Fig. 42a and b). At autopsy the internal organs of the dead animal showed no signs of necrosis but histopathological examination of the heart showed the muscles immediately adjacent to the heart chamber slightly damaged (Fig. 44a). This damage was not widespread and resembled an early necrosis. In addition the adipose tissue immediately adjacent to the heart showed a heavy cellular infiltration (Fig. 44C) whereas the adipose tissue surrounding the heart showed normal lymphnodes (Fig.44b).

Histopathological examination of abscesses showed massive invasion by neutrophils (Fig. 43a). On closer examination the lesions were seen to be inflammatory, heavily necrotic and showing deposition of a coloured granule which was found in all section. This appearance has not been described before and remains unknown in origin (Fig. 43b x 400). The inoculum volume used for induced experimental infection in this study was typically well within the range used by others (Weinstein, et.al., 1974; Hill, et.al., 1974; Onderdonk, et.al., 1976 and 1977; Bartlett, et.al., 1978. McConville, et.al., 1981). All of these previous studies were concerned with the inducement of intraabdominal abscesses using anaerobes and the inoculum either contained mixed cultures ot certain additives.

B.asaccharolyticus was found by Mayrand, et.al.,

(1980) to cause a fatal infection within 18 - 24 h, when 0.5 ml of this species (OD_{660} = 1) suspended in supplemented thioglycolate medium and combined with four non-pathogenic helper organisms was injected subcutaneously into a guinea-pig groin. Their results were variable; the same inoculum on different occasions giving rise to two types of infection, one localized and the other a spreading infection. Death in the latter case was probably due to bacteremia and peritonitis. They also attributed the difference between the two types of infection to a variable growth rate of the strains tested.

Unlike Mayrand, et.al., (1980) the infection produced in the current study was similar in all repeats and resulted in death within 60 - 72 h. This result always required the combination of B.asaccharolyticus and B.fragilis injected in the same animal and the use of relatively high doses of both cultures. A possible explanation for the fatal infection is this high inoculum level used and to a lesser extent to the presence of B.fragilis. B.fragilis has been shown to possess catalase (Table 6) and superoxide dimutase (Carlsson, et.al., 1977; Gregory, et.al., 1977; Hewitt & Morris, 1975 and Tally, et.al., 1977). These two membrane associated enzymes could have helped to establish the infection in its early and critical stage which is believed to be the major problem with the induced infection using pure Bacteroides species, especially B.asaccharolyticus. Although B.fragilis and B.asaccharolyticus were introduced into different thighs, their capability of producing various

enzymes (Table 11) could soon join the two infected area into a severe infection which could have taken place within less than 24 h. This time scale was apparent from the animal's impeded movement of the hind leg in addition to the obvious accumulation of large amount of pus enclosed by the skin and its continuous discharge by a fistula. After this stage the infection spread rapidly to various parts of the body and was followed by death probably caused by heart failure due to toxin production (Rowley, 1971). The toxic effects arise from the capsular antigens and from endotoxin of both species which has been found to be lethal to mice when administered in high doses (Mansheim, et.al., 1978 and 1979; Gorbach, 1979). The endotoxin of both species, however, was shown to be chemically distinct from endotoxin of facultative Gram-negative bacteria in that it lacks two important core sugars, Ketodeoxoctonate and heptose (Hofstad, 1968). It also lacks the predominant fatty acid component of lipid A, B-hydroxymyristic acid as well as having low potency in biological testing.

The present study further clarifies the significance of the presence of selected bacteria on the type of infection caused by otherwise pure strains of B.assacharolyticus. A mixed inoculum (0.5 ml) containing E.coli; Streptococcus faecalis; Clostridium sporogenes and and B.asaccharolyticus at a final concentration of 10^5 cell.ml^{-1} was tested with the results (Fig. 42c) showing similar type of infection to that caused by 10^{10} cell.ml^{-1} of pure B.asaccharolyticus culture. Further tests with the inoculum lacking B.asaccharolyticus failed to produce

an infection establishing that this species is a key agent in the above mixed infection.

Of particular interest was the observation that the type of infection caused by B.asaccharolyticus at an inoculum level of 10^{10} cell.ml^{-1} was similar to that caused by 10^5 cell.ml^{-1} of B.asaccharolyticus with the added organisms. This could imply that although B.asaccharolyticus is virulent in pure culture and causes local infections in small doses (10^5 cell.ml^{-1}, Fig. 41a) severe infection was found to be produced only by either a high, pure, bacterial challenge (approximately 10^{10} cell.ml^{-1}, Fig. 42c) or the addition of certain species to the inoculum (Fig. 42c). The need of 10^{10} cell.ml^{-1} of pure B.asaccharolyticus strain could well be an indication of a low level pathogenicity of this species which could be related in part to low toxicity of its endotoxin (Mansheim, et.al., 1978 and 1979) or to sensitivity of this species to oxygen at a newly infected site which might cause the initial high inoculum numbers to be reduced. The increased virulence of B.asaccharolyticus in the presence of other bacteria could well be attributed to bacterial synergy. Many studies have documented the synergistic effect of facultatively aerobic intestinal bacteria in experimental infections (Altemeir, 1938; Meleney, et.al., 1932; Hite, et.al., 1948; Weinstein, et.al., 1974 and Onderdonk, et.al., 1976) an abscess formation in rats implanted with colonic bacteria has been shown to be related to the presence of anaerobes as part of the inoculum.

There are several possible mechanisms which could explain the increased virulence of B.asaccharolyticus.

One possibility is the production of certain enzymes by the other organisms which help the pathogen to invade the tissue. For example the production of collagenase by various Clostridium species which seems to be more potent than that produced by Bacteroides species (Macdonald & Gibbons, 1962). Another synergistic mechanism is the production of critical growth factors by one organism that permits the survival of B.asaccharolyticus at the infected site, such as the production of vit.K by E.coli (Dam, et. al., 1941). The third possibility for the synergistic effect of aerobic-anaerobic combination depends on lowering local oxygen concentrations and the oxidation reduction potential by aerobes (Fildes, 1929 and Mergehagen, et.al., 1958). The resultant physical condition are appropriate for replication and invasion by anaerobes. Such environmental factors are known to be critical for anaerobic growth in vitro and may apply with equal relevance to in vivo, experimental animal studies. Finally, Gram-negative anaerobes (Bacteroides) markedly impair the phagocytosis and killing of aerobes.

The inhibitory effect of Bacteroides on phagocytosis appears to be due to an interaction between a heat stable, cell associated factor present in Bacteroides intact cells with a heat labile component in the serum necessary for phagocytosis. Stability of this factor is more pronounced with B.melaninogenicus species (115°C for 20 min.) than B.fragilis (Ingham, et.al., 1977). This effect might account for the results obtained in experimental animals, whereby infection could only be produced when B. asaccharolyticus was present in the inoculum.

The histopathological examination of abscesses caused by mixed cultures, showed a normal superficial skin layer with normal epithelial layer, hair follicles and glands (Fig. 45, a, X10). The dermis showed heavy neutrophils infiltration. Magnification of this area (Fig. 45b x 400) shows the severity of the infection with severe necrosis of muscle cells and the massive infiltration of neutrophils. Higher magnification of the same area (Fig. 45 C, x 1000) shows the presence of some Gram-positive rods (Cl.sporogenes) from the mixed inoculum used.

In comparing the results of this study and that of Mayrand, et.al., (1980), it was found that typically, 7 days after inoculation, infection was detected that involved the area of the original injection and which did not cause death. Death may occur with longer incubation because all animals showed severe decline in their eating pattern. In addition the type of infection was quite consistent.

The results show that B.asaccharolyticus is capable of causing severe infection when injected in pure culture. However, a high number of cells were necessary, whereas a relatively small inoculum of the same species would cause a similar severe infection if mixed with selected species.

Autopsy specimens can yield invaluable information when the examination is performed promptly and aseptically and when cultures are plated immediately and accompanied by smears. This work showed that routinely, the kidney, spleen, liver and lung were negative unless distinct lesions are cultured or appreciable portion of the organ is diseased. If an excess of 6 h elapsed between death and the examination, heart blood cultures and the tissue

will reflect postmortmortem or perimortem invasion by organisms from the large bowel and so confuse the picture.

Although characterization of Bacteroides species has progressed considerably (Chapter 1, page 10), there is still a need for more rapid and prompt methods for this detection. Direct GLC analysis of clinical specimens has been shown to provide a rapid presumptive diagnosis of anaerobic infection (Gorbach, et.al., 1976; Phillips, et.al., 1976; Wüst, 1977, Edson, et.al., 1982). This approach was used with pus material obtained from experimental infections with selected Bacteroides species (Fig 1 and 2).

The direct GLC analysis of pus derived from pure B.fragilis infection gave a good separation and detection of the characteristic VFAs produced by this species using the adopted extraction procedure for pure cultures. For n-VFAs however the resultant chromatogram was not reliable for qualitative or for quantitative purposes. The VFAs from B.melaninogenicus ss. macacae infection (Fig. 39C) gave reasonable separation and were identifiable when compared with standard acid mixture. They could not, however, be quantified due to peak tailing. Methylated n-VFAs on the other hand, were not detected under the GLC condition employed in this study.

GLC analysis of pus material from the mixed bacterial infection (Fig. 42C) gave poorly resolved chromatogram for VFAs; n-VFAs were not detected.

The results could be attributed to various reasons such as inadequate extractions of all fatty acids from the pus samples perhaps due to the presence of other materials in the pus which could interfere with the extraction and

detection of some SCFAs.

Column contamination was noted repeatedly after the injection of these samples which is related to the impurities of these samples. It was also found that when pus material was emulsified in CMG and incubated for 10 - 12 h a much more resolved chromatogram was obtained for all SCFAs. The resulted chromatogram, although only good for qualitative purposes was more reliable than that obtained from direct pus analysis and could provide information within 10 h of sampling. The VFAs chromatogram was greatly improved by this pretreatment of the pus material; n-VFAs were detected but showed a poor separation with unsymmetrical peaks and excess peak tailing.

Although direct analysis of clinical samples is an established technique, it has been subjected to criticism due to the report of some false positive and negative results (Eykyne & Phillips, 1976; Watt, et.al., 1982).

Wüst (1977) in his quantitative direct analysis of blood culture has stated that the presence of S has to be interpreted with caution as facultatively anaerobic and aerobic bacteria were capable of producing this metabolite. He also found that IV and S were most diagnostic for the presence of Bacteroides species. Watt, et.al., (1982) on the other hand found that the presence of S does not necessarily correlate with the presence of anaerobic bacteria in the sample. Thus, concentrating on the detection of VFAs, they found that the presence of A is correlated with the presence of aerobic organisms, whereas the presence of a mixture of VFAs correlated with the presence of anaerobic organisms either alone or in combination

with aerobes. They also reported that a certain proportion of VFA-positive samples gave negative results on culturing.

Both studies used the VPI manual sample preparation procedure which was also used in the present study. This approach needs modification before it would be suitable for quantitative or semi-quantitative analysis of pus material. The false positive results reported by the previous authors using direct GLC analysis of pus material could be possibly contributed to column contamination due to impurities of the samples.

Methods for bacterial characterization ranged from simple biochemical or serological tests to highly specialized, technique comparing the DNA structures of organisms (Chap. 1, page 10). Most of these classical methods involved the measurement of only one discriminatory feature. Alternatively, recent chemotaxonomic approaches such as SCFA by gas chromatography (Fig. 7 - 26) have provided measurements on multiple discriminatory features in a single run, but are generally based on chemical derivatization and tend to be time consuming. Meuzelaar (1974) listed the requirement for the ideal identification technique:

(i) Applicability to wide range of microorganisms

(ii) Specificity - ideally any novel identification method should provide information concerning the biochemical differences between microorganisms. In practice these differences are not restricted to a single class of compound and so the identification technique has to compromise between universal

applicability and high specificity.

(iii) Reproducibility

(iv) Speed

(v) Sensitivity: preferably allowing the analysis of single colony from culture plate.

PGLC is one technique which may have many of these requirements with the clear advantages of simplicity and speed of sample preparation and its wide applicability for identification and differentiation of microorganisms (Chapter I, table 1).

An attempt at classification of Bacteroides species using PGLC is presented in Fig. 34 and shows a plot of 12 Bacteroides group means, relative to the first canonical variate axes for all 53 samples using 39 peaks and displaying 79% of the total generalised variation. The results shows B.asaccharolyticus (9 and 10), B.melaninogenicus ss. intermedius (7) and B.melaninogenicus ss. macacae (11) to be extremely well differentiated from the rest of the species tested. It also show the degree of variation between these three species, where maximum variation was noticed between B.asaccharolyticus (9 and 10) B.melaninogenicus ss. macacae (11). With B.melaninogenicus ss. intermedius (7) somewhat appropriately occupying an intermediate position. A comparison of the results for these 3 species in relation to their biochemical behaviour and fatty acid production shows B.asaccharolyticus and B.melaninogenicus ss. macacae to have similar fatty acid profiles consisting of A, P, IB, B. IV, L and S which were produced in lower quantities by the latter species

(Fig. 17 - 19, Fig. 16). Variations also exist in biochemical behaviour of these two species. B.asaccharolyticus is non-fermentative whereas B.melaninogenicus ss. macacae is weakly fermentative (Table 3 and 5). Moreover they have been found to be different genetically and serologically (Slots & Genco, 1980; Coykendall, et.al., 1980).

B.melaninogenicus ss. intermedius seem to be quite different from the above two species in its fatty acid profile where A, IV, L and S were the only acids produced (Fig. 15) and in its biochemical behaviour where more fermentative activity were detected in this species (Table 3 and 5). This species was also found genetically different from the B.asaccharolyticus and B.melaninogenicus ss. macacae (Shah, et.al., 1976; Coykendall, et.al.,1980).

PGLC mapping of the two B.asaccharolyticus strains studied W50 (9) and B536 (10) (Fig. 34) shows variation between the two strains which necessitated their separation into two types. Shah & Collins (1980) reported the presence of two centres of variation within the species. B.asaccharolyticus which is represented by strain W50 (low G + C ratio) which possesses MK-9 (menaquinone with nine isoprene units) as the major isoprenologue with high amount of C_{15} and C_{16} fatty acid and strain B536 (high G + C ratio) which contain major amounts of MK-10 and lower levels of the C_{16} fatty acids. Coykendall, et.al. (1980) confirmed the heterogenicity of these strains and reported that oral strains of B.asaccharolyticus (represented in the present study by strain W50) have a lower DNA base ratio than the non-oral strain

(represented by B536)

The PGLC results on the species of the first set (9, 10, 11, 7, Fig. 34) shows complete agreement with the previous authors and clearly demonstrates the extent of variation between the different species.

Fig. 34 shows B.ruminicola ss. brevis (NP333 , 12) and B.vulgatus (6) to differ drastically from the species of the first and third sets. Strain NP333 is an oral isolate previously designated as B.oralis [illegible]; Duerden, et.al., 1976). It shares many phenotypic characters with B.ruminicola ss. brevis and has a similar G + C ratio (Reddy & Bryant, 1977; Shah, et.al., 1976) was found to be unrelated to the rumen species of B.ruminicola ss. brevis based on the results of a DNA/DNA reassociation and lipid analysis studies of Shah, et.al., 1976, 1979 and Shah & Collins, 1980 who recommended that strains NP333 formed a separate, previously unrecognized species from the oral cavity. *(1),(4),(10),(11) Although strain NP333 is the only test strain received as B.ruminicola ss. brevis, the relationship between this oral strain and the rumen strains can be easily tested using PGLC as has been shown for the species of the first set.

The pyrolysis results (Fig. 34) show strain NP333 in regard to its variation and differences from the other species to be intermediary between the black pigmented and the non-pigmented species. It has few common characteristics with the non-pigmented species (failure to produce pigment on blood agar and its ability to ferment

carbohydrates). At the same time it shares few other characteristics with the pigmented species (failure to produce catalase and the inhibition of this strain's growth by the presence of bile salts in the growth medium) (Table 6). In addition, its fatty acid profile is similar to that of B.melaninogenicus ss. melaninogenicus (Fig. 12) and both species are considered to be the least productive strains (Fig. 25). The second set also includes B.vulgatus (6) and Fig. 34 shows this species to be quite well differentiated from strain NP333 and from the closely related non-pigmented species. These comprise the members of the third set which were found to be undifferentiated from each other on the results of biochemical profile (Table 6). Additionally others have found B.vulgatus, B.fragilis and B.thetaiotaomicron to possess a closely related fatty acid profile with branched-C_{15} constituting the major acids (Shah & Collins, 1980), (8),(9).

B.fragilis (NCTC 8560 and 9343, 4 and 5), B.thetaiotaomicron (1), B.ovatus (2), B.distasonis (3) constitute the members of the third set. Variation clearly does exist between some members of this set which is sufficient to separate them into distinct group. This applies to three members of this set, B.ovatus (2) B.fragilis NCTC 8560 (5) and B.thetaiotaomicron (1). These three species have been very difficult to differentiate by means of biochemical tests (Table 2 and 4) and fatty acid profile (Fig. 9, 7 and 10). B.fragilis is the only species in this set that is of direct interest to clinicians and researchers due to its pathogenic potential

(Fig. 39a and Fig. 42 a and b). The rest of the species exist as harmless commensals, so very little work has been done on differentiating them from each other. Fig. 34 shows PGLC to be capable of differentiating them and further more it was also capable of differentiating heterogenous strains of B.fragilis, NCTC 9343 and 8560 (4 and 5). Strain 9343 always gave a more complex pyrogram than strain 8560. By applying canonical variate analysis to the data, this variation was sufficient to separate them into two groups (4 and 5). B.distasonis (3) on the other hand seemed to share some characteristics with B.fragilis, NCTC 8560 (4) which made their separation difficult i.e. within the cell fragments analysed by PGLC more similarities exist between B.distasonis (3) and B.fragilis (4) than between the two heterogenous strain of the same species (4 and 5).

B.melaninogenicus ss.melaninogenicus (8) seems to be quite differentiated and separated from the other 11 Bacteroides strains studied. Although this species is quite differentiated from B.asaccharolyticus and B.melaninogenicus ss. macacae from their fermentative behaviour (Table 6) and fatty acid profile (Fig. 14 and 25), it shares many phenotypic characters with B.melaninogenicus ss. intermedius (Holdeman & Moore, 1974, Harding, et.al., 1976 and Shah, et.al., 1976). Shah & Collins (1980) found that the production of indole by B.melaninogenicus and its failure to ferment lactose were the only reliable tests for differentiation of this subspecies from subspecies melaninogenicus. They were successfully able to differentiate the two subspecies by their unsaturated fatty acid profiles in which that of B.melaninogenicus ss.

melaninogenicus was found to contain a high proportion of C_{16} in comparison with the C_{14} present in the other subspecies. The PGLC plot (Fig. 34) provides supportive evidence that variation exists between these two subspecies.

Of particular interest was the position of B.melaninogenicus ss. melaninogenicus (8) in the PGLC plot where it shows this strain (VPI 9085) to have a maximum variation from all the black pigmented species studied (first set) and is apparently more related to the non-pigmented species (3rd set). This species has been known for its saccharolytic activity which differentiates it from most of the black pigmented species (Table 6). It also has a simple fatty acid profile (Fig. 14) which shows more similarity with the non-pigmented species profile than the complex pattern produced by B.asaccharolyticus and B.melaninogenicus ss. macacae.*(3),(10), Shah & Collins (1980) showed strain VPI 9085 has a similar isoprenoid quinone composition to B.fragilis (NCTC 8560) and B.thetaiotaomicron but different unsaturated fatty acid profile.

A problem thus does exist within the genus Bacteroides in that despite the highly specific technique employed for their identification (Chapter 1, page 10), the taxonomic position of certain species is still confused and unclear. For example, B.oralis species had been described by Shah, et.al., (1979) as a repository for a long and varied collection of strains which could not be accommodated elsewhere. B.oralis and B.melaninogenicus ss. melaninogenicus (VPI 9085) are distinguished from each other almost exclusively on the basis of pigment production (Sutter, et.al., 1972a,Holdeman & Moore, 1974).

Strain VPI 9085 and reference strain of B.oralis (G9a-C_2) which has been found to produce pigment intermittently (Shah, et.al., 1976) were shown by numerical taxonomy (Sundqvist, 1976) to be closely related thus emphasing the need for more fundamental methods for classification of these species.

Although the specific identities of the peaks on a pyrogram are not known to us, these peaks represent a chemical fragment or components and the PGLC results (Fig. 34) seem to point out clearly to the presence of some basic variation within the cells of strain VPI 9085 (8) which differentiates its pyrolytic fragments from those of B.melaninogenicus cells (7). This was expressed on the PGLC plot as a maximum distance between the two subspecies. This strain, although known to produce black pigment on blood agar, might not necessarily belong to a subspecies of B.melaninogenicus and it could belong to a separate new species of black pigmented Bacteroides.

A similar result was noted with B.melaninogenicus ss. macacae (12) where the PGLC plot (Fig. 34) shows this strain to be quite different from the black pigmented species. This finding correlates extremely well with the taxonomic information presented by Coykendal, et.al., (1980) who proposed the separation of this subspecies from the B.melaninogenicus group and named it as B.macacae.

Comparison of the classification of these 12 Bacteroides strains using PGLC with the present conventional system shows that a close correlation exist between the two methods except with B.melaninogenicus ss. melaninogenicus (VPI 9085).

The canonical variate plot of the PGLC data (Appendix 1) for the third canonical variate versus the first accounts for 16% of the variation which exists between these species and is presented in Fig. 35. Fig. 34 and 35 together account for 95% of the variation present within the species tested. Although Fig. 35 accounts for only 16% of the variation, it still separates the species to fit in within the discussion of the previous plot (Fig. 34) with the exception that B.ovatus (2) and B.fragilis (NCTC 9343,5) are now difficult to differentiate. Heterogenous strains of B.asaccharolyticus 9 and 10 shows minimum variation but B.fragilis strains 4 and 5 still shows a big variation from each other. It also shows that by eliminating large proportion of these variations, a slight degree of relatedness between B.asaccharolyticus (9 and 10) with B.macacae (11) is noticed. Fig. 35 shows the sensitivity of PGLC in detecting even small degrees of variation. It also show how successful it is in differentiating most species.

Canonical variate analysis is a discriminatory analysis based on dividing the samples into groups and then finding functions which best separate these groups. It directly compares peak heights to obtain differences between groups. It is sensitive and once a stable plot has been obtained the calculation to allocate an unknown can be performed on a pocket calculator or a minicomputer. This stage in the identification of the samples can be extremely fast and once the unknown pyrogram has been digitised, the time taken to obtain a presumptive

identification with a computer is measured in seconds. In addition, storage of canonical variates plots takes less computer space than sets of peak heights of standard strain and would allow the use of relatively small computers in conjunction with PGLC. Generally this technique work best when differentiating groups that partially overlap in their properties (Sneath, 1978b). The canonical variate approach to identification of unknowns is not the only one available and is probably too sophisticated for the simple allocation of groups to the genus level.

Each canonical variates plot is a unique identification base determined by three factors.

(i) Number of groups

(ii) Number of samples

(iii) Number of peaks used to discriminate the groups.

Any change in these factors will alter the plot and Fig. 36 a and b is an example of the effect of these factors on the plot. Fig. 36 a and b show a plot the means of 8 Bacteroides species, relative to third and first canonical variate axis, respectively for all 41 samples using 39 peaks and displaying 95% of the total generalised variation. Fig. 36b shows different allocation of the eight species from their position in Fig. 34. It also shows most species to be quite differentiated from each other, with the exception of B.ovatus (2) and B.distasonis (3). B. fragilis strains 4 and 5 seem to be well differentiated from each other. Although the allocation of species different (Fig. 35 and 36b) the PGLC on classification of these species has

not been effected since the new plot carries the same characteristic features of the first plot mentioned earlier. It is also noticed that the use of more than the first two canonical variate did not improve the differentiation in both plots.

The number of factors having the potential to effect the reproducibility of PGLC has put to over 30, Gutteridge, 1979. Some of these factors are listed in Table (A.19) and need to be strictly standardized. In all the PGLC work reported in this thesis these factors were standardized as far as possible. Preliminary work in the present study showed the use of blood agar changed the pyrogram drastically. This is probably due to the presence of blood which would encourage pigment production by some Bacteroides species with a consequent change in the resulted pyrogram. As a result all species were grown on VitK-haemin supplement BM medium without added blood which supported an excellent growth of all species and the yielding of non-pigmented colonies by the pigment producing Bacteroides.

Although using B.fragilis and B.asaccharolyticus strains in the exponential phase of growth (Fig. 27) gave good result in the infectivity assay, it was not successful with PGLC analysis. Species in the stationary phase (96 h) gave more reliable and reproducible programs thus confirming the valuable effect of incubation time on pyrolysis. Reiner & Ewing (1968) and Reiner, et.al.(1969) found that certain characteristic peaks remain constant inspite of the changes in growth condition, whereas Oxborrow, et.al. (1977)reported that the pyrogram of Bacillus species was drastically effected by changes in

growth conditions. Gutteridge & Norris (1980) confirmed both previous observations and suggested that the changes in growth media produced no unique products but changed the peak heights ratios of the pyrolysate which is used for the discrimination of microorganisms. They also found that the effects of changes in incubation time and temperature seem to depend on the organism under study in that they were minimal for E.coli but severe for P. putida and S.aureus. They attributed these differences to variation in growth rate and metabolic activity of the three bacteria such as production of extracellular substances, pigment and secondary metabolites.

Factors concerned with the microbiological parameters of pyrolysis are easily controlled, as are those concerned with instrumentation. Technical factors are however less easily controlled, for example, those concerned with the actual pyrolysis process and the performance and replacement of the analytical column. B.fragilis, NCTC 8560, (Fig. 29) was used routinely to monitor the performance of the PGLC system. Preliminary work with a newly packed column gave poor resolution of the standard strain. The new column was brought to its optimum performance with maximum reproducibility and efficiency within three months of commissioning (Fig. 29 - 33). Over long period of use, column performance gradually deteriorates, associated with discolouration of the column top part and loss of resolution in pyrograms.

Gradual poisoning of the analytical column is the most serious factor affecting long-term reproducibility.

Quinn (1974), Needleman & Stuchbery (1977) and Gutteridge & Norris (1979) emphasise this effect and suggested that the introduction of microprocessor controlled gas chromatograph would greatly improve the reproducibility of PGLC.

In this study the upper part of the column was replaced when necessary and a period of two weeks recommissioning was employed.

Inter-laboratory reproducibility is an ideal which is extremely difficult to obtain with complex fingerprints unless the analytical equipment can be exactly duplicated. Nevertheless the major pyrolysis products identified using pyrolysis-mass spectrometry have been found similar by various authors (Simmonds, 1970; Medley, et.al., 1975; Quinn, 1976 and Gutteridge, et.al., 1979). Since they used different pyrolysers the pyrolysis process appears to be sufficiently reproducible and that only the separation and detection of these products that produces different pyrogram. Finally the assessment of pyrograms by mathamatical and not visual techniques also helps to improve reproducibility (Macfie & Gutteridge, 1978; Macfie, et.al., 1978).

Curie-point pyrolysis is preferred for microbiological identification of microorganisms since the pyrolysed specimen has direct access to the column (i,e. no problems encountered with dead volume). Samples can also be taken directly from culture plate and long-term reproducibility is easily achieved.

The present study represent the first and only report on the application of PGLC in the microbial taxonomy of Bacteroides species. The real value of PGLC lies in being a rapid identification technique by which a sample

can be analysed directly from a culture plate. It is however essential to point out that the impact of PGLC on identification of microorganisms is dependent on the use of discriminatory data analysis such as the canonical variate analysis applied in this study. The use of this mathematical technique does however require a prior knowledge of the groups and seeks to discriminate on this basis. Thus it should not be used for developing new taxonomies but it can be used to test suspected relationships and subsequently to improve taxonomies. One of the most exciting aspects of PGLC is the ability to superimpose an existing classification on pyrolysis data from unknown strains thus confirming their identification. This ability of rapidly identifying unknowns was put to test using pus samples resulting from three types of induced infection (pure strains of B.fragilis (NCTC 8560) B.asaccharolyticus (W50) and mixed bacterial infection using E.coli, Streptococcus faecalis, Clostridium sporogenes and B.asaccharolyticus). The resulted pyrogram from direct pyrolysis of pus sample from infection induced with pure strains showed visual similarity to that obtained with pure cultures (Fig. 29 and 37). This visual similarity probably relates to the large number of pure cells present in the pus sample. Visual comparison have been used by some authors for differentiation purposes below the species level which is surprising as such conclusion cannot be established by the presence or absence of a single peak and required the consideration of a number of peaks (Fingerprint area) (Reiner, 1965 ; Reiner & Ewing, 1968; Reiner, et.al., 1973 and

Emswiller & Kotula, 1978). Visual comparison of the fingerprint area of the pure culture pyrograms (Fig. 29) and pus pyrograms (Fig. 37) presented in this study gave an early indication to the presence of the same specific organism in the pus sample, but this would need more confirmation by later data analysis.

Comparison of the data in Table A.4 with A.16 for B.fragilis and A.9 with A.17 for B.asaccharolyticus showed the expected higher yield of the characteristics peaks by the pure strain compared with that from pus analysis.

Identification has long been regarded as the last stage of taxonomy but in direct analysis of pus samples using PGLC the situation is reversed as we were able to identify specific organism present within the abscess by visual examination of its pyrograms without the delay encountered using the conventional methods. However, a clear distinction must be drawn between the speed offered by direct SCFA analysis where result could be obtained within 12 h of incubation and the 96 h of incubation needed for pyrolysis culture sample to give a reliable and reproducible pyrogram. In this respect SCFA analysis is the most rapid method, but this technique was not sensitive and rapid when applied to pus samples as explained by the results of the present study and that of Watt, et.al., (1982), and Edson, et.al., (1982). Thus the application of PGLC to pus analysis may be considered to provide the specific identification for pure infections with minutes compared with hours and days by the conventional method.

No conclusions, however, could be drawn from visual comparison of the pyrogram resulted from mixed bacterial infections with those derived from pure culture of the individual organisms. This implies massive peak overlapping, since similarities in cell structure between the four different species would normally yield peaks common to some or all species. This would be impossible to detect visually but canonical variate analysis could provide a means of showing the characteristic peaks shared between various organisms and the peaks that differentiate them. If the nature of these peaks needed to be defined pyrolysis-mass spectrometry offers a rapid and reliable technique.

Comparison of the data derived from pyrograms of pure samples of E.coli (A.13), St.faecalis (A.14) and Cl.sporogenes (A.15) with the data derived from Bacteroides species (A.1 - A.12) shows drastic differences both qualitatively and quantitatively. If these three different species were to be plotted together with the twelve Bacteroides strains, it could give misleading results. Canonical variate analysis is sensitive to various aspects including (a) small number of samples, (b) when the organisms under study are substantially different and (c) data processing problems when dealing with large numbers of samples (the 12 different organisms tested here are just within the limit for this mathematical technique).

The role of canonical variate analysis in the identification of the unknowns derived from experimental

infections would be in imposing these results on a canonical variate plot of B.fragilis, B.asaccharolyticus, E.coli, Cl.sporogenes and St.faecalis pure cultures. This would establish the relationship between them. For example, the CVA plot for a pus sample would be compared with pre-existing plots for pure organisms, relative locations noted and from this the identity of the organism present in the abscesses determined. As for mixed infection the positions of the unknown sould be possibly situated in between the different species involved in the infection or it might tend to reflect the characteristics of one species more than the others.

The unknown data (Table A.16 - A.18) would have small amounts of their peaks contributed by the abscess itself (white blood cell ... etc.). So for accurate evaluation of the unknown, these would have to be deducted from data in Table A.16 - A.18 before canonical variate analysis could be employed. 1% Kaolin and Killed bacterial suspensions both failed to produce sterile abscesses under the experimental condition employed in this study and consequently the mathematical evaluation of these unknown data was not possible.

In comparison to other techniques such as lipid analysis (Shah & Collins, 1980) and DNA hybridization (Coykendall, et.al., 1980), PGLC requires no sample preparation and needs only a simple chromatograph which is readily available to most dignostic and research laboratories and which when fitted with a pyrolysis head would offer rapid, extremely sensitive and inexpensive method for the identification of microorganisms.

The variety of techniques found useful in the differentiation of the 13 strains of Bacteroides used in the present study is summarized in Fig. 46.

Overall PGLC was found to be invaluable in many different aspects of this study, where it successfully confirmed the relationships between 12 Bacteroides strains and divided them into separate sets with black pigmented species (except B.melaninogenicus s.s. melaninogenicus) on one end of the scale and the non-pigmented species on the other and with B.ruminicola ss. brevis and B.vulgatus taking an intermediate position. In addition it successfully differentiates the closely related B.fragilis, B.thetaiotaomicron, B.ovatus and B.vulgatus from each other. PGLC was also successful in differentiating heterogeneous strains of B.fragilis and B.asaccharolyticus and finally was found to be extremely rapid and sensitive in identifying organisms involved in pure anaerobic infection.

Fig. 46 Summary of various techniques used in differentiation of Bacteroides species

Bacteroides Species

Non-pigmented Colonies on blood agar at 7 days of incubation

Pigmented Colonies on blood agar at 7 days of incubation

	B.fragilis	B.thetaiotaomicron	B.distasonis	B.ovatus	B.vulgatus	B.ruminicola ss.brevis	B.melaninogenicus ss. melaninogenicus	B.melaninogenicus ss. intermedius	B.macacae	B.asaccharolyticus
Growth in 20% bile	+	+	+	+	+	-	-	-	-	
Catalase	+	+	+	V	-	-	-	-	+	
Indole	-	+	-	+	-	-	-	+	+	
Glucose	+	+	+	+	+	+	+	+	+	
Minnitol	-	-	-	+	-	-	-	-	-	
Lactose	+	+	+	+	+	+	+	-	+	
Fatty acid profile	A,P,L,S	A,P,L,S	A,P,L,S	A,P,L,S	A,P,L,S	A,L,S	A,L,S	A,IV,L,S	A,P,IB,B,IV L,S	A,P,IB,IV,L,S
S:A ratio at 12 in CMGW	*9343 2.61	5.02	3.60	5.16	6.74	4.79	3.62	4.85	0.42	*B537 1.07
Total acid production in CMG at 96h (µmoles. ml^{-1})	NCTC 8560 98 / NCTC 9343 128	158	176	158	154	97	84	187	135	W50 115 / B537 127 / B536 84
Total acid production in PYG at 96h (µmoles. ml^{-1})	89	110	N/T	N/T	N/T	N/T	N/T	N/T	N/T	103 / 95 / N/T / 83
Differentiation using PGLC	NCTC 8560: U.D. from B.distasonis; NCTC 9343: W.D.	W.D	U.D from NCTC 8560	W.D.	W.D.	W.D.	W.D.	W.D.	W.D.	W50 W.D. / B536 W.D.
Infectivity assay	Pathogenic in Pure culture at (10^8 cell. ml^{-1})	N/T	N/T	N/T	N/T	N/T	N/T	N/T	Pathogenic in pure culture (10^8 cell. ml^{-1})	Pathogenic at 10^5 cell. ml. Severe infection resulted: Pure 10^{10} cells. ml^{-1}; 10^5 cells. ml^{-1} in presence of helper organisms

N/T = strains not tested

W.D = well differentiated

U.D = undifferentiated

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APPENDIX I

Table A.1

B.thetaiotaomicron (NCTC 10582)

Relative heights, Range and Average relative heights with peak No. 13 fixed at 100

Peak No	$R.H_1$	$R.H_2$	$R.H_3$	$R.H_4$	$R.H_5$	$R.H_6$	Range	Average height ± SD
1	14.2	13.7	16.4	12.12	16.3	17.5	5.38	15.03± 1.84
2	5.4	6.2	5.85	3.30	2.24	5.06	3.96	4.67± 1.40
3	2.8	2.2	2	1.73	1.89	3.71	1.98	2.38± 0.6
4	10	10.9	10.1	8.97	12.06	14.1	5.13	11.02± 1.06
5	3.8	2.4	4.5	3.62	2.58	4.39	2.1	3.54± 0.8
6	3.3	5.5	6.42	2.51	3.96	5.74	3.91	4.57± 1.4
7	18.5	15.5	10.7	11.3	13.44	15.5	7.8	14.15± 2.6
8	59.5	40.7	35.7	43.3	50	55.06	23.8	47.3 ± 8.2
9	19.5	18.8	19.4	14.8	16.03	20.6	5.8	18.1 ± 2.06
10	14.7	17.7	13.4	10.8	12.75	18.5	7.7	14.6 ± 2.70
11	3.09	5.18	3.85	3.62	4.31	5.06	2.09	4.18± 0.75
12	7.6	7.7	7.14	6.45	6.89	7.09	1.25	7.14± 0.42
13	100	100	100	100	100	100	0	100 ± 0
14	1.9	2.96	3.14	2.20	2.93	2.36	1.24	2.58± 0.45
15	5.4	5.92	4.42	3.14	3.44	6.41	3.27	4.78± 1.22
16	5.7	6.85	6	5.66	3.96	6.41	2.89	5.76± 0.90
17	11.4	10.7	10.5	8.81	8.62	11.4	2.78	10.23± 1.12
18	3.3	4.81	4	3.46	3.27	3.71	1.54	3.75± 0.53
19	5.7	7.77	6.28	5.03	4.13	7.43	3.64	6.05± 1.27
20	5.2	4.25	5.57	4.40	3.44	4.05	2.13	4.48± 0.71
21	17.6	19.07	18.5	14.6	20.6	17.9	6	18.04± 1.81
22	3.3	5.18	4.71	3.46	2.93	4.05	2.25	3.93± 0.79
23	73.8	62.9	70	68.2	62.06	61.8	12	66.46± 5.34
24	23.8	20.3	18.5	18.8	18.96	21.2	5.3	20.26± 1.84
25	0.95	-	1.01	0.94	0.51	-	1.01	0.56± 1.01
26	111.9	107.4	91.4	82.4	85	101.3	29.5	96.5 ±15.1
27	3.09	2.92	3.42	2.36	3.62	3.71	1.35	3.18± 0.46
28	3.8	4.07	3.28	2.67	3.10	4.05	1.4	3.49± 0.51
29	13.5	11.2	12.8	10.2	12.7	14.5	4.3	12.48± 1.42
30	6.9	5.74	7	5.19	5.68	8.10	2.91	6.43± 0.99
31	4.2	5.37	5.14	3.93	3.44	5.06	1.93	4.52± 0.70
32	0.47	-	0.72	0.62	0.51	0.67	0.72	0.47± 0.18
33	2.3	2.22	2.28	1.73	1.72	2.02	0.58	2.04± 0.42
34	-	-	-	-	-	-	-	-
35	14.2	12.4	11.42	11.66	9.65	12.5	5.54	11.97± 1.8
36	-	-	-	-	-	-	-	-
37	24.04	25.9	26.4	22.04	25.8	29.3	12	25.57± 3.6
38	0.86	0.99	2.42	1.88	2.82	2.70	4.82	1.95± 1.1
39	10.9	10.7	10.7	7.40	6.89	10.1	4.01	9.44± 1.6

$R.H_1$ - $R.H_6$ are repeat at different time.

Table A.2

B.ovatus (ATCC 8483)

Relative heights, Range and Average relative heights with peak No. 13 fixed at 100

Peak No	$R.H_1$	$R.H_2$	$R.H_3$	$R.H_4$	$R.H_5$	Range	Average height ± SD
1	10.8	13.7	11.6	10.2	9.8	3.9	11.22 ± 1.38
2	3.5	4.5	5.3	3.5	3	2.3	3.96 ± 0.82
3	2.4	3.3	2.7	2.4	2	1.3	2.56 ± 0.43
4	8.06	10.3	10	8.7	9.3	2.2	9.27 ± 0.82
5	3.3	3.8	4.7	3.6	3.4	1.4	3.76 ± 0.50
6	2.4	3.8	3.6	2.2	2	1.8	2.8 ± 0.74
7	10.4	10.9	10.7	10	10	0.9	10.4 ± 0.36
8	33.3	48.4	64.4	30.4	44.3	34	44.2 ± 12.03
9	14.4	18	20.7	16	13.9	6.8	16.6 ± 2.50
10	12.4	15	16	12.2	12.5	3.8	13.6 ± 1.57
11	4.7	5	4.7	4.4	4.7	0.6	4.7 ± 0.18
12	6.9	7.7	6.2	7.3	5.7	2	6.76 ± 0.72
13	100	100	100	100	100	0	100 ± 0
14	2.1	2.5	3.3	3.3	2.3	1.2	2.7 ± 0.50
15	3.7	3.9	2.4	2.2	2.5	1.7	2.94 ± 0.71
16	7.9	13.3	12.2	11.3	6.9	7.4	10.32 ± 2.82
17	7.1	10	9.1	7.3	7	3	8.10 ± 1.22
18	2.1	2.3	2.4	2.2	2	0.4	2.2 ± 0.14
19	6.5	8.3	8.7	7.1	6.8	2.2	7.48 ± 0.86
20	2.9	3.3	4.7	4.4	2.5	2.5	3.56 ± 0.85
21	17.1	18.8	15.1	15.3	15.7	3.7	16.4 ± 1.38
22	1.9	2.3	4.0	3.8	2.7	2.1	2.94 ± 0.82
23	58.1	64.1	51.1	48.9	52.3	15.2	54.9 ± 5.51
24	25	25	31.1	28.4	27.7	6.1	27.4 ± 2.29
25	0.95	1.1	6.0	5.1	2	6	3.05 ± 2.05
26	59.7	81.3	91.1	78.9	55.7	35.4	73.3 ± 13.4
27	1.61	2	2.7	2.4	2.3	1.1	2.20 ± 0.37
28	2.3	3.3	3.8	4	2.7	1.7	3.22 ± 0.64
29	7.7	11.3	16.7	14.4	8	9	11.62 ± 3.52
30	5.0	6.4	6.4	6	5.9	1.4	5.94 ± 0.51
31	3.4	4.1	5.6	4.4	4.5	2.2	4.4 ± 0.71
32	0.5	0.6	2.4	2	0.7	1.7	1.24 ± 0.79
33	1.5	1.7	4.2	3.8	1.6	2.5	2.56 ± 1.18
34	0.6	1.1	2.2	2.4	0.5	1.8	1.36 ± 0.79
35	11.7	10.2	14.9	12.9	11	4.7	12.4 ± 1.64
36	-	-	2.4	2.2	-	2.4	0.92 ± 1.1
37	18.1	23.4	21.6	19.3	18.9	5.3	20.2 ± 1.95
38	3.2	7.2	21.1	18	-	21.1	12.3 ± 7.3
39	3.5	11.1	16.7	13.6	9.1	8.2	11.8 ± 3

RH_1 and RH_2 = replica $RH_{2,3\ \&\ 4}$ are different repeat.

Table A.3

B.distasonis (ATCC 8503)

Relative heights, Range and Average relative heights with peak No. 13 fixed at 100

Peak No	$R.H_1$	$R.H_2$	$R.H_3$	$R.H_4$	$R.H_5$	Range	Average height ± SD
1	9.8	10.5	10.4	8.8	9	1.7	9.7 ± 0.69
2	3.3	5.2	3.2	3.5	4	2.0	3.84± 0.73
3	1.3	2.1	2.2	1.2	1.7	1.0	1.70± 0.40
4	9.6	9.7	8.8	10.6	9.5	1.8	9.64± 0.57
5	2.9	2.9	2.2	3	3.1	0.9	2.82± 0.31
6	2.2	4.1	3.6	2.4	2.6	1.9	2.98± 0.73
7	9.3	12.9	12.8	8.8	8.6	4.3	10.48± 1.94
8	34.2	41.3	52.2	37.9	59.5	25.3	45.02± 9.41
9	12.7	12.5	12.4	12.6	13.3	1.1	12.7 ± 0.31
10	11.2	12.1	11.3	14.5	11.9	3.2	12.2 ± 1.20
11	2.7	5.55	6.1	2.7	4.3	5.2	4.74± 2.01
12	64.	6.1	4.6	4.4	6.7	2.3	5.64± 0.95
13	100	100	100	100	100	0	100 ± 0
14	2.5	2.2	2.8	2.9	2.6	0.7	2.60± 0.24
15	2.2	3.9	4.2	3.03	2.1	2.8	2.76± 1.09
16	24.4	22.2	15.1	12.1	19	10.1	18.56± 4.50
17	7.8	8.09	8.1	7.1	8.6	1.0	7.93± 0.49
18	1.8	2.2	2.8	1.5	1.9	1.3	2.04± 0.44
19	5.6	10.5	9	5	6.4	5.5	7.30± 2.10
20	3.1	2.3	3.8	2.4	4.5	2.1	3.22± 0.83
21	15.6	15.1	15.9	10.3	20.2	9.9	15.42± 3.14
22	3.3	4.3	3	2.4	2.9	1.9	3.18± 0.63
23	34.5	57.1	60.9	31.1	66.7	35.6	50.06±14.4
24	27.3	30.2	24.6	30.3	25.7	5.7	27.62± 2.31
25	1.6	0.56	.7	3.8	1.4	3.8	1.82± 0.97
26	50.9	71.4	77.5	52.2	81	30.1	66.5 ±10.6
27	4.2	-	1.2	2.3	3.3	4.2	2.87± 1.19
28	2.5	2.9	3.3	2.4	3.6	1.2	2.94± 0.45
29	11.3	10.2	10.1	10	9.5	1.3	10.22± 0.59
30	3.5	4.8	5.8	4.2	4.3	2.3	4.62± 0.76
31	3.5	2.5	4.1	2.7	4.5	2.0	3.46± 0.77
32	0.7	-	0.9	1.5	0.7	1.5	0.76± 0.32
33	1.1	-	2.2	2.6	1.0	2.6	1.72± 0.69
34	-	-	0.9	1.5	0.5	1.5	0.96± 0.41
35	11.1	10.2	9.9	11.4	7.9	3.5	10.1 ± 1.23
36	-	-	-	1.5	-	1.5	1.5
37	19.6	20.6	26.1	22.7	26.0	6.5	2.3 ± 2.68
38	-	2.69	5.1	10.6	2.4	10.6	5.19± 3.29
39	8.5	7.6	8	8.6	8.8	1.2	8.3 ± 0.43

$R.H_1$ & H_3 = replica $R.H_4$ & $R.H_5$ = replica

$R.H_2$ = a repeat

Table A.4

B.fragilis (NCTC 8560)

Relative heights, Range and Average relative heights with peak No. 13 fixed at 100

Peak No	$R.H_1$	$R.H_2$	$R.H_3$	$R.H_4$	$R.H_5$	$R.H_6$	Range	Average height ± SD
1	9.62	8.51	8.24	13.5	11.6	10	5.36	10.26 ± 1.85
2	3.77	4.44	3.33	5.16	3.78	4.23	1.83	4.11 ± 0.58
3	4.15	2.03	1.57	3	1.89	1.73	2.58	2.39 ± 0.9
4	11.5	11.1	10.5	9.83	8.64	7.30	4.2	9.81 ± 1.45
5	3.77	2.22	2.63	5	2.97	2.88	2.78	3.24 ± 0.91
6	2.07	4.44	1.57	3.16	2.43	2.5	2.37	2.69 ± 0.91
7	4.15	6.11	6.31	8.83	9.18	5.96	5.03	6.75 ± 1.74
8	57.5	51.8	21.05	38.3	36.7	28.6	36.4	42.3 ± 14
9	17.5	14.2	11.7	19.3	11.9	13.2	7.6	14.6 ± 2.8
10	9.24	12.2	7.54	13	8.80	11.1	5.66	10.3 ± 0.72
11	3.77	4.07	4.91	5.5	3.33	4.61	2.17	4.36 ± 0.72
12	6.03	5.37	5.26	7.16	5.23	5.28	1.93	5.73 ± 0.69
13	100	100	100	100	100	100	0	100
14	2.64	2.03	1.40	3	2.16	2.30	1.6	2.25 ± 0.49
15	2.26	2.22	0.87	2	1.89	2.11	1.39	1.89 ± 0.47
16	17.9	12.7	8.77	14.1	6.48	7.30	11.4	11.20 ± 4.06
17	10	8.14	7.19	12.8	8.10	8.46	5.61	9.11 ± 1.84
18	2.64	2.03	2.10	4	2.70	1.92	2.08	2.56 ± 0.70
19	3.77	4.25	2.98	5.33	4.86	4.42	2.35	4.26 ± 0.75
20	3.77	3.51	3.33	5	3.78	3.46	1.44	3.80 ± 0.55
21	13.2	11.1	10.7	20	14.3	15.1	9.3	14.06 ± 3.08
22	3.58	1.11	1.40	2.83	2.70	1.73	2.47	2.22 ± 0.87
23	45.2	42.5	42.2	66.6	56.7	42.3	14.5	49.2 ± 9.2
24	21.1	20	25.4	32.5	24.3	18.1	7.1	23.56 ± 4.6
25	4.71	2.03	2.28	4.5	1.35	-	4.71	2.97 ± 1.36
26	71.6	75.9	54.3	93.3	70.2	30.7	39	74.2 ±11.70
27	2.83	0.92	2.10	2.83	1.89	-	2.83	2.11 ± 0.70
28	3.01	2.77	1.92	4.16	2.43	2.69	2.24	2.83 ± 0.68
29	13.5	8.33	8.07	14.1	8.37	7.11	6.99	9.91 ± 2.78
30	3.96	3.70	2.98	4.83	3.78	3.07	1.85	3.72 ± 0.61
31	5.84	5.18	4.21	7.5	4.59	4.80	3.29	5.35 ± 1.08
32	2.26	4.25	4.91	6.33	3.51	2.30	2.65	3.92 ± 1.44
33	3.96	1.48	1.40	0.83	-	0.38	3.96	1.34 ± 1.24
34	2.45	0.92	0.87	1.66	-	-	2.45	0.48 ±00.64
35	10	6.66	6.84	11.6	5.94	3.07	9.23	7.35 ± 2.77
36	1.88	-	-	-	-	-	1.88	±
37	20.3	17.9	15.6	20	22.4	18.07	6.8	19.04 2.15
38	16.9	5.37	4.03	9.5	2.14	3.14	12.8	6.86 ± 5
39	10.9	7.59	7.54	11.6	8.9	9.23	4.01	9.29 ± 1.52

$R.H_1$ & $R.H_5$ = Replica $R.H_{2,3,4 \& 6}$ = different repeats

Table A.5

B. fragilis (NCTC 9343)

Relative heights, Range and Average relative heights with peak No. 13 fixed at 100

Peak No	$R.H_1$	$R.H_2$	$R.H_3$	$R.H_4$	$R.H_5$	Range	Average height ± SD
1	26.09	14.2	14.3	17.02	14.1	11.9	17.14 ± 4.60
2	7.80	9.52	4.81	7.44	4.56	4.96	6.82 ± 1.88
3	2.19	1.07	0.96	1.27	1.25	1.23	1.34 ± 0.43
4	12.9	14.2	11.8	12.7	10.8	3.4	12.48 ± 1.13
5	6.09	7.1	3.85	5.10	3.62	3.48	5.15 ± 1.32
6	6.34	7.1	3.73	6.38	3.62	3.48	5.43 ± 1.46
7	26.09	30.9	14.4	27.6	14	16.9	22.5 ± 7.03
8	48.04	32.1	41.5	41.4	48.7	45.9	42.3 ± 15.7
9	27.8	28.09	18.07	22.5	18.7	10.02	23.1 ± 4.14
10	23.6	30.2	9.63	21.4	12.7	17.5	19.50 ± 7.40
11	8.78	11.4	4.27	4.89	3.87	7.53	6.64 ± 2.95
12	16.2	22.6	14.4	14.4	8.81	13.7	15.28 ± 4.4
13	100	100	100	100	100	0	100 ± 0
14	2.92	4.76	4.21	1.91	2.12	2.85	3.18 ± 1.1
15	2.53	3.57	1.32	1.91	3.25	7.94	2.51 ± 0.83
16	10.4	16.07	10.8	8.51	9.37	7.56	11.03 ± 2.64
17	12.4	14.2	11.4	6.38	11.7	10.1	11.21 ± 2.06
18	9.26	5.35	4.21	10.8	4.25	6.59	6.77 ± 2.73
19	9.63	8.33	5.30	7.65	6	4.33	7.38 ± 1.56
20	7.19	3.57	4.45	4.25	5	3.44	4.89 ± 1.23
21	22.4	9.52	10.8	16.3	11.2	12.8	14.04 ± 4.77
22	9.75	5.35	4.63	11.7	2.87	7.55	6.86 ± 3.31
23	52.9	48.8	49.3	56.3	55	36.5	52.4 ± 2.99
24	36.5	27.3	36.1	40.4	37.5	13.1	35.6 ± 4.32
25	7.80	2.97	5.20	6.27	5.12	4.83	5.49 ± 1.57
26	134.1	78.5	75.9	106.3	90	58.2	96.9 ± 21.4
27	3.65	1.54	2.40	3.51	1.87	2.11	2.59 ± 0.85
28	5.85	3.57	3.55	5.21	3	2.85	4.23 ± 1.09
29	24.3	11.3	15.6	17.2	16.2	13	16.92 ± 4.20
30	9.75	7.02	7.22	10.4	7.5	3.38	8.37 ± 1.40
31	6.09	5.23	4.81	5.31	6.25	1.44	5.53 ± 0.54
32	3.90	1.42	2.16	3.16	2.25	2.48	2.66 ± 0.93
33	5.26	3.92	3.13	4.36	2.75	2.61	3.90 ± 0.92
34	2.92	-	2.28	-	-	2.92	1.04 ± 0.32
35	36.5	19.6	19.2	32.9	17.5	17.3	25.14 ± 7.91
36	3.17	0.59	3.07	2.76	2.12	2.58	2.34 ± 0.94
37	31.7	21.07	21.08	24.4	25	10.63	24.65 ± 3.88
38	26.8	13.09	20.4	22.3	22.5	13.7	21.01 ± 4.48
39	10.4	8.33	8.61	12.1	11.6	3.77	10.20 ± 1.52

$R.H_1$ & $R.H_4$ = replica $R.H_{2,3\ \&\ 5}$ = different repeats

Table A.6

B.vulgatus (WBM137)

Relative heights, Range and Average relative heights with peak No. 13 fixed at 100

Peak No	R. H_1	R. H_2	R. H_3	Range	Average Relative height ± SD
1	13.8	12.4	15.9	3.5	14.03 ± 1.43
2	2.75	4.15	4.43	1.48	3.77 ± 0.64
3	1.02	1.38	1.95	0.93	1.45 ± 0.38
4	8.36	11.2	9.33	2.84	9.64 ± 1.17
5	3.67	8.84	4.74	1.07	4.08 ± 0.46
6	3.26	2.38	3.04	0.88	2.89 ± 0.37
7	18.3	10.8	11.9	7.5	13.6 ± 3.3
8	57.1	41.5	47.9	15.6	48.8 ± 6.4
9	14.6	17.4	18.5	3.9	16.8 ± 1.64
10	13.06	13	11.3	1.76	12.4 ± 0.81
11	3.26	3.23	4.63	1.4	3.70 ± 0.65
12	6.73	4.76	8.24	3.48	6.57 ± 1.42
13	100	100	100	0	100
14	2.04	1.69	3.09	1.13	2.27 ± 0.59
15	2.24	1.07	3.09	2.02	2.13 ± 0.82
16	4.08	2.15	5.15	3	3.79 ± 1.24
17	5.51	2.46	10.3	7.84	6.09 ± 3.22
18	7.95	7.84	11.8	3.96	9.19 ± 1.84
19	6.73	8.15	5.15	3	6.67 ± 1.22
20	3.97	4.07	6.18	2.21	4.74 ± 1.01
21	15.5	12.4	18.5	6.1	15.4 ± 2.49
22	5.51	2.15	2.06	3.45	3.24 ± 1.60
23	42.8	49.2	76.2	33.4	56.06 ± 14.4
24	17.5	18.4	26.8	9.3	20.9 ± 4.18
25	1.09	4.23	3.09	4.23	2.80 ± 0.57
26	69.3	68.4	100	31.6	79.2 ± 14.6
27	2.44	2	5.15	3.15	3.19 ± 1.39
28	2.44	1.84	4.12	2.28	2.8 ± 0.96
29	8.97	14.6	15.4	6.43	12.9 ± 2.86
30	3.26	4.46	7.21	3.95	4.97 ± 1.65
31	2.85	4.30	6.18	3.33	4.44 ± 1.36
32	0.56	2.15	1.54	2.15	1.84 ± 0.3
33	0.96	2.61	2.06	2.61	2.33 ± 0.27
34	1.52	2.15	2.57	2.57	2.36 ± 0.21
35	7.75	12.9	10.3	5.15	10.31 ± 2.10
36	0.45	1.69	0.51	1.69	1.1 ± 0.59
37	20	18.3	29.8	11.5	22.7 ± 5.06
38	1.02	7.23	5.67	6.21	4.64 ± 2.63
39	7.34	8.46	9.27	1.93	8.35 ± 0.79

R.H_1 - R.H_3 = different repeats

Table A.7

B.melaninogenicus SS. intermedius (T588)

Relative heights, Range and Average relative heights with peak No. 13 fixed at 100

Peak No	$R.H_1$	$R.H_2$	$R.H_3$	$R.H_4$	$R.H_5$	Range	Average height ± SD
1	15.7	21.2	19.07	12.8	16.7	8.4	17.09 ± 2.87
2	12.85	15.3	21.03	12.4	23.02	24.6	16.92 ± 10.1
3	2.38	4.69	4.07	3.2	3.48	2.3	3.56 ± 0.78
4	10.4	22.1	24.4	9.6	17.4	14.8	16.78 ± 5.90
5	2.38	3.12	3.33	2.4	2.55	0.95	2.75 ± 0.39
6	3.33	9.37	6.66	2.8	5.11	6.57	5.45 ± 2.38
7	22.8	39.06	29.6	20.4	17.9	21.1	25.95 ± 7.60
8	119.04	146.8	162.9	80	93.02	66.8	120.3 ± 31.2
9	32.3	43.7	36.2	24.4	25.5	18.2	32.4 ± 7.12
10	13.3	21.8	31.4	14.8	37.2	23.9	23.7 ± 9.30
11	11.9	16.5	12.9	8.8	11.1	7.7	12.24 ± 2.52
12	8.57	19.06	17.7	10.4	14.1	10.4	13.96 ± 4.04
13	100	100	100	100	100	0	100
14	1.90	4.37	2.22	2.8	2.79	2.47	2.81 ± 0.84
15	3.33	5.31	4.44	2	3.02	2.44	3.62 ± 1.1
16	114.2	181.2	162.9	104	155.8	77.2	143.6 ± 29.5
17	33.3	59.3	68.5	36	74.4	41.1	54.3 ± 16.7
18	4.28	8.43	14.4	4.4	9.06	10.1	8.11 ± 3.7
19	6.19	8.12	6.48	5.6	6.04	2.52	6.48 ± 0.86
20	5.71	10.3	8.51	5.6	9.30	4.7	7.88 ± 1.90
21	19.5	28.1	28.1	19.2	25.5	8.9	24.08 ± 3.97
22	1.90	3.43	4.07	2.8	2.79	2.17	2.99 ± 0.72
23	71.4	121.8	103.7	64	88.3	57.8	89.34 ± 21
24	3.33	6.56	3.14	2.4	2.79	4.16	3.64 ± 1.49
25	7.14	14.06	15.1	17.2	14.4	12.06	13.58 ± 3.39
26	40.4	71.8	51.8	40.4	41.8	31.6	49.24 ± 12
27	-	1.40	1.85	2.4	1.86	1.86	1.50 ± 0.35
28	3.80	6.56	-	3.2	-	6.56	2.71 ± 1.46
29	7.61	15.6	14.07	15.2	11.6	7.99	12.8 ± 2.95
30	6.19	12.5	7.03	7.2	6.97	6.31	7.97 ± 2.28
31	5.71	8.43	7.40	4.8	7.44	3.63	6.75 ± 1.31
32	3.80	10.6	8.70	4.8	-	10.6	5.58 ± 2.78
33	-	4.68	3.70	4.4	3.02	4.68	3.16 ± 0.64
34	-	3.12	-	1.6	-	3.12	0.94 ± 0.76
35	6.66	23.4	22.5	12.4	13.9	16.7	15.8 ± 6.34
36	-	-	-	-	-	-	-
37	26.1	48.4	38.5	24	32.5	24.4	33.9 ± 8.8
38	-	7.5	-	2.8	-	7.5	2.06 ± 2.35
39	7.61	10.9	9.62	6	7.44	4.9	8.31 ± 1.73

$R.H_1$ - $R.H_5$ are different repeats

Table A.8

B.melaninogenicus SS. melaninogenicus (VPI 9085)

Relative heights, Range and Average relative heights with peak No. 13 fixed at 100

Peak No	$R.H_1$	$R.H_2$	$R.H_3$	$R.H_4$	$R.H_5$	$R.H_6$	Range	Average height ± SD
1	11.7	18.1	22	11.1	14	19.2	10.9	16.01 ± 4.02
2	5.56	4.15	4.5	3.33	5.33	2.59	2.97	4.24 ± 1.04
3	4.55	3.96	3.5	5.18	4	4.44	1.68	4.27 ± 0.53
4	6.32	10.1	9.5	7.03	5.66	8.51	4.44	7.85 ± 1.63
5	7.08	5.66	5.75	5.18	7	4.81	2.27	5.91 ± 0.85
6	1.77	2.61	2	-	1	6.29	6.29	2.27 ± 1.85
7	6.32	4.90	5	7.40	5.33	7.77	2.87	6.12 ± 1.13
8	32.1	64.1	70	29.2	32	41.4	40.8	44.8 ± 16.2
9	20.7	21.6	18.2	13.7	30.6	11.8	11.8	19.43 ± 6.10
10	13.2	11.1	12.5	10.3	12.3	14.07	3.77	12.24 ± 1.25
11	5.06	4.52	5.25	4.44	5	6.29	1.85	5.09 ± 0.60
12	6.58	5.66	13.2	5.18	5.66	6.66	7.54	7.15 ± 2.75
13	100	100	100	100	100	100	0	100 ± 0
14	2.27	2.64	2.5	2.22	3	2.22	0.78	2.47 ± 0.28
15	3.03	2.26	2.5	1.48	3	2.59	1.55	2.47 ± 0.52
16	11.1	7.16	11.7	5.92	6.66	9.25	5.78	8.63 ± 2.20
17	10.6	9.43	11.7	7.77	11.3	7.40	4.4	9.70 ± 1.65
18	4.30	2.26	4.7	2.96	5.33	2.96	3.07	3.75 ± 1.09
19	8.86	5.66	5	4.44	7.33	4.44	4.42	5.95 ± 1.62
20	6.58	7.16	8	3.70	7.66	7.77	4.3	6.81 ± 1.46
21	12.6	22.2	28.5	17.03	15.6	28.1	15.9	20.6 ± 6.09
22	0.50	1.50	1.25	-	5	1.11	5	1.87 ± 1.59
23	117.7	109.4	117.5	85.1	120	122.2	37.1	111.9 ± 12.6
24	29.8	30.9	24.2	16.6	32	11.8	20.2	24.2 ± 7.62
25	6.83	7.92	2	-	8.66	-	8.66	6.35 ± 2.59
26	98.7	79.2	105	59.2	123.3	74.07	64.1	89.9 ± 21.3
27	1.77	4.15	7	3.33	-	3.33	4.15	3.91 ± 1.72
28	2.78	3.39	4	2.96	3.66	4.07	1.29	3.47 ± 0.48
29	18.2	21.1	20	8.88	19	8.88	12.2	16.01 ± 5.11
30	8.35	7.54	8.5	5.92	8.66	5.92	2.74	7.48 ± 1.15
31	10.8	7.92	8.5	6.66	11.3	6.29	5.01	8.57 ± 1.90
32	2.53	2.26	-	-	3.33	-	3.33	2.70 ± 0.45
33	4.05	4.52	3.75	2.22	5.66	-	5.66	4.04 ± 1.11
34	-	-	-	-	-	-	-	-
35	15.5	17.3	15.7	11.1	19	6.66	12.3	14.2 ± 4.14
36	2.27	-	-	-	-	-	2.27	2.27
37	35.4	33.5	40	23.3	41	32.9	17.7	34.3 ± 5.80
38	21.7	18.8	14.25	9.24	25.6	7.69	18.8	16.21 ± 8.06
39	12.6	13.5	13	8.51	17	7.77	9.23	12.06 ± 3.12

$R.H_2$ & $R.H_3$ = replica

$R.H_{1,4,5.6}$ = different repeat

Table A.9

B.asaccharolyticus (W50)

Relative heights, Range and Average relative heights with peak No. 13 fixed at 100

Peak No	R. H_1	R. H_2	R. H_3	Range	Average Relative height ± SD
1	14.01	13.68	17.34	3.66	15.01 ± 1.65
2	16.62	13.63	12.51	4.11	14.25 ± 1.73
3	4.51	2.42	2.53	2.09	3.15 ± 0.96
4	12.89	17.41	15.66	4.52	15.32 ± 1.86
5	4.52	2.26	2.16	2.36	2.98 ± 1.08
6	6.41	4.77	4.33	2.08	3.24 ± 1.85
7	27.45	24.85	21.74	5.71	24.68 ± 2.33
8	63.6	131.8	139.4	75.8	111.6 ± 34.08
9	26.5	30.8	32.4	5.9	29.9 ± 2.49
10	22	19.7	25.8	6.1	22.5 ± 2.51
11	12.71	10.31	8.66	4.05	10.56 ± 1.66
12	14.72	12.63	16.72	4.09	14.69 ± 1.67
13	100	100	100	0	100 ± 0
14	1.50	4.21	2.16	2.71	2.62 ± 1.15
15	3.92	2.86	6.03	3.17	4.27 ± 1.31
16	112.5	101.7	109	10.8	107.7 ± 4.49
17	38.2	41.6	46.8	8.6	42.2 ± 3.53
18	8.81	7.82	11.63	3.81	9.42 ± 1.61
19	4.68	4.24	7.16	2.92	5.36 ± 1.28
20	5.33	6.72	9.13	3.88	7.06 ± 1.56
21	24.51	25.99	31.88	7.37	27.46 ± 3.18
22	1.47	2.03	3.22	1.75	2.24 ± 0.72
23	97.7	114.9	91.3	23.6	101.3 ± 9.96
24	2.23	6.32	4.11	4.09	4.22 ± 1.67
25	10.92	12.03	15.33	4.41	12.76 ± 1.87
26	37.9	40.9	46.3	8.4	41.7 ± 3.47
27	1.55	1.15	1.92	0.77	1.54 ± 0.31
28	4.78	5.57	6.81	2.03	5.72 ± 0.83
29	15.43	13.65	12.95	2.48	14.01 ± 1.04
30	8.68	4.92	5.72	3.76	6.44 ± 1.61
31	7.35	5.46	8.31	2.85	7.04 ± 1.18
32	3.21	7.67	5.41	4.46	5.43 ± 1.82
33	2.98	2.23	5.32	3.09	3.51 ± 1.31
34	1.91	3.55	2.16	1.64	2.54 ± 0.72
35	28.62	22.49	21.82	6.8	24.31 ± 3.05
36	3.09	2.21	2.29	0.38	2.53 ± 0.39
37	31.49	26.64	34.63	7.99	30.92 ± 3.28
38	6.95	6.02	9.71	3.69	7.56 ± 1.56
39	11.26	9.33	12.71	3.38	11.1 ± 1.38

The 3 runs represent 3 different repeats ($R.H_1$ - $R.H_3$)

Table A.10

B.asaccharolyticus (B536)

Relative heights, Range and Average relative heights with peak No. 13 fixed at 100

Peak No	R. H_1	R. H_2	R. H_3	Range	Average Relative height ± SD
1	15.82	12.95	15.66	2.87	14.81 ± 1.31
2	12.91	15.48	15.29	2.57	14.56 ± 1.16
3	4.01	3.82	1.11	2.90	2.98 ± 1.32
4	15.16	17.90	15.54	2.74	16.2 ± 1.21
5	3.16	2.10	1.67	1.49	2.31 ± 0.62
6	5.90	6.98	5.48	1.5	6.12 ± 0.63
7	27.41	25.11	24.34	3.07	25.62 ± 1.30
8	64.88	137.76	148.06	83.18	116.9 ± 37.02
9	30.31	27.92	24.57	5.74	27.6 ± 2.35
10	27.39	21.66	23.85	5.73	24.3 ± 2.36
11	14.21	9.33	10.57	4.88	11.37 ± 2.07
12	15.86	11.57	13.91	4.29	13.78 ± 1.75
13	100	100	100	100	100 ± 0
14	2.73	2.59	3.92	1.33	3.08 ± 0.59
15	3.54	3.93	4.59	1.05	4.02 ± 0.43
16	126.5	101.6	107.0	24.9	111.7 ± 10.6
17	51.9	39.1	40.9	12.8	43.96 ± 5.65
18	9.29	11.67	9.97	2.38	10.31 ± 1
19	6.76	3.87	4.41	2.89	5.01 ± 1.25
20	9.44	7.63	6.96	2.48	8.01 ± 1.04
21	28.32	22.41	25.17	5.91	25.3 ± 2.41
22	4.12	2.84	2.37	1.75	3.11 ± 0.73
23	122.5	98.4	101.9	24.1	107.6 ± 10.61
24	5.82	4.34	4.49	1.48	4.88 ± 0.66
25	16.19	10.35	14.29	5.84	13.61 ± 2.43
26	46.4	48.8	38.63	10.17	44.61 ± 4.34
27	1.92	3.02	1.54	1.48	2.16 ± 0.62
28	4.99	5.64	7.43	2.44	6.02 ± 1.03
29	14.1	11.2	12.8	2.9	12.7 ± 1.18
30	8.41	6.64	6.31	2.1	7.12 ± 0.92
31	6.32	7.83	7.05	1.51	7.06 ± 0.61
32	6.75	5.52	6.06	1.23	6.11 ± 0.50
33	3.42	2.56	2.45	0.97	2.81 ± 0.43
34	5.32	3.89	0.66	4.66	3.29 ± 1.94
35	31.46	23.66	23.84	7.80	26.32 ± 3.63
36	2.54	2.19	1.21	1.33	1.98 ± 0.56
37	35.99	29.34	29.65	6.65	31.66 ± 3.06
38	10.44	8.13	6.99	3.45	8.52 ± 1.43
39	10.66	8.43	10.79	2.36	9.96 ± 1.08

The 3 runs represent 3 different repeats ($R.H_1$ - $R.H_3$)

Table A.11

B.melaninogenicus SS. macacae (ATCC 33141)

Relative heights, Range and Average relative heights with peak No. 13 fixed at 100

Peak No	R. H_1	R. H_2	R. H_3	Range	Average Relative height ± SD
1	18.24	20.42	18.1	2.32	18.92 ± 1.06
2	10.17	8.19	9.33	1.98	9.23 ± 0.81
3	4.62	3.44	3.16	1.46	3.74 ± 0.63
4	13.96	11.62	11.56	2.40	12.38 ± 1.11
5	5.13	3.88	3.44	1.69	4.15 ± 0.71
6	4.22	4.86	2.86	2	3.98 ± 0.83
7	16.12	14.54	18.63	4.09	16.43 ± 1.68
8	76.7	81.9	89.5	12.8	82.7 ± 5.25
9	26.9	22.6	26.4	4.3	25.3 ± 1.92
10	15.84	17.34	19.92	4.08	17.70 ± 1.68
11	10.63	7.46	7.74	3.17	8.61 ± 1.43
12	10.38	9.59	12.64	3.05	10.87 ± 1.29
13	100	100	100	0	100 ± 0
14	2.68	2.22	1.76	0.92	2.22 ± 0.37
15	3.47	2.13	3.65	1.52	3.08 ± 0.67
16	87.5	75.7	67.2	20.3	76.8 ± 8.32
17	33.5	26.5	37.8	11.3	32.6 ± 4.65
18	13.39	12.33	10.55	2.84	12.09 ± 1.17
19	6.40	6.32	5.84	0.56	6.18 ± 0.24
20	8.21	7.38	6.43	1.78	7.34 ± 0.72
21	26.94	20.29	20.93	6.65	22.72 ± 2.99
22	3.21	3.53	2.32	0.32	3.02 ± 0.51
23	97.6	114.4	91.5	22.9	101.16 ± 9.68
24	14.57	13.34	12.32	2.25	13.41 ± 0.91
25	28.39	29.92	25.35	4.57	27.88 ± 1.89
26	64.68	67.95	76.62	11.94	69.75 ± 5.03
27	2.16	2.54	1.99	0.55	2.23 ± 0.22
28	5.32	3.94	3.31	2.01	4.19 ± 0.83
29	14.31	13.52	15.67	2.15	14.5 ± 0.88
30	8.82	7.34	7.27	1.55	7.81 ± 0.71
31	8.78	7.13	7.52	1.65	7.81 ± 0.70
32	5.66	5.11	4.20	1.46	4.99 ± 0.60
33	3.83	4.64	3.59	1.05	4.02 ± 0.44
34	2.01	1.51	0.74	1.27	1.42 ± 0.52
35	14.93	13.64	18.47	4.83	15.68 ± 2.04
36	-	-	-	-	-
37	33.89	39.33	33.64	5.69	35.62 ± 2.62
38	8.69	13.08	10.24	4.39	10.67 ± 1.31
39	12.68	8.28	9.52	4.40	10.16 ± 1.85

The 3 runs represent 3 different repeats ($R.H_1$ - $R.H_3$)

Table A.12

B.ruminicola SS. brevis (NP333)

Relative heights, Range and Average relative heights with peak No. 13 fixed at 100

Peak No	R. H_1	R. H_2	R. H_3	Range	Average Relative height ± SD
1	19.22	15.50	16.61	3.72	17.11 ± 1.55
2	8.14	13.29	10.31	5.15	10.58 ± 2.11
3	4.12	1.36	1.87	2.76	2.45 ± 1.19
4	18.47	13.91	11.74	6.73	14.70 ± 2.80
5	3.24	3.91	5.71	2.47	4.28 ± 1.04
6	4.87	6.98	4.47	2.51	5.44 ± 1.10
7	22.37	23.67	27.85	5.48	24.63 ± 2.33
8	85.7	107.3	82.4	24.9	91.3 ± 11.04
9	23.63	28.92	25.3	5.29	25.95 ± 2.20
10	20.14	24.94	19.72	5.22	21.60 ± 2.36
11	8.36	11.02	11.34	2.98	10.24 ± 1.33
12	12.69	13.72	16.41	3.72	14.72 ± 1.56
13	100	100	100	0	100 ± 0
14	1.52	4.39	3.67	2.87	3.19 ± 1.21
15	2.73	4.29	4.02	1.56	3.68 ± 0.68
16	76.4	97.62	89.5	21.22	87.84 ± 8.74
17	38.32	46.81	43.45	8.49	42.86 ± 3.49
18	7.85	9.36	6.43	2.93	7.88 ± 1.19
19	8.31	7.42	5.81	2.50	7.18 ± 1.03
20	6.41	5.55	7.48	1.93	6.48 ± 0.78
21	18.62	15.49	21.87	6.38	18.66 ± 2.60
22	5.67	5.61	4.11	1.56	5.13 ± 0.72
23	49.74	53.88	64.83	15.09	56.15 ± 6.36
24	17.02	21.68	18.72	4.66	19.14 ± 1.92
25	12.16	9.09	8.39	3.77	9.88 ± 1.63
26	54.92	53.75	70.85	17.1	59.84 ± 7.79
27	1.69	2,71	2.74	1.05	2.38 ± 0.48
28	5.08	3.43	3.22	1.86	3.91 ± 0.83
29	12.1	11.71	15.03	3.32	12.94 ± 1.48
30	6.77	5.01	4.39	2.38	5.39 ± 1
31	6.54	3	3.24	3.54	4.26 ± 1.61
32	3.15	6.91	3.86	3.76	4.64 ± 1.63
33	3.21	6.42	2.79	3.63	4.14 ± 1.62
34	2.11	2.36	1.11	1.25	1.86 ± 0.54
35	20.02	27.47	22.89	7.45	23.46 ± 3.06
36	1.07	1.84	0.96	0.88	1.29 ± 0.39
37	26.43	30.78	32.82	6.39	30.01 ± 2.66
38	15.26	11.93	12.62	3.33	13.27 ± 1.43
39	8.01	12.86	8.74	4.85	9.87 ± 2.13

R.$H_{1,2\ \&\ 3}$ represent different repeats

Table A.13

E.coli (NCTC 9001)

Relative heights, Range and Average relative heights with peak No. 13 fixed at 100

Peak No	$R.H_1$	$R.H_2$	$R.H_3$	Range	Average Relative height ± S D
1	12.90	13.46	11.30	2.16	12.55 ± 0.91
2	2.37	3.85	2	1.85	2.74 ± 0.79
3	3.27	4.47	2.99	1.48	3.57 ± 0.64
4	14.72	18.82	12.65	6.17	15.39 ± 2.56
5	-	-	-	-	-
6	2.90	3.08	2.23	0.85	2.73 ± 0.36
7	9.45	10.98	8.79	2.19	9.74 ± 0.91
8	34.54	48.39	29.86	18.53	37.59 ± 7.86
9	12.72	14.92	11.73	3.19	13.12 ± 1.33
10	7.09	11.54	8.11	4.45	8.91 ± 1.90
11	4.75	6.51	4.31	2.2	5.19 ± 0.90
12	12.90	13.85	11.47	2.38	12.74 ± 0.97
13	100	100	100	100	100 ± 0
14	2.36	3.58	2.49	1.22	2.81 ± 0.54
15	3.45	4.87	3.76	1.42	4.02 ± 0.60
16	2.36	3.91	2.83	1.55	3.03 ± 0.64
17	6	8.32	7.19	2.32	7.17 ± 0.94
18	4.28	5.98	4.73	1.7	4.99 ± 0.71
19	-	-	-	-	-
20	-	-	-	-	-
21	10.18	13.44	11.96	3.26	11.86 ± 1.33
22	1.27	3.85	2.14	2.58	2.42 ± 1.07
23	17.45	19.53	18.80	2.08	18.59 ± 0.86
24	10.36	11.39	10	1.39	10.58 ± 0.58
25	-	-	-	-	-
26	38.18	48.42	44.85	10.24	43.81 ± 4.24
27	-	-	-	-	-
28	3.63	4.75	4.19	1.12	4.19 ± 0.45
29	2	6.09	3.34	4.09	3.81 ± 1.70
30	0.91	2.11	1.68	1.2	1.56 ± 0.49
31	1.81	2.34	2.02	0.53	2.05 ± 0.21
32	-	-	-	-	-
33	-	-	-	-	-
34	-	-	-	-	-
35	-	-	-	-	-
36	18.18	23.46	21.65	5.28	21.09 ± 2.19
37	-	-	-	-	-
38	2.36	4.39	3.39	2.03	3.38 ± 0.82
39	-	-	-	-	-

$R.H_{1, 2 \& 3}$ represent different repeats

Table A.14

Streptococcus feacalis (NCIB 6783)

Relative heights, Range and Average relative heights with peak No. 13 fixed at 100

Peak No	$R.H_1$	$R.H_2$	$R.H_3$	Range	Average Relative height ± S D
1	25.27	29.34	19.61	9.73	24.74 ± 3.98
2	8.33	13.40	7.17	6.23	9.63 ± 2.70
3	10	12.50	7.89	4.61	10.13 ± 1.88
4	7.77	9.56	5.93	3.63	7.75 ± 1.48
5	7.22	11.31	10.76	4.09	9.76 ± 1.81
6	4.72	4.81	2.64	2.17	4.05 ± 1.00
7	11.66	13.86	14.16	2.5	13.22 ± 1.11
8	66.6	78.31	35.89	42.42	60.26 ± 17.8
9	21.11	19.86	20.75	1.25	20.57 ± 0.52
10	7.51	7.42	5.96	1.55	6.96 ± 0.71
11	4.72	8.16	7.61	3.44	6.83 ± 1.50
12	5.27	7.34	6.46	2.07	6.35 ± 0.84
13	100	100	100	100	100 ± 0
14	1.66	3.11	0.99	2.12	1.92 ± 0.88
15	3.05	7.38	4.16	4.33	4.86 ± 1.83
16	13.15	18.79	14.11	5.64	15.35 ± 2.46
17	17.22	26.32	19.03	9.1	20.85 ± 3.93
18	4.72	5.39	3.19	2.2	4.43 ± 0.92
19	5	6.42	5.71	1.42	5.71 ± 0.57
20	-	-	-	-	-
21	8.05	11.21	10.32	3.16	9.86 ± 1.33
22	2.50	3.40	2.84	0.9	2.91 ± 0.37
23	29.16	39.44	31.96	10.28	33.52 ± 4.33
24	4.72	6.57	5.99	2.03	5.82 ± 0.83
25	-	-	-	-	-
26	17.2	25.79	22.16	8.59	21.71 ± 3.52
27	2.22	4.09	3.11	1.87	3.14 ± 0.76
28	-	-	-	-	-
29	0.83	2.56	1.99	1.73	1.79 ± 0.71
30	1.66	3.05	0.66	2.39	1.79 ± 0.98
31	-	-	-	-	-
32	-	-	-	-	-
33	2.22	3.15	0.93	2.17	2.11 ± 0.88
34	1.94	4.78	4.13	2.84	3.63 ± 1.22
35	1.86	2.99	2.16	1.13	2.33 ± 0.47
36	8.88	19.73	11.41	10.85	13.34 ± 4.63
37	-	-	-	-	-
38	4.16	7.32	6.53	3.16	6 ± 1.34
39	-	-	-	-	-

$R.H_1$, 2 & 3 represent different repeats

Table A.15

Clostridium sporogenes (NCIB 532)

Relative heights, Range and Average relative heights with peak No. 13 fixed at 100

Peak No	$R.H_1$	$R.H_2$	$R.H_3$	Range	Average Relative height ± S D
1	13.75	9.51	14.26	4.75	12.50 ± 2.12
2	4.16	3.19	4.32	1.13	3.89 ± 0.49
3	3.75	4.43	6.42	2.67	4.86 ± 1.13
4	1.25	1.05	1.99	0.94	1.43 ± 0.40
5	8.33	8.11	7.19	1.14	7.87 ± 0.49
6	-	-	-	-	-
7	9.08	7.16	11.08	3.92	9.10 ± 1.60
8	38.75	25.95	33.11	12.8	32.60 ± 5.23
9	9.58	10.99	11.42	1.84	10.66 ± 0.78
10	5.83	5.71	6.72	1.01	6.08 ± 0.45
11	10	10.11	10.9	0.9	10.33 ± 0.40
12	10.83	8.97	12.24	3.27	10.68 ± 1.33
13	100	100	100	100	100 ± 0
14	3.75	2.59	4.11	1.52	3.48 ± 0.64
15	6.66	5.74	7.11	1.37	6.50 ± 0.57
16	1.66	0.56	1.79	1.23	1.33 ± 0.55
17	20.17	21.44	22.04	1.87	21.21 ± 0.77
18	-	-	-	-	-
19	7.5	7.43	8.51	1.08	7.81 ± 0.49
20	2.08	1.01	3.17	2.16	2.08 ± 0.88
21	27.08	28.59	29.07	1.99	28.24 ± 0.84
22	3.75	3.56	6.26	2.7	4.52 ± 1.23
23	-	-	-	-	-
24	58.75	50.79	63.32	12.53	57.62 ± 5.17
25	13.75	12.42	14.25	1.83	13.47 ± 0.77
26	14.16	11.03	13.39	3.13	12.86 ± 1.33
27	108.33	121.9	111.9	13.57	114.04 ± 5.74
28	-	-	-	-	-
29	-	-	-	-	-
30	75	59.66	61.41	15.34	65.35 ± 6.85
31	4.16	3.09	5.17	2.08	4.14 ± 0.84
32	4.58	3.96	5.49	1.53	4.67 ± 0.62
33	7.08	6.45	7.79	1.34	7.10 ± 0.54
34	-	-	-	-	-
35	6.25	5.74	6.94	1.2	6.31 ± 0.49
36	8.33	7.04	7.89	1.29	7.75 ± 0.53
37	37.91	39.16	40.58	2.67	39.21 ± 1.09
38	24.58	23.83	27.71	3.88	25.37 ± 1.68
39	-	-	-	-	-

$R.H_{1, 2 \& 3}$ represent different repeats

Table A.16 Experimental Infection

B.fragilis (NCTC 8560) pure infection

Direct analysis of pus material giving the relative height, range and average with peak No. 13 fixed at 100

Peak NO	$R.H_1$	$R.H_2$	$R.H_3$	$R.H_4$	Range	Average Relative height ± SD
1	11.17	9.84	8.15	13.29	5.14	10.61 ± 1.88
2	1.78	1.92	2.03	2.63	0.85	2.09 ± 0.32
3	0.94	1.38	1.73	2.39	1.45	1.61 ± 0.53
4	3.41	4	3.84	6.64	3.23	4.47 ± 1.26
5	0.64	0.76	0.98	1.11	0.74	0.87 ± 0.18
6	1.47	1.07	1.57	2.27	1.2	1.59 ± 0.43
7	3.94	4.30	4.15	5.16	1.25	4.38 ± 0.46
8	18.82	20	38.63	40.05	21.23	29.37 ± 9.98
9	4.72	3.38	3.12	5.28	2.16	4.12 ± 0.90
10	3.54	2.46	2.37	4.36	1.99	3.18 ± 0.80
11	1.47	1.53	1.89	2.11	0.64	1.75 ± 0.26
12	-	-	-	-	-	-
13	100	100	100	100	100	100 ± 0
14	2.35	1.38	1.46	2.03	0.97	1.80 ± 0.40
15	2.29	1.52	2	2.40	0.88	2.05 ± 0.34
16	4.23	3.68	4.12	5.23	1.55	4.31 ± 0.56
17	2.64	1.69	1.89	2.81	1.12	2.25 ± 0.47
18	1.76	1.84	0.89	2.16	1.27	1.66 ± 0.47
19	-	-	-	-	-	-
20	-	-	-	-	-	-
21	7.35	8.46	9.33	10.7	3.35	8.96 ± 1.22
22	1.17	2.22	1.11	2.30	1.19	1.7 ± 0.56
23	15.8	19.2	21.31	26.61	10.81	20.73 ± 3.92
24	4.70	4.92	3.25	5.73	2.48	4.65 ± 0.89
25	-	-	-	-	-	-
26	28.23	24.98	32.51	41.28	16.3	31.75 ± 6.11
27	-	-	-	-	-	-
28	-	-	-	-	-	-
29	2.05	2.76	2.89	3.78	1.73	2.87 ± 0.61
30	0.76	0.46	0.95	1.94	1.48	1.02 ± 0.55
31	0.82	1.07	2.14	2.98	2.16	1.75 ± 0.86
32	0.88	0.76	1.30	2.26	1.5	1.3 ± 0.58
33	-	-	-	-	-	-
34	-	-	-	-	-	-
35	-	-	-	-	-	-
36	-	-	-	-	-	-
37	8.88	8.23	9.04	11.62	3.39	9.44 ± 1.29
38	5.47	5.92	4.03	6.89	2.86	5.57 ± 1.03
39	-	-	-	-	-	-

$R.H_{1\ \&\ 2}$ are replica $R.H_{3,4}$ = different repeats

Table A.17 Experimental Infection

B.asaccharolyticus (W50) pure infection

Direct analysis of pus material giving the relative height, range and average with peak No. 13 fixed at 100

Peak No	$R.H_1$	$R.H_2$	$R.H_3$	Range	Average Relative height ± SD	
1	65.50	62.92	40.73	24.77	56.38	11.11
2	5.17	3.38	3.62	1.79	4.05	0.79
3	3.44	1.89	2.51	1.55	2.61	0.63
4	6.20	2.47	4.85	3.73	4.50	1.54
5	2.06	1.68	2.20	0.52	1.98	0.21
6	6.55	3.03	5.18	3.52	5.54	1.68
7	7.24	3.25	6.14	3.99	5.54	1.68
8	175.80	105.60	138.24	70.2	139.8	28.68
9	27.58	19.58	24.15	8	23.77	3.27
10	13.79	9.50	11.31	4.29	11.53	1.75
11	6.20	3.96	4.81	2.24	4.99	0.92
12	6.55	5.05	4.41	2.14	5.33	0.89
13	100	100	100	100	100	0.[illegible]
14	3.44	3.25	5.51	2.26	4.06	1.02
15	3.54	2.69	2.89	0.85	3.04	0.36
16	55.86	38.65	46.53	17.21	47.01	7.03
17	26.20	32.93	40.14	13.94	33.09	5.67
18	8.27	3.70	6.28	4.57	6.08	1.87
19	3.44	1.68	4.81	3.13	3.31	1.28
20	4.13	3.42	3.13	1	3.56	0.42
21	23.44	16.85	19.51	6.59	19.93	2.70
22	6.89	4.49	3.27	3.62	4.88	1.50
23	48.27	38.08	69.31	31.23	51.88	13
24	8.82	5.61	7.46	3.21	7.29	1.31
25	8.27	5.76	8.18	2.51	7.40	1.16
26	100	80.78	87.43	19.22	89.40	7.96
27	-	-	-	-	-	
28	-	-	-	-	-	
29	10.34	7.86	9.25	2.48	9.15	1.01
30	5.86	7.68	6.44	1.82	6.66	0.75
31	7.72	5.05	5.98	2.67	6.25	1.10
32	4.13	6.74	3.63	3.11	4.83	1.36
33	-	-	-	-	-	
34	-	-	-	-	-	
35	13.37	11.23	9.39	3.98	11.33	1.62
36	-	-	-	-	-	
37	48.62	37.07	29.17	19.45	38.28	7.98
38	18.27	19.01	11.89	7.12	16.39	3.19
39	48.27	42.69	40.33	7.94	43.76	3.32

All runs represent 3 different repeats ($R.H_1$ - $R.H_3$)

Table A.18

Direct analysis of pus material giving the relative height, range and average with peak No.13 fixed at 100

Mixed anaerobic Infection, B.asaccharolyticus (W50), E.coli (NCTC 9001), Streptococcus feacalis (NCIB 6783), Clostridium sporogenes (NCIB 532)

Peak No	$R.H_1$	$R.H_2$	$R.H_3$	Range	Average Relative height ± S D
1	36.74	42.85	40.11	6.11	39.9 ± 2.49
2	7.88	10.35	8.13	2.47	8.87 ± 1.11
3	10.78	8.92	10.12	1.86	9.94 ± 0.76
4	15.68	10.35	11.41	5.33	12.48 ± 2.30
5	1.73	2.14	1.89	0.41	1.92 ± 0.16
6	9.80	7.85	6.58	3.22	8.07 ± 1.32
7	15.68	16.78	14.15	2.63	15.53 ± 1.07
8	18.43	25.21	17.09	8.12	20.24 ± 3.55
9	33.33	27.85	42.62	14.77	34.6 ± 6.09
10	17.64	16.78	18.81	2.03	17.74 ± 0.83
11	9.80	7.85	11.31	3.46	9.65 ± 1.41
12	11.70	7.70	7.35	4.35	8.91 ± 1.97
13	100	100	100	100	100 ± 0.
14	8.82	8.92	10.8	1.98	9.51 ± 0.91
15	6.86	6.07	7.17	1.1	6.7 ± 0.46
16	15.68	15	16.86	1.86	15.84 ± 0.76
17	9.80	11.07	8.79	2.28	9.88 ± 0.93
18	-	-	-	-	- -
19	-	-	-	-	- -
20	-	-	-	-	- -
21	48.09	43.92	59.71	15.79	50.57 ± 6.68
22	5.88	8.21	4.79	3.42	6.29 ± 1.42
23	62.74	78.58	81.30	18.56	74.20 ± 8.18
24	17.61	17.40	18.80	1.40	17.93 ± 0.61
25	11.80	16.42	12.46	4.62	13.56 ± 2.04
26	112.35	142.81	153.32	40.97	136.16 ± 17.3
27	6.88	7.85	8.10	1.22	7.61 ± 0.52
28	-	-	-	-	-
29	9.80	13.57	11.73	3.77	11.7 ± 1.53
30	5.92	7.50	6.66	1.58	6.69 ± 0.64
31	13.88	15	11.10	3.9	13.32 ± 1.63
32	7.84	6.78	8.77	1.99	7.79 ± 0.81
33	3.92	3.21	4.13	0.92	3.75 ± 0.39
34	9.90	12.14	11.98	2.24	11.34 ± 1.02
35	17.84	22.85	25.41	7.57	22.03 ± 3.14
36	47.05	60.71	56.73	13.66	54.83 ± 5.73
37	-	-	-	-	-
38	22.60	35.71	29.57	13.11	29.29 ± 5.35
39	-	-	-	-	-

All runs represent 3 different repeats. ($R.H_1$ - $R.H_3$)

Table A - 19 (After Gutteridge, 1979)

Parameters affecting Pyrolysis gas liquid chromatography

A. Microbiology

1. Nature of medium (-type-pH-Eh)
2. Nature of growth (liquid or gel) medium
3. Time and temperature of incubation
4. Type of incubation (aerobic/anaerobic)
5. Number of subcultures
6. Physiological state of organisms (sporulated/non sporulated)

B. Pyrolysis

1. Pretreatment of Curie-point wires
2. Centralisation of wires in high frequency coil
3. Position of sample on wires
4. Pyrolysis temperature
5. Total pyrolysis time
6. Sample size

C. Column

1. Type of stationary phase
2. Column degradation

D. Chromatographic conditions

1. Injector temperature
2. Detector temperature
3. Initial oven temperature
4. Final oven temperature
5. Programming rate
6. Duration of initial and terminal constant temperature periods
7. Flow rate of carrier gas
8. Nature of carrier gas
9. Flow rates of detector air and hydrogen supplies
10. Leaks in high pressure couplings
11. Recorder speed and sensitivity
12. Maintenance of flame ionization detector sensitivity.