An in vitro study of the interactions of bacteria from the human colon.

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An *In Vitro* Study of the Interactions of Bacteria from the Human Colon

by

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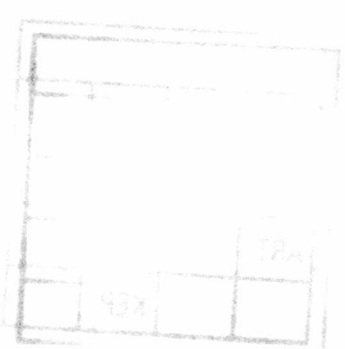
An *in vitro* model system inoculated with human faeces was used to study the fermentation of mixtures of bran, cabbage, turnip, carrot and apple dietary fibres. The results suggested that the mixture in which a dietary fibre is supplied influenced its digestibility even though the chemical composition of the different mixtures was very similar. The pentose fractions of the fibre were the least digestible. Starch-, xylan- and pectin-degrading bacteria were enumerated and the majority of isolates were capable of more than one activity. These results highlight the potential for metabolic diversity amongst the predominant bacteria *in vivo*. Total numbers of bacteria were relatively constant throughout the experiments and close to estimates of bacterial numbers in the human colon. The volatile fatty acids, acetate, propionate and butyrate were the major bacterial end-products and were produced in molar ratios close to those observed *in vivo*. Small amounts of methane and hydrogen were produced during all experiments but there was no evidence of a product-precursor relationship. It is postulated that the production of acetate from H_2 and CO_2 during homoacetic acid fermentation was the major hydrogenotrophic reaction taking place. Nitrate was reduced to N_2 during dissimilatory nitrate reduction and nitrite only appeared as a transient intermediate when nitrate concentrations increased. Ammonia was always produced in the fermentor as a by-product of the fermentation of amino acids. Sulphate was reduced to H_2S and this process was inhibited by increased concentrations of nitrate.



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To my parents and Mel for their love and support.

CONTENTS

SECTION 1

INTRODUCTION

1.1 Diet and Colonic Disease

The relationship between diet and predominantly 'western' diseases has aroused considerable interest largely as a result of the pioneering studies of Burkitt and his co-workers (Painter and Burkitt, 1971; Burkitt et al., 1972; Trowell, 1972; 1973). Despite nearly two decades of clinical, experimental and epidemiological research no unequivocal evidence has been produced which establishes the precise aetiology of these diseases although they are frequently referred to as 'diet linked' in the literature.

Disorders of the large intestine such as colo-rectal cancer, diverticular disease, irritable bowel syndrome, constipation and diarrhoea are conditions with an obvious intestinal and hence possibly dietary link but ischaemic heart disease, atherosclerosis, cholelithiasis, certain mineral deficiencies and abnormal glucose metabolism are also amongst those illnesses believed to have a dietary involvement. The association of diet with the development and treatment of disease is the subject of recent reviews by Weisburger (1985), Heatly (1985) and Kay (1982). The general acceptance of dietary manipulation in preventative medicine and clinical treatment is reflected in the routine dietary management of most of these conditions and in recent UK dietary recommendations (N.A.C.N.E.) which stress the potential benefits of certain dietary modifications.

'Western-type' diseases have therefore been associated with 'western-type' diets typically high in saturated fats and low in dietary fibre. An average daily fibre intake of 20g in the UK compares with estimates of between 38-150g in parts of Africa and the Indian sub-continent (Bingham and Cummings, 1980). In general, it is thought that diets high in meat products, consequently high in fat, predispose populations towards colonic disease and that high fibre intakes somehow diminish the pathological effects of such diets. The protein moeity of meat might also be implicated as nitrogenous compounds are prominent amongst the potential carcinogens or procarcinogens cited in the literature. Available information, however, suggests that dietary protein per se is probably of little consequence as a cause of large bowel disease as it is efficiently digested and absorbed by the small intestine. Protein metabolites produced in situ may be more important in the aetiology of diet linked diseases.

Dietary fat is implicated in large bowel disease principally through it's modulatory effect on the colonic concentration of circulating bile acids which may act as tumor promotors, carcinogens or procarcinogens subsequent to bacterial transformation and/or act directly on the colonic mucosa.

1.2 Dietary Fibre

Human digestive secretions do not contain enzymes capable of digesting the main constituents of dietary fibre namely

cellulose, hemicellulose, pectin and lignin. A wide range of plant materials composed of different amounts, forms and proportions of these polymers is collectively referred to as dietary fibre. Starch, an abundant plant polymer is not included in the definition. Indeed the major fraction of fibre is referred to as non-starch polysaccharide (N.S.P.) which on further analysis can be divided into cellulose and non-cellulosic polysaccharide (N.C.P.). N.C.P. includes hemicellulose, pectin, storage polysaccharides like inulin, guar and some other plant gums and mucilages.

Both *in vitro* and *in vivo* animal model systems have been used to investigate the effect of dietary fibre on large bowel function and contents. Conclusive evidence has been limited by the extent of individual variation (Wyman *et al.*, 1978) and the difficulties of validation of model systems. Nyman *et al.* (1986) recently produced an appraisal of studies using laboratory rats. An inherent problem common to all of these investigations is deciding which features best characterise the 'normal' large bowel with regard to structure, function and contents. The inclusion of purified dietary fibres or fibre-rich foods in the diets of subjects or *in vitro* feedstocks should be evaluated in relation to the physical and chemical composition of these fibres. Davies *et al.* (1986) suggest that a mixture of fibres may provide most relevant results given that consumption of fibres from a variety of sources is the norm in western countries. Further, studies have revealed that preparation procedures

(Schweizer et al., 1983), particle size (Broadribb and Groves, 1978), previous diet (Ehle et al., 1982) as well as the original source of the fibre will alter the behaviour of a fibre in the large bowel.

1.3 Microbial digestion of Fibre

In addition to the physico-chemical composition, the evaluation of a fibre's potential for altering colonic response requires previous knowledge of the extent to which that fibre is digested by the colonic microflora (discussed later). Non-cellulosic polysaccharides eg pectins, xylans and guar gums are extensively metabolised by the colonic microflora whereas cellulose degradation is often less complete being dependent upon structural factors such as the degree of crystallinity and association with lignin. Concentrated sources of fibre, such as bran, resist degradation primarily due to the extent of lignification (approximately 3% in this case). Evidence in support of the degradation of fibre and other substrates by the colonic microflora is conclusive. Southgate and Durnin (1970) found that of 20g of fibre consumed daily only 5-10g was recovered in faeces. Analysis of ileal and colonic contents from accident victims confirms the disappearance of high molecular weight carbohydrates from the colon (Vercellotti et al., 1978). Combining results from Stephen and Cummings (1980a) and J Banwell (unpublished results cited ibid) 15%, 25% and 33% increases in bacterial cell output were observed on

feeding 14.2g bran, 19.3g carrot and 18.3g cabbage respectively. Further, bacteria capable of digesting plant polysaccharides have been isolated from faeces (Bayliss and Houston, 1984; Dekker and Palmer, 1981; Salyers, 1981 and Salyers, 1983) and ^{14}C-labelled cellulose metabolites were measured in faecal fractions and breath samples ($^{14}CO_2$) Kelleher et al. (1984).

1.4 Water holding capacity, Faecal Bulking and the Microflora

Overlooking the potential for bacterial degradation of a fibre and the concomitant increase in bacterial cell mass probably accounts for anomalous results recorded for the effects of fibres on bowel function, particularly faecal bulking.

The water holding capacity (W.H.C.) is a property of a given fibre measured in vitro which is assumed to increase the volume of colonic contents in vivo. It has therefore been used as an index to predict a fibres' bulking effect - the greater the W.H.C. the greater the effect on faecal volume. However, Stephen and Cummings (1979) observed an inverse relationship between the W.H.C. values of seventeen different fibres and faecal bulking.

McBurney et al. (1985) assert that these apparently contradictory observations arise from experimental procedures which take no account of the microbial digestion of fibre in vivo. W.H.C. may be of greater value in predicting potential

digestibility in that the most extensively degraded fibres tend to be those with high W.H.C. values.

McBurney et al. (1985) propose that potential water holding capacity P.W.H.C. is a more accurate indicator of faecal bulking capacity as it is measured after microbial fermentation of the fibre and includes a calculation of the W.H.C. of unfermentable fibre organic matter and microbial organic matter.

Stephen and Cummings (1980a) estimate the microbial contribution to faecal mass at 55% of the dry weight and 75% of the wet weight. Any dietary component providing a substrate for the microflora may indirectly create a bulking effect due to the resultant increase in microbial biomass. Recently Kurpad and Shetty (1986) concluded from their studies that water holding capacity was the most important factor in faecal bulking and maintained that any increase in microbial biomass as a result of the fermentation of a fibre would be counterbalanced by a loss of water secondary to the absorption of volatile fatty acids (discussed later).

Experimental studies must therefore assess the potential effects of fibres largely unchanged with passage through the gut and those fermentable sources which may exert little direct effect on large bowel structure and function but which may have the most profound effects on the microflora and its metabolic end-products.

1.5 Unabsorbed Carbohydrate

In a healthy individual fibre forms the bulk of the material entering the colon. Although there is increasing evidence that a proportion of the 'digestible fraction' may also escape normal host enzymic digestion. Recent work by Wolver et al. (1984) demonstrated that 8% and 17% of bread and lentil starch was unabsorbed and subsequently fermented in the colon. Seventeen out of eighteen normal subjects malabsorbed 10-20% of the starch from white flour (Anderson et al., 1981) and malabsorption of lactose in pre-term infants appears to be the norm (Maclean and Fink, 1980). Bond et al. (1980) conclude that the colon plays an important role in the salvaging of unabsorbed carbohydrate, in their study 2-4% of ingested sucrose, reducing the osmotic activity of the sugar which could otherwise lead to extreme bouts of diarrhoea and that this function appears to depend upon bacterial metabolism of the carbohydrate. In relation to faecal bulking Shetty and Kurpad (1986) found increased faecal weights and faecal nitrogen attributable to increased microbial biomass on diets supplemented with corn starch. The importance of unabsorbed carbohydrate is discussed by Cummings et al. (1986).

1.6 The Action of Dietary Fibre in the Colon

Eastwood and Kay (1979) formulated a hypothesis to explain

the action of dietary fibre in the gastrointestinal tract which related the observed physiological responses to the physical and chemical structure of fibre. Stephen and Cummings (1980b) summarise the effects of dietary fibre as follows: fibre causes an increase in stool output, dilution of colonic contents, a faster rate of passage through the gut and changes in the colonic metabolism of minerals, nitrogen and bile acids. The consequences of these changes are generally believed to be beneficial to man but it is possible to envisage certain adverse aspects occurring simultaneously. Much of the current debate regarding the relative merits of increased intakes of dietary fibre in normal individuals is speculative and therefore, the potentially negative effects of dietary fibre cannot be discounted. The issues for and against inclusion of increased levels of dietary fibre are discussed by Stephen (1981).

The most convincing experimental verification of the beneficial effects of fibre is in the finding that it acts by increasing faecal volume and softening stools making defaecation easier. Increased stool output and decreased retention times are related to the bulking effect of fibre or as mentioned earlier microbial biomass. Kien et al. (1981) conclude that a critical volume of faeces must accumulate in the colon which prompts colonic evacuation. It would be reasonable to assume that the increased volumes of digesta caused by the presence of certain fibres would more often be sufficient to reach this critical volume thereby increasing

stool output and decreasing apparent retention time. However a simple cause and effect relationship may not exist between faecal weight and transit time. Shetty and Kurpad (1986) suggest that transit time may be intrinsic. The combined effect of increased volume and decreased transit time is to dilute colonic contents. Increasing the amount of fibre in the diet was shown to increase the dilution of a faecal marker (Southgate et al., 1978). Dilution of colonic contents may in effect mean reducing colonic exposure to toxic substances. Many potentially harmful compounds are produced or chemically transformed by bacteria eg H_2S; ammonia, nitrite and bile acids. The concept of dilution therefore hinges upon the diluent (fibre) not being digested otherwise, increased fermentation could well increase the concentration of toxic metabolites. Bran is the most commonly prescribed fibre in cases of constipation and other colonic disorders as it is found to be the most resistant to digestion. Dilution of microbial metabolites also applies to the major fermentation products acetic, propionic and butyric acids. Butyrate was shown to be a preferred energy source for isolated colonocytes (Roediger, 1980) and further evidence is consistent with absorption of the short chain volatile fatty acids (SCFA) by passive non-ionic diffusion. Decreasing the colonic concentration of SCFA would therefore lead to decreased rates of absorption.

Certain minerals including zinc, copper, calcium, manganese, magnesium, iron, phosphorous and cadmium are thought to bind

to fibrous materials most notably the phytate component. *In vitro* studies confirm the ability of wheat and maize bran to bind iron (Reinhold *et al.*, 1981 and Simpson *et al.*, 1981). Whole grain cereals contain large quantities of these minerals and so there may be in some instances, compensation in terms of net delivery of nutrient to the body. The effects of dietary fibre on mineral absorption are reviewed by James (1980). Fibre has been shown to bind bile salts *in vitro* (Floren and Nilsson, 1982) and there is a suggestion that as well as binding essential minerals, fibre may also sequester heavy metals.

The development of faecal fractionation techniques has shown that most of the energy, minerals, fat and nitrogen are in fact located in the microbial fraction but are doubtless of dietary origin (Stephen and Cummings, 1980).

1.7 Effect of Fibre on Colonic Structure

Evidence is accumulating which involves dietary fibre with changes in the gross structure of the colon and with alterations at cell level.

Cassidy *et al.* (1981) using scanning electron micrographs of rat colonic mucosa observed topographical characteristics suggestive of increased rates of cell loss with feeding bran, cellulose, pectin and alfalfa. Hageman and Stragand (1977) noted altered cell turnover kinetics upon fasting and

refeeding in rats fed normal and low residue diets and Jacobs and White (1983) showed that the mechanism by which wheat bran influenced large bowel growth varied according to the anatomical segment of the intestine. Watters et al. (1985) reported significant differences in the tensile strength and width at burst of the colon of Africans as compared with Europeans and suggest that the greater strength of the former group may be influenced by environmental factors such as diet. Finally, Staniogias and Pearce (1985) found that the source of neutral detergent fibre in the diet of growing pigs markedly influenced both length and weight of the distal colon.

Diet-induced alterations in cell turnover kinetics such that defoliation is increased or decreased would have a pronounced effect on the availability of endogenous substrates.

1.8 Endogenous Substrates

Evidence for the contribution of endogenous substrates to the colonic fermentation is largely circumstantial due in part to the difficulties involved in quantifying the different sources. Energy balance studies derived from either a knowledge of the amount of methane and carbon dioxide gases produced by the colonic microflora or estimates of the daily production of bacterial cells both conclude that the average fibre intake of approximately 20g could not sustain the observed turnover of bacterial cells. McNeil (1984)

estimates that 50-60g of hexose would be required for this purpose and that the apparent deficit of available carbohydrate (30-40g) is made up primarily by unabsorbed sugars and starch. Stephen et al. (1983) also postulate that approximately 20% of the Western dietary starch intake (300-400g) is unabsorbed and fills the 'carbohydrate gap'. McNeil (1984) assumes that carbohydrate from mucus will contribute only a "small part" to the deficit and alludes to the possible use of protein as energy and carbon sources for bacterial growth. Potential endogenous substrates include, desquamated mucosal cells, immuno-globulins and glycoproteins present in the form of mucins and in saliva and gastric juices. Although the concentration of glycoprotein in saliva and gastric juices is low, an average person produces 1-1.5L of saliva and 2L of gastric juice per day and therefore the total amount may be significant due to the large volumes involved. The concentration of glycoprotein in mucin is higher but quantitative assessment of the total glycoprotein available from this and other sources has not been reported. Wolin and Miller (1983a) however assert that endogenous substrates are probably a source of "large amounts" of fermentable organic matter and of particular importance when considering colonic gas production. Miller et al. (1984) illustrate the point by demonstrating long term production of methane gas by a bacterial population in a woman with a left hemicolectomy and end colostomy with mucous fistula. The large number of bacteria present in the isolated sigmoid colon were effectively cut off from the normal faecal stream

and consequently supported only by non-dietary substrates.

Wolin (1981) obtained a comparable substrate deficit in his results by estimating that 16g dry weight of bacteria produced per day required 66.4g of hexose. Salyers (1983) assumed the bacterial contribution to faeces to be approximately 30%, considerably lower than the values of Stephen and Cummings (1980a) and consequently the estimated daily hexose required to maintain a steady state population was 27-36g/day. Clearly dietary fibre intakes of approximately 20g could not furnish these amounts of hexose.

Estimates of the amount of substrate other than dietary fibre which must be required to sustain the colonic microbial population are in general agreement but the relative contribution of unabsorbed dietary carbohydrate and endogenous substances towards that requirement is as yet unresolved.

Additional evidence for the degradation of endogenous substrates comes from the investigation of colonic bacteria capable of using these materials. Although mucin-degrading faecal bacteria have been identified (Salyers et al. 1977a) their overall significance as a functional group within the colonic microflora may be small and vary with the availability of dietary substrates.

Variyam and Hoskins (1981) note that of 342 strains tested by

Salyers (1977a) and Salyers (1977b) which together comprise approximately 70% of the total faecal flora only eleven strains from three genera were capable of utilizing either porcine gastric mucin or bovine submaxillary mucin. The authors do, however, demonstrate degradation of mucin carbohydrate and to a lesser extent mucin protein moeities using faecal incubation systems. Bacteroides strains were amongst those capable of using mucin. Given that the Bacteroides comprise approximately 20% of the normal colonic microflora, their numerical predominance may confer greater functional significance upon the few strains isolated so far.

Salyers et al. (1977a) found that Bacteroides species can also degrade a wide range of plant polysaccharides which, implies that their activity may be modulated by the availability of dietary fibre.

In contrast Bayliss and Houston (1984) found that mucin-degrading organisms isolated from faeces had a very restricted ability to utilize complex polysaccharides and their constituent monosaccharides suggesting that the presence of plant polysaccharides was unlikely to affect the use of colonic mucin. Stanley et al. (1986) found that one mucin-degrading isolate from the pig colon was unaffected by inclusion of glucose and furthermore, experimental evidence suggested that complete degradation in vivo was a cooperative effort.

Many workers view the stability of the colonic microflora as evidence for a major role of endogenous substrates in sustaining the population as they would be available more or less continuously. Alternatively bacteria dependent solely upon dietary substrates might have to survive prolonged periods of starvation ie while the host is not feeding. A bacterial strategy for survival under circumstances of starvation is discussed by Wachenheim and Hespell (1985) in relation to Ruminococcus flavefaciens a ruminal cellulolytic species.

Recent evidence (Read et al. 1986) indicated that the colon appeared to fill in a linear fashion with approximately 16% of the residue from a solid test meal entering every hour. The test meal was therefore delivered into the colon over a period of between 8-10 hours. It is possible that the interval between feeds is such that the first portion of a food bolus arrives at the ileo-caecal valve shortly after the last portion of the previous meal enters the colon, thereby minimizing periods of bacterial starvation.

1.9 Colonic Conservation of Microbial Metabolites

The volatile fatty acids acetate, propionate and butyrate are the major anions found in the solute fraction of normal stool water. In addition to small amounts of isobutyrate, isovalerate and valerate these acids are the major end-products of the bacterial fermentation of dietary and endogenous substrates. McNeil et al. (1978) used a dialysis

bag technique to measure rectal absorption of acetate, propionate and butyrate from forty six subjects. Absorption rates of around 8.5μmol.cm^{2}.hr^{-1} were recorded which compared well with rates recorded in animal studies. The authors estimate that a daily fibre intake of 20g might account for 100 kJ energy but note that this contribution may be greatly increased for individuals in countries where fibre forms a much greater proportion of the diet. Ruppin et al. (1980) found that 60% of the absorbed VFA was in the unionized form and absorption was concentration dependent and associated with luminal accumulation of bicarbonate. A sodium-hydrogen exchange may furnish the hydrogen ions involved in the hydration of CO_2 in the luminal solution or mucosal cell. Roediger and Moore (1981) demonstrated enhanced sodium uptake with 20mm n-butyrate using an isolated human colon perfused through the vascular bed and infer that an absence of volatile fatty acids in the colon could be one factor leading to diminished sodium absorption in the colon of man.

Roediger (1980) found that suspensions of isolated colonocytes used butyrate in preference to glucose as a major energy source. The fate of absorbed volatile fatty acids has not been established in man but in typical mammalian systems acetic and butyric acids are usually employed in triglyceride synthesis while propionic acid is involved in glycogen synthesis and plays a role in ketogenesis.

Grossklaus (1983) suggests that colonic absorption of

nutrients eg VFA may explain the "unmeasured energy" reported by Webb et al. (1980) and Webb (1980). These reports concluded that the more indigestible the food supply was the higher the "unmeasured energy". Their reports assumed that the indigestible fraction of the diet was 'lost' and hence dietary energy supplies could not account for energy expended.

In addition to volatile fatty acids the microflora produce B-vitamins and folate but it is not known if they are used by the host.

Eggum et al. (1984) suggest that methionine and lysine formed by bacteria in the rat hind gut might be absorbed and thus improve the quality of dietary protein.

In conclusion although the human colonic microflora is not essential for survival the conservation of dietary materials may provide the host with an additional energy supply proportional to the intake of dietary fibre. In well nourished countries the primary consideration may be the localized production of respiratory substrates (VFA) fundamental to the metabolic welfare of the colonic mucosa, whereas in undernourished populations colonic conservation of nutrients from high fibre diets may furnish a considerable proportion of the total energy requirements of an individual.

The metabolic activities of the colonic microflora are

obviously an integral part of the study of the inter-relationships between diet, the microflora and the well-being of the host.

1.10 Microbial Metabolism

The human colon contains a large and diverse population of anaerobic and facultatively anaerobic bacteria. It is thought the population may be of the order 10^{11} - 10^{13} per gram faeces and contain as many as four hundred different species.

Methodological problems have restricted direct sampling of the population which is required for accurate measurement of numbers and investigation of the existance of sub-populations inhabiting microniches (flocs or consortia) within the lumen contents, in different regions of the gut or attached to the gut wall. As yet no one has firmly established the existence of a distinct mucosal population which might not be represented in faeces but the matter is still under investigation (Croucher et al., 1983 and Robinson, 1984).

Although the stability of the microflora is emphasized in the literature, defining a 'normal' colonic microflora and assessing the significance of any diet-induced changes within the context of individual variation is a formidable task. Microbiological assessment following dietary alterations in normal individuals, in subjects with intestinal disease and between different populations is reviewed by Hentges (1980),

Holdeman et al. (1976) and Bornside (1978). Recently Wyatt et al. (1986) observed a rapid increase in the numbers of bacteria capable of using gum arabic during ingestion of that substrate.

An alternative approach to the investigation of the microflora is to concentrate on the metabolic reactions taking place and then try to attribute these activities to a particular organism or group of organisms. This often involves a study of the metabolic end products, the substrates from which they were derived and the interactions between the bacteria necessary for the fermentation. In addition assaying for specific enzymes can indicate levels of functional activity during an experimental period.

An appreciation of the nutritional inter-dependence and the cooperativity of the colonic population is the key to an understanding of their functional capabilities in vivo. Cooperation may involve the provision of soluble and gaseous end-products which are the carbon and energy sources for other organisms. Likewise, the growth factor and nitrogen requirements of certain bacteria are met by the excretion of branched chain fatty acids (Allison, 1978) and the release of ammonia by ureolytic organisms (Suzuki et al., 1979). The removal of hydrogen by hydrogenotrophs which may in turn shift the redox balance within the hydrogenogen and even scavenging of oxygen by facultative organisms enabling growth of strict anaerobes are further examples of the

inter-dependence between species. Gottschal and Szewzyk (1985) were able to demonstrate growth of a strictly anaerobic sulphate reducing bacterium under aerated conditions in co-culture with a facultative anaerobe.

Competition for carbon, energy and possibly nitrogen sources is also a feature of any fermentation in which any one may be limiting and the survival of an individual organism may depend upon the ability to adapt to different sources and concentrations of nutrients which it shares with other species. Metabolic versatility amongst the major groups of colonic bacteria may explain the apparent stability of the population as a whole.

Growth of the colonic microflora is unlikely to be nitrogen limited. Ammonia, often the preferred nitrogen source for colonic organisms (Varel et al., 1974; Forsyth and Parker, 1985) accumulates in faecal extracts (2% of total faecal-N: Kurzer and Calloway, 1981) and hence cannot be completely exhausted in the colon. In addition, nitrate and amino acids are present in the colonic milieu but the extent of assimilatory nitrate reduction and deamination of amino acids is not known. Maczulak et al. (1985) found that of the fifty isolates tested from the equine caecum none had a unique requirement for urea or ammonia since peptones, amino acids or both supported growth.

Macfarlane et al. (1986) presented evidence of substantial

proteolysis in faeces and found that the predominant proteolytic bacteria were Bacteriodes spp. and Propionibacterium spp. Wrong and Vince (1984) using N^{15} enrichments found that over 90% of faecal nitrogen was derived from non-urea sources. The role of bacteria in ammonia metabolism in man is discussed by Visek (1979). In general, investigators have not considered the possibility of microbial fixation of molecular nitrogen in the colon. So far, of the few species of anaerobic eubacteria tested Clostridium, Propionispira, Desulfovibrio and Desulfotomaculum species were capable of diazotrophic growth. Recently, Bomar et al. (1985) demonstrated N_2-fixation by a member of the archebacteria Methanosarcina barkeri. There is no reason to assume that M.barkeri is unique amongst the archebacteria or that N_2- fixation is not widespread amongst the anaerobic eubacteria. The significance of this reaction in environments with adequate supplies of fixed nitrogen is not clear.

An outline of the principal fermentation routes is shown in fig. (1-10-1)

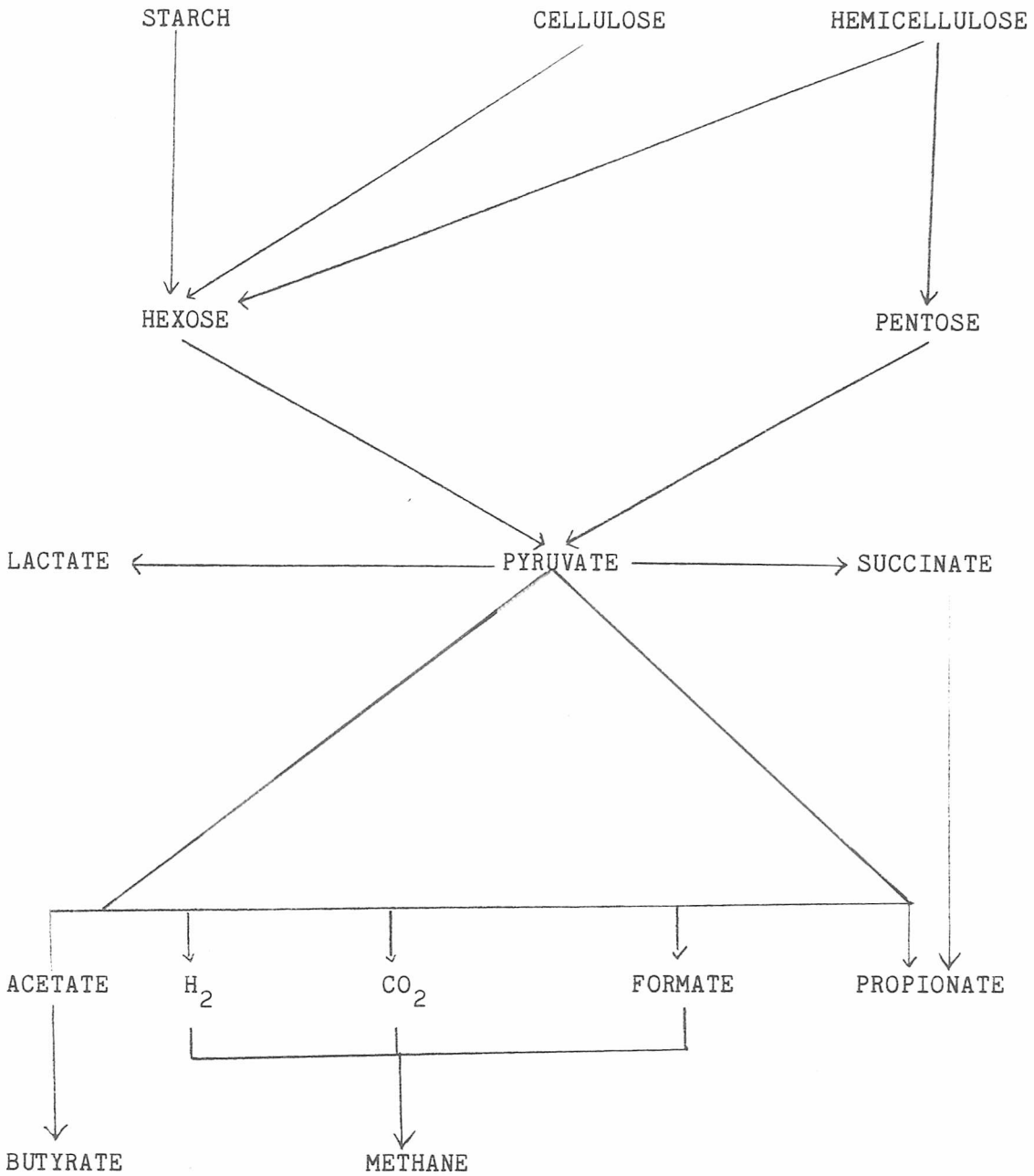


Fig 1-10-1 (Adapted from Wolin and Miller, 1983b)

Starch, cellulose and hemicellulose are degraded by bacterial extracellular enzymes to oligomers, dimers and free sugars.

Studies on a number of bacteria producing these enzymes have indicated that they are cell associated Riley (1984) Ohmiya

et al. (1985) and Gherardini (1985). Degradation by cell associated enzymes requires close proximity between the cell and substrate which in turn has the advantage of placing the organism in the best position for absorption of the products. The importance of juxta-positioning between hydrogen producing and methanogenic bacteria within flocs or consortia was high-lighted by Conrad et al. (1985). Bacterial colonization of fibrous materials in the rumen is well documented (Akin and Barton, 1983).

Soluble sugars, lactate, formate, ethanol and succinate do not usually accumulate in the colonic environment. Under normal circumstances the volatile fatty acids acetate, propionate and butyrate are the major soluble products along with H_2, CO_2, CH_4 (in some individuals) and possibly N_2 and H_2S.

Details of the pathways leading to formation of fermentation end products are given by Wolin and Miller (1983a).

Although very little soluble carbohydrate is available in the colon, saccharolytic species incapable of degrading the major polymers co-exist with polymer fermenting species. Lactate, formate, ethanol, methanol and succinate produced during primary digestion are metabolised as quickly as they are formed. Schink and Pfennig (1982) demonstrated growth on succinate by Propionigenium modestum present in human saliva and Genthner et al. (1981) describe a rumen and sewage sludge

strain of Eubacterium limosum growing on methanol, or H_2-CO_2. Eubacterium species are amongst the predominant species in the colon and methanol is released by degradation of pectins. Lactate is fermented by members of the Enterobacteriaceae and by sulphate-reducing bacteria. Bereens and Romond (1977) isolated sulphate-reducing bacteria from human faeces which metabolised lactate and pyruvate and the predominant human colonic methanogenic species Methanobrevibacter smithii can oxidise formate (Miller et al., 1982). In addition the uptake of monomers of xylan by Bacteroides species has been shown to be 'leaky' that is, disaccharides formed by an intracellular enzyme escape into the medium (Kurmitza and Salyers unpublished results). The scope for cross-feeding is further advanced by the findings of Russel (1985) in which non-celluloytic rumen organisms were identified which used cellodextrin preparations and of Giuliano and Khan (1984) in which a new isolate Bacteriodes cellulosolvens produced cellobiose and glucose from cellulose but could not metabolise glucose. Murray (1986) described a mutualistic relationship between Bacteroides cellulosolvens and Clostridium saccharolyticum in which B. cellulosolvens fermented 33% more cellulose in co-culture than in monoculture. The non-cellulolytic species obtained soluble sugars produced by B. cellulosolvens and in return removed a toxic secondary metabolite. Cross-feeding between Bacteroides amylophilus, Megasphaera elsdenii and Ruminococcus albus resulted in a pattern of succession where stimulation of growth of B. amylophilus by inclusion of

starch resulted in growth of amino acid-dependent M. elsdenii and then branched chain fatty acid-dependent R. albus in a medium devoid of both amino acids and branched chain fatty acids (Miura, 1980). Later studies demonstrated the stimulatory effect of starch on cellulose digestion by a mixed culture of rumen bacteria (Miura et al., 1983). Rode et al. (1981) reported a syntrophic association between co-cultures of Eubacterium limosum a methanol, H_2-CO_2 utilizing species and Lachnospira multiparus a pectin fermenting organism.

The interactions of microbial populations in cellulose fermentation is discussed by Wolin and Miller (1983b).
Although the presence of cellulolytic bacteria is inferred from the disappearance of cellulose in the colon, from faecal incubations and cellulase assay systems, only one species has been isolated and identified. Anaerobic cellulolytic organisms are often difficult to maintain in laboratory culture. Betian et al. (1977) isolated a cellulolytic Bacteroides species from human faeces and Bacteroides are also amongst the predominant cellulolytic genera in the rat (Macy et al., 1982) pig (Varel et al. 1984) and rumen (Wolin and Miller 1983b).

Salyers (1979) lists the substrate specificities of the major genera present in the human colon and shows that many species particularly the Bacteroides are quite versatile in their range of fermentable substrates. Versatility amongst

polysaccharide degrading bacteria may be a general feature of anaerobic fermentations in which the primary substrates are constantly changing. Sleat et al. (1984) isolated a new anaerobic cellulolytic species Clostridium cellulovorans from a mesophilic digester which fermented woody biomass. The organism could use cellulose, xylan, pectin and a variety of mono- and disaccharides as growth substrates. Fusobacterium polysaccharolyticum isolated from the ovine rumen, ferments cellulose, starch and xylan in preference to a range of hexose and pentose sugars (Van Gylswyk, 1980). In contrast, Jensen and Canale-Paraola (1985) describe three pectinolytic isolates which they propose should be termed pectinophilic due to their severely limited ability to use other compounds.

As mentioned earlier, hydrogen, carbon dioxide and in some cases methane, are produced by the colonic fermentation These gases are excreted in flatus or passed in the blood stream to the lungs. Clinicians use the evolution of breath hydrogen to test for carbohydrate malabsorption, intestinal bacterial overgrowth and as an indicator of abnormal upper intestinal transit.

The metabolic end-products of bacteria which produce hydrogen (hydrogenogens) may be substantially altered by changes in the external partial pressure of hydrogen. In anaerobic environments three diverse bacterial groups consume extra-cellular hydrogen namely the methanogens, sulphate reducers and acetogens. As a result these groups often

compete for transferred hydrogen. The nature of interspecies hydrogen transfer reactions varies in extent from organisms which derive a metabolic advantage from the removal of H_2 usually by switching to energetically favourable fermentation pathways which operate only whilst the partial pressure of hydrogen is low, to organisms incapable of growth in the absence of a hydrogenotroph eg Syntrophomonas wolfei (McInerney et al., 1981) and Syntrophomonas wolinii (Boone and Bryant, 1980). Both organisms degrade short chain volatile fatty acids in co-culture with either a sulphate-reducing bacterium or methanogen.

Volatile fatty acid utilization is not however confined to syntrophy. Acetate is completely degraded to CO_2 by Desulfotomaculum acetoxidans (Widdel and Pfennig, (1977) and Desulfobacter postgatei (Widdel and Pfennig, 1981). D. acetoxidans has been isolated from the rumen and the latter species from brackish water and marine mud sediments. Zehnder et al. (1980) isolated an acetate decarboxylating methanogen from digested sludge which was unique in its ability to form methane without oxidizing hydrogen. A new organism Desulfobulbus propionicus isolated from marine and fresh water mud samples is capable of an incomplete oxidation of propionate to acetate (Widdel and Pfennig, 1982).

These organisms are characterized by extremely long generation times (up to 9 days) and as such are unlikely to proliferate in the normal human colon. It should be noted

however that there is a tendency to ignore bacterial species present in numbers less than 10^6/g wet weight of faeces. Zehnder et al. (1980) point out that under natural conditions 'the fastest growing and most efficient organism is not necessarily the ecologically most important one'.

An essential feature of fermentations is the internal reoxidation of reduced pyridine nucleotides. NADH produced during glycolysis is reoxidized via coupling to the formation of reduced fermentation products. In many organisms this may be the only means of maintaining a recycling pool of electron carriers and as such they act as 'electron sink' products. Examples are formate, lactate, ethanol and succinate as shown in fig. 1-10-2.

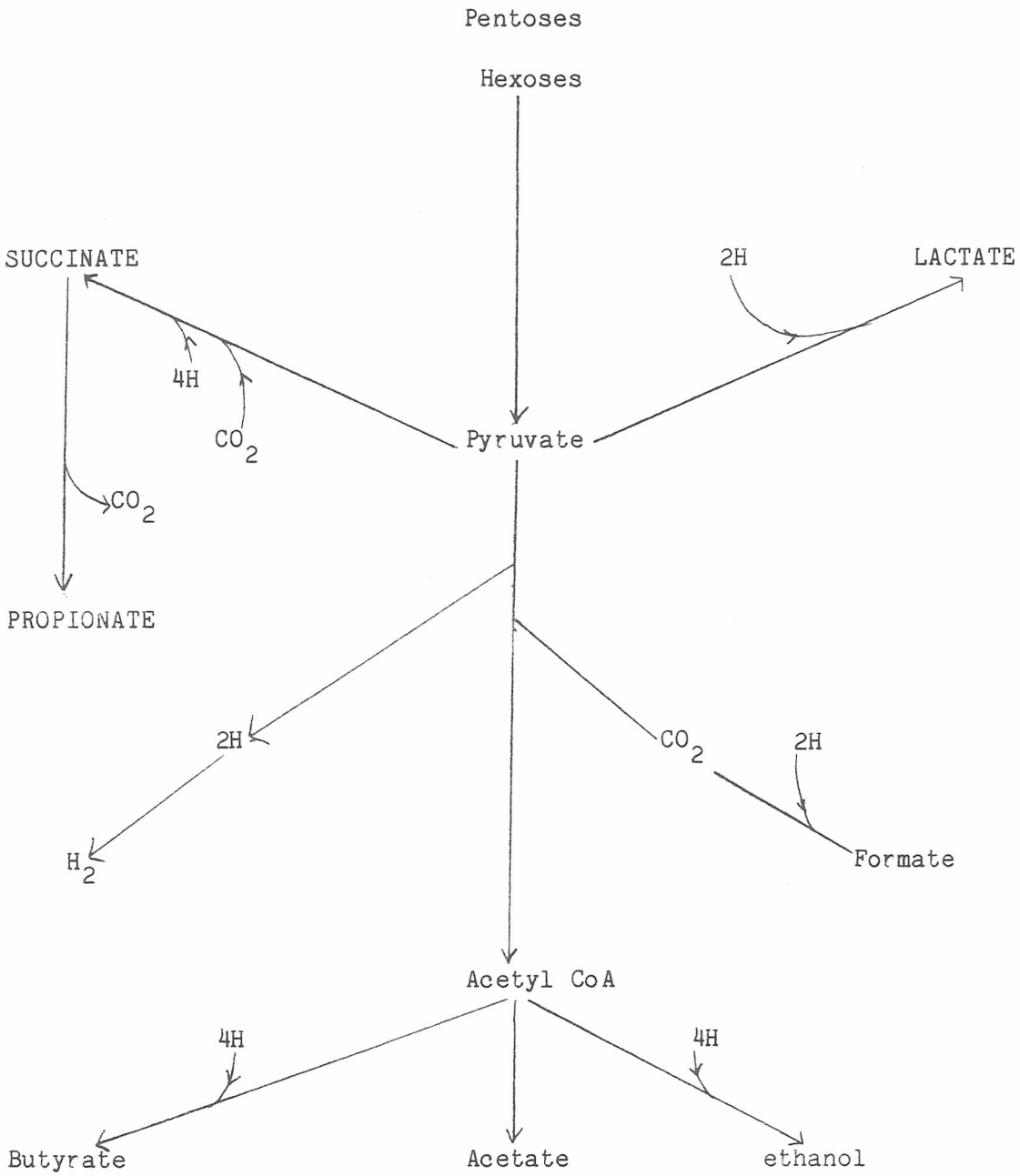


Fig. 1-10-2 (From Miller and Wolin, 1979)

In a large number of anaerobic organisms an alternative mechanism furnishes the generation of molecular hydrogen from NADH. The reaction is thermodynamically unfavourable being inhibited by the accumulation of hydrogen.

$NADH + H^+ \rightleftharpoons NAD^+ + H_2 \quad \Delta G'^{\circ} = + 18.0$ KJ.

If hydrogen is continuously removed as for example when

hydrogenotrophic methanogens or sulphate-reducing bacteria are present, the reaction can proceed in the forward direction. Thus, the uninhibited system is present in many organisms but only operative while hydrogen is continuously removed.

The importance of hydrogen inhibited and uninhibited systems in relation to glucose fermentation products can be illustrated by the pathways of Ruminococcus albus shown in Fig.1-10-3.

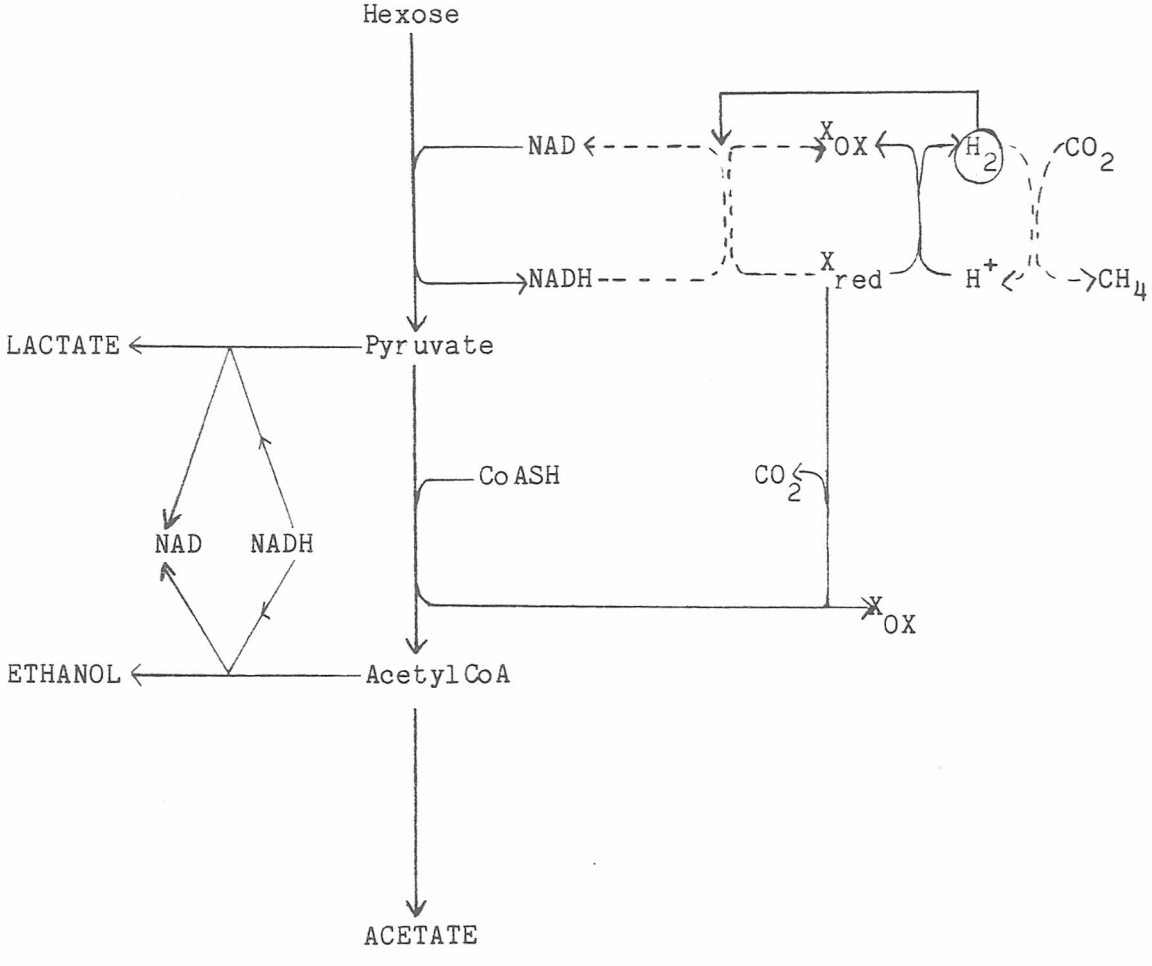


Fig 1-10-3 (From Wolin and Miller, 1983a)

Catalytic amounts of NADH generated during glycolysis must be reoxidized by the formation of reduced end-products from

acetyl CoA or pyruvate. AcetylCoA is reduced to acetaldehyde which is in turn reduced to ethanol with NADH. Hydrogen is formed from pyruvate in a ferredoxin dependent reaction uninhibited by H_2. Under these circumstances ethanol, acetate, H_2 and CO_2 are produced. If hydrogen is continuously removed from the environment, the inhibited systems are released. Reducing equivalents are no longer available for the production of ethanol and acetate accumulates. In a cross-feeding experiment using R. albus and the hydrogenotroph Wolinella succinogenes (previously Vibrio succinogenes), acetate concentrations in the co-culture doubled (Iannottii et al., 1973).

The formation of acetate from AcetylCoA is associated with the production of 1 ATP. Organisms which can redirect reducing equivalents into H_2 gas production may be at an advantage with regard to energy conservation.

The roles of some sulphate reducers, methanogens and acetogens appear to be interchangeable with regard to the direction of flow of transferred hydrogen, that is they may act as either hydrogenogen or hydrogenotroph depending upon the substrate and other species present. Desulfovibrio species growing on ethanol and CO_2 in the absence of sulphate, produced acetate and hydrogen when a hydrogen oxidizing methanogenic bacterium was present (Bryant et al., 1977). In the presence of sulphate Desulfovibrio species can also act as a hydrogen utilizing sink in co-culture with

Syntrophomonas spp. as noted earlier. Further, Phelps et al, (1985) demonstrated sulphate-dependent interspecies hydrogen transfer between Methanosarcina barkerii and Desulfovibrio vulgaris during co-culture metabolism of acetate or methanol. Hydrogen consumption by D.vulgaris altered the internal carbon and electron flow of M.barkeri such that approximately 42% of the available hydrogen equivalents derived from methanol or acetate were transferred to and were utilized by D.vulgaris to reduce sulphate. As a direct consequence, methane formation in co-cultures was approximately two thirds that observed in pure cultures. In addition to carbohydrate fermentation hydrogenotrophs may also influence amino acid metabolism. Nanninga and G ottschal (1985) found that the degradation of serine, alanine, valine and leucine was accelerated considerably by active sulphate reduction.

Many workers have noted the production and/or consumption of trace amounts of H_2 during growth of Desulfovibrio species on organic electron donors in the presence of sulphate. Likewise, Methanosarcina species grown on acetate produce and consume H_2 (Lovley and Ferry, (1985).) Odom and Peck (1981) proposed hydrogen cycling as a general mechanism for energy coupling in Desulfovibrio species. The authors formulated a hypothesis which involved diffusion of cytoplasmic H_2 (produced by a cytoplasmic hydrogenase from reducing equivalents generated during oxidation of lactate or pyruvate) into the periplasmic space. A periplasmic hydrogenase then oxidized this H_2 generating a proton motive

force and the reaction was coupled to electron transport linked phosphorylation and sulphate reduction. Theoretically, hydrogen might leak from the periplasmic space if for example there was a faster rate of H_2 production in the cytoplasm compared with H_2 oxidation in the periplasm. In contrast to the obligate hydrogen cycling model of Odom and Peck (1981), Lupton et al. (1984) presented evidence in which the periplasmic hydrogenase plays only a minor role during growth on organic electron donors in the presence of sulphate. In this model H_2 is not an intermediate product of lactate or pyruvate oxidation. Reducing equivalents are transferred directly from cytoplasmic carriers to membrane bound carrier proteins which subsequently reduce sulphate to sulphide. Cytoplasmic hydrogenase acts principally in the control of the redox state of electron transfer compounds during metabolism by using excess carrier-bound electrons to reduce protons to hydrogen gas which then diffuses into the periplasmic space. Periplasmic hydrogenase may reoxidise this H_2 conserving reducing power, or the H_2 may appear as trace H_2 in the medium. In Methanosarcina a deficit of reducing power may prompt uptake of extracellular H_2 mediated by the periplasmic hydrogenase. According to the model of Lupton et al. (1984) the major role of cytoplasmic hydrogenase in both Methanosarcina and Desulfovibrio growing on organic electron donors in the presence of sulphate appears to be internal redox control whereas, the principal role of periplasmic hydrogenase is likely to be during growth using H_2 as an energy source.

Wolin and Miller (personal communication) have suggested that in the colon, hydrogen may be used to reduce CO_2 in a homoacetogenic fermentation which could function as an alternative to hydrogen utilization by methanogenic bacteria.

The hydrogen may be derived directly within the cell from carbohydrate fermentation or acetate may be produced by a distinct H_2-CO_2 utilizing population. Acetobacterium woodii, Peptostreptococcus productus, Eubacterium limosum and Clostridium aceticum can grow autotrophically using H_2 and CO_2 (Balch et al., 1977; Lorowitz and Bryant, 1984; Genthner et al., 1981 and Owaki and Hungate, 1977).

Preliminary results (Wolin and Miller personal communication) support the view that homoacetogenic fermentation accounts for the high proportions of acetate seen in faecal extracts of non-methanogenic individuals.

Although Methanobrevibacter smithii is the methanogen most often isolated from human faecal samples (Miller and Wolin, 1982; 1983 and Miller et al. 1982) smaller numbers of a mixotrophic methanol, H_2-Acetate utilizing methanogen Methanosphaera Stadtmaniae are frequently encountered (Miller and Wolin, 1985) and Misawa et al. (1986) isolated an antigenically unique methanogen from human faeces.

The metabolic pathways and the mechanism(s) of energy generation in the methanogens are not well understood. Jones

et al. (1985) present evidence of a common pathway for carbon dioxide reduction to methane and Lancaster (1986) has outlined a unified scheme for carbon and electron flow coupled to ATP synthesis by substrate level phosphorylation.

Methane is not generated by mammalian tissues and is generally believed to be biologically inert in such tissue. Zhender and Brock (1979; 1980) have demonstrated biological methane oxidation in anaerobic systems but attempts to identify the organisms involved have been unsuccessful (Alpern and Reeburgh, 1985).

The significance of methane production by approximately one third of individuals is not clear. Weaver et al. (1986) noted that a greater number of patients with diverticulosis produced methane and Haines et al. (1977) found a higher incidence of methane producers in patients with cancer of the large bowel compared with patients with non-malignant large bowel disease and healthy subjects. Evidence of this sort runs contrary to earlier reports of higher proportions of methane producers in populations least disposed to large bowel disease. There seems little doubt that the real value of dietary fibre is inextricably linked to the metabolism of the colonic microflora. The colonic response to the inclusion of dietary fibre will at the same time be a function of the bacterial metabolism of dietary and endogenous substrates and both are dependent upon the composition of the microflora.

1.11 The principal aim of the work undertaken in the present study was to establish an *in vitro* model system which simulated certain aspects of the human colonic fermentation of a mixture of dietary fibres. The fibres used were isolated from bran, carrot, cabbage, turnip and apple. The bran content was kept constant and the mixture was varied by deleting in turn one of the other four fibre types. Previous work by Wolin and Miller (1981) indicated that faecal bacteria fed semi-continuously with a fibre suspension similar in composition to that of ileal chyme provided a 'reasonable facsimile' of the colonic fermentation.

A similar model was employed in the present study and used to describe and quantify the major attributes of the fermentation of the different mixtures of dietary fibres with a view to demonstrating an overall balance between substrate utilization and product formation. In addition an investigation of the bacterial population was carried out. Total numbers of bacteria and bacteria capable of degrading certain constituents of dietary fibre were enumerated and the major groups present were identified to genus level.

Some preliminary studies were made of the metabolism of nitrogen and sulphur by the mixed bacterial populations in the fermentor and of the autotrophic metabolism of H_2 and CO_2 in enrichment cultures isolated from the fermentor.

SECTION 2

MATERIALS AND METHODS

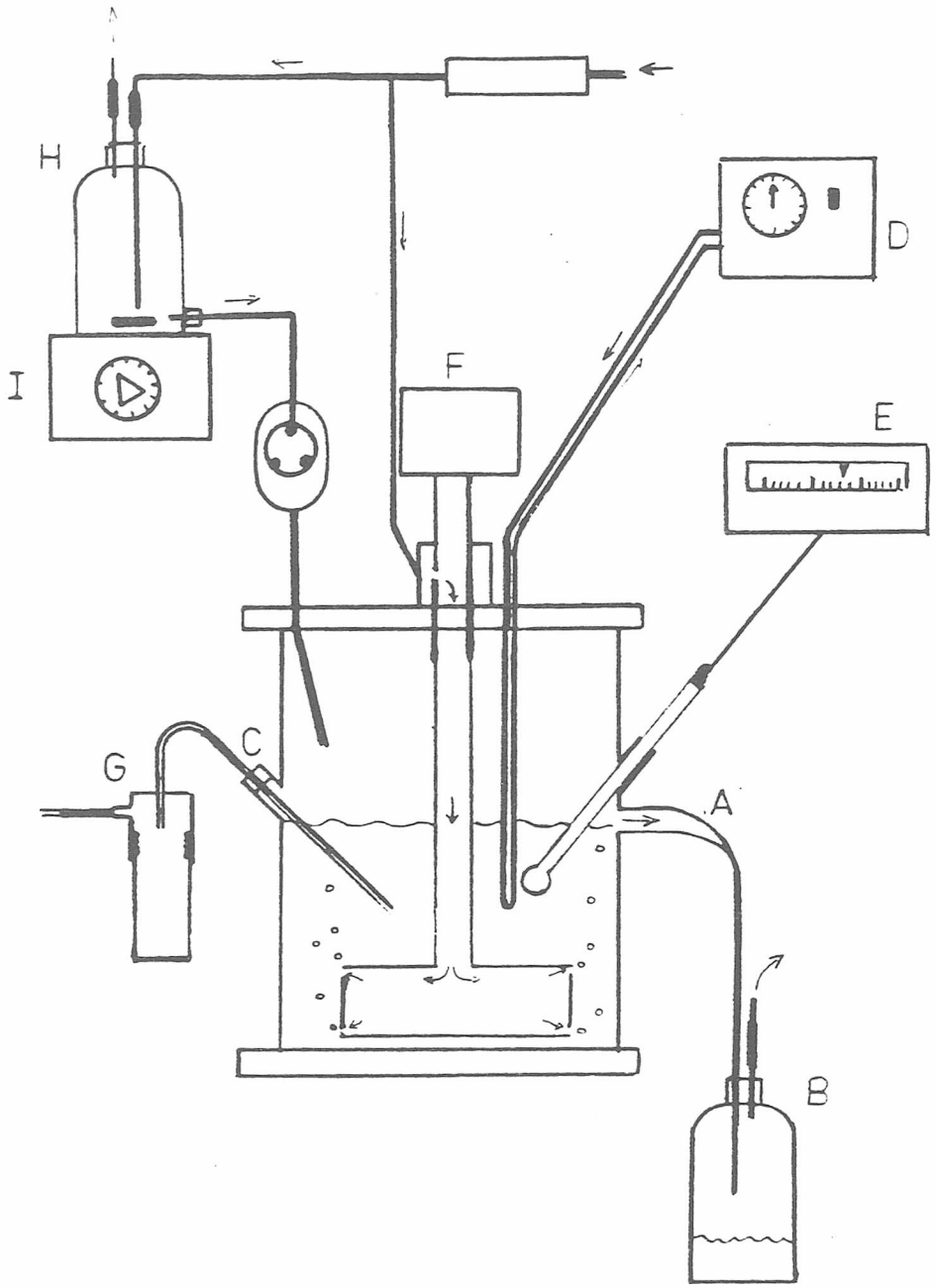
2.1 Gas Supply

All routine anaerobic work including operation of the fermentor was carried out under an atmosphere of oxygen free carbon dioxide (O-F CO_2). Traces of oxygen in the CO_2 were removed by passing the gas over copper turnings heated to 370°C in a Gallenkamp tube furnace. When an atmosphere of hydrogen and carbon dioxide was required an 80%:20% mixture was used. This cylinder was fitted with a Deoxo-Hydrogen purifier which effectively removed any traces of oxygen.

2.2 The Fermentor

An L.H. Engineering type LHE 1/1000 laboratory fermentor of 1 litre capacity was used throughout the experiments (see figure 2.a). The glass vessel was fitted with a side arm (A) which served as an outlet for gas and allowed the liquid culture to overflow maintaining a volume of 670 ml in the vessel. Liquid effluent was collected in a 10L aspirator bottle (receiver vessel B) fitted with an expanding bung closure from which gaseous effluent was expelled to the outside. A second glass porthole (C) was used for inoculation and sampling. The fermentor unit was equipped with temperature (D) pH (E) and stirrer-speed (F) controls. All experiments were conducted at 37°C at a stirrer speed of approximately 200 r.p.m. and no external pH control was necessary to maintain the culture at between pH 6.7-7.1. The fermentor was gassed continuously at approximately 200 ml.

Figure 2a The Fermentor



min^{-1}. L.H. Engineering also supplied a universal sampling hood (G) which facilitated anaerobic sampling of up to 20 ml volumes, a sterilizable bacteriological gas filter housing and stainless steel 'steri-connectors' which provided air-tight junctions in the butyl-rubber supply and effluent lines. Contamination of the feedstock reservoir and the fermentor vessel was prevented by inclusion of glass wool filters at suitable positions in gas supply lines. The feedstock was contained at room temperature in a 5 litre aspirator bottle (reservoir H) fitted with an expanding bung closure. After autoclaving the reservoir was gassed continuously with O-F CO_2 and stirred on a large scale magnetic stirrer (I) (L.H. Engineering) using a 'Polygon' giant teflon coated follower (108 x 27mm).

2.2(1) Inoculation

Before the first inoculation approximately 650 ml of stirred medium was pumped into the sterile pre-gassed fermentor using a peristaltic pump and allowed to reach a temperature of $37^{o}C$.

The faeces used throughout the study were from the same adult female subject who followed an ad-lib ovo-lacto vegetarian diet. Freshly voided faeces were collected in a sterile Stomacher bag under an atmosphere of O-F CO_2 and approximately 10 ml transferred into a pre-gassed cut-end 10 ml pipette using a vacuum pump. The pipette was then fitted with an O-F CO_2 filled bulb and the faeces washed into the

fermentor by flushing with fermentor contents. During inoculation, the effluent weir was clipped creating a positive gas pressure in the vessel prior to opening the porthole thereby maintaining anaerobic conditions in the vessel. This inoculation procedure was found to be the most effective in sustaining strict anaerobes. An alternative method in which the faecal sample was comminuted in anaerobic dilution solution using a Colworth Stomacher was unsuccessful in transferring methanogenic bacteria to the fermentor.

Except in circumstances of accidental washout, experiments were continuous. Pre-existing cultures were not discarded. When a new feedstock was connected to the fermentor either the pre-existing culture was sustained or a fresh faecal inoculum was added to it. Each 5L reservoir lasted approximately 2 weeks.

2.2(2) Feeding Regimes

A peristaltic pump delivered three 100 ml feeds each over a period of two minutes at 9.00am, 1.00pm and 5.00pm. In situations where 'overnight' experiments were carried out the fermentor could be switched onto a 9.00pm 1.00am and 5.00am feeding routine so that day time samples gave the corresponding night time values.

9.0 ml of Anaerobic dilution solution containing various concentrations of KNO_3 was added to the fermentor immediately after the morning feed on several days during an experiment

in which all of the fibres were included in the feedstock. On the last day of the same experiment, a single dose of lactulose was added to the fermentor to a final concentration of 0.5% v/v.

2.2(3) Sampling

When possible, samples were taken immediately prior to a feed and were either stored frozen for later biochemical analysis or maintained anaerobic for immediate use in microbiological work.

2.3 General Microbiology

Examination of wet preparations from isolated colony, fermentor, or broth cultures was performed by phase contrast microscopy using a Reichert Biovar microscope (X100 phase objective and X10 ocular lens). For Gram-stained preparations a X100 oil immersion objective lens was used.

2.4 Evaluation of Minimal Media and Anaerobic Technique

A pure culture of *Methanobrevibacter smithii* was kindly donated by Dr Colin Stewart of the Rowett Research Institute. This culture was sub-cultured into several minimal media both using the Hungate technique (Hungate 1950) and in an anaerobic chamber. Tubes were boosted to twice atmospheric pressure with H_2:CO_2 (80%:20%) and incubated in a shaking

incubator for 10 days. Broths were checked for turbidity and the headspace gas analysed for methane.

2.5 Anaerobic Dilution Series

Dilutions of 10^{1} to 10^{11} were performed in triplicate from the same fermentor sample. Two sets were used to inoculate media in a laminar air flow cabinet and the third reserved for the inoculation of plates and minimal media in an anaerobic chamber.

The above procedure was also followed for the preparation of faecal dilutions.

2.6 Amino Acid Utilization

Several of the dilution series were incubated under O-F CO_2 and tubes showing visible turbidity were presumed to contain bacteria growing on amino acids. Growth was recorded between 10^{1} - 10^{11}.

2.7 Total Viable Counts

2.7(1) Most Probable Numbers

Triplicate samples of 1.0 ml volume from the 10^{8} - 10^{11} dilutions were inoculated into non-selective (N-S.) liquid medium. Tubes were examined after 5 days using turbidity as evidence of growth. Numbers were estimated using M.P.N. tables.

2.7(2) Roll Tubes

Duplicate samples of 1.0 ml volume from the 10^8 - 10^{11} dilutions were inoculated into N-S Agar kept molten at 42°C. The tubes were then spun on a roller machine and incubated. Colonies growing in the agar film were counted at between 3-7 days.

2.7(3) Anaerobic Plate Counts

Spread plates of N-S medium were prepared using duplicate samples of 0.2 ml volume from the 10^6 - 10^9 dilutions of faecal and fermentor samples. Plates were incubated overnight in the anaerobic chamber and then in anaerobic jars within the chamber. Colonies were counted after 5 days. Individual colonies from these plates were selected for end product analysis and antibiotic sensitivity testing. (See 2-11 and 2-12).

2.8 Aerobic Plate Counts

2.8(1) Total Counts

Agar plates of N-S medium were spread with duplicate 0.5 ml volumes of the 10^4 - 10^8 dilutions. Colonies capable of growth in aerobic conditions were enumerated after 4 days incubation.

2.8(2) Number of Enterobacteriaceae

Violet Red Bile Agar pour plates were inoculated with 0.5 ml of dilutions 10^4 - 10^8 and incubated for 4 days. Members of

the Enterobacteriaceae were identified by the production of deep pink/red colonies.

2.9 Growth of Bacteria in Minimal Media

Several types of liquid minimal media were used to try to promote growth of organisms capable of growth using combinations of H_2, CO_2, formate, methanol and acetate. Duplicate 1.0 ml volumes from 10^1 - 10^6 dilutions were used to inoculate tubes in the anaerobic chamber. The gas space was filled with either H_2:CO_2(80%:20%) at twice atmospheric pressure (or O-FCO_2 at atmospheric pressure) and the tubes incubated horizontally in a Gallenkamp orbital shaker.

Utilization of gas was measured either by the decrease in headspace gas pressure or the analysis of headspace gas on a gas chromatograph. This was carried out for cultures prepared from both faecal and fermentor samples.

2.9(1) End-Product Analysis of Cultures in Minimal Media

Cultures in which the use of H_2 and CO_2 was evident were analysed for volatile fatty acids, ethanol and methanol as described in section (2-15). Several of the cultures were maintained by sub-culturing and tested repeatedly.

2.10 Testing Cultures for Starch, Xylan, Pectin and Cellulose Digestion

2.10(1) Starch

Starch Roll tubes were inoculated with 0.5 ml of 10^5 - 10^9 dilutions. After 4 days tubes were flooded with dilute iodine solution which revealed areas of clearing around starch-hydrolysing colonies.

2.10(2) Xylan

Xylan roll tubes were inoculated as for starch. Clear zones surrounding xylanolytic colonies were visible after 4 days.

2.10(3) Pectin

Roll tubes were inoculated as above. Pectinolytic colonies were identified after 3 days by flooding roll tubes with a 40% aqueous 'Cetavalon' solution which creates an opaque complex with undigested pectin.

2.10(4) Cellulose

Cellulose agar plates, roll tubes and broths were used to try to cultivate cellulolytic colonies from fermentor cultures and faecal samples. Pour plates, spread plates and inoculated plates with an agar layer spread over the surface were prepared using 0.5 ml of 10^1 - 10^4 dilutions. Plates were incubated in the anaerobic chamber for one month. Broths containing a strip of filter paper or ball milled filter paper were likewise inoculated from 10^1 - 10^4 fermentor and faecal dilutions. Faecal samples were also inoculated into cabbage dietary fibre broths.

Plates or roll tubes were flooded first with a 0.2% (aq) solution of Congo Red for 15 minutes and secondly with 1 M NaCl for a further 15 minutes (see method Teather and Wood, 1982). Cellulolytic colonies create clear zones in the stain. In liquid culture the disintegration of a cellulose strip or the disappearance of a cellulose sediment indicates activity.

2.11 End Product Analysis of Plate Count Isolates

Single colonies isolated from non-selective medium plates (see 2-7(3)) were grown in N-S broths and analysed for volatile fatty acids, ethanol and methanol.

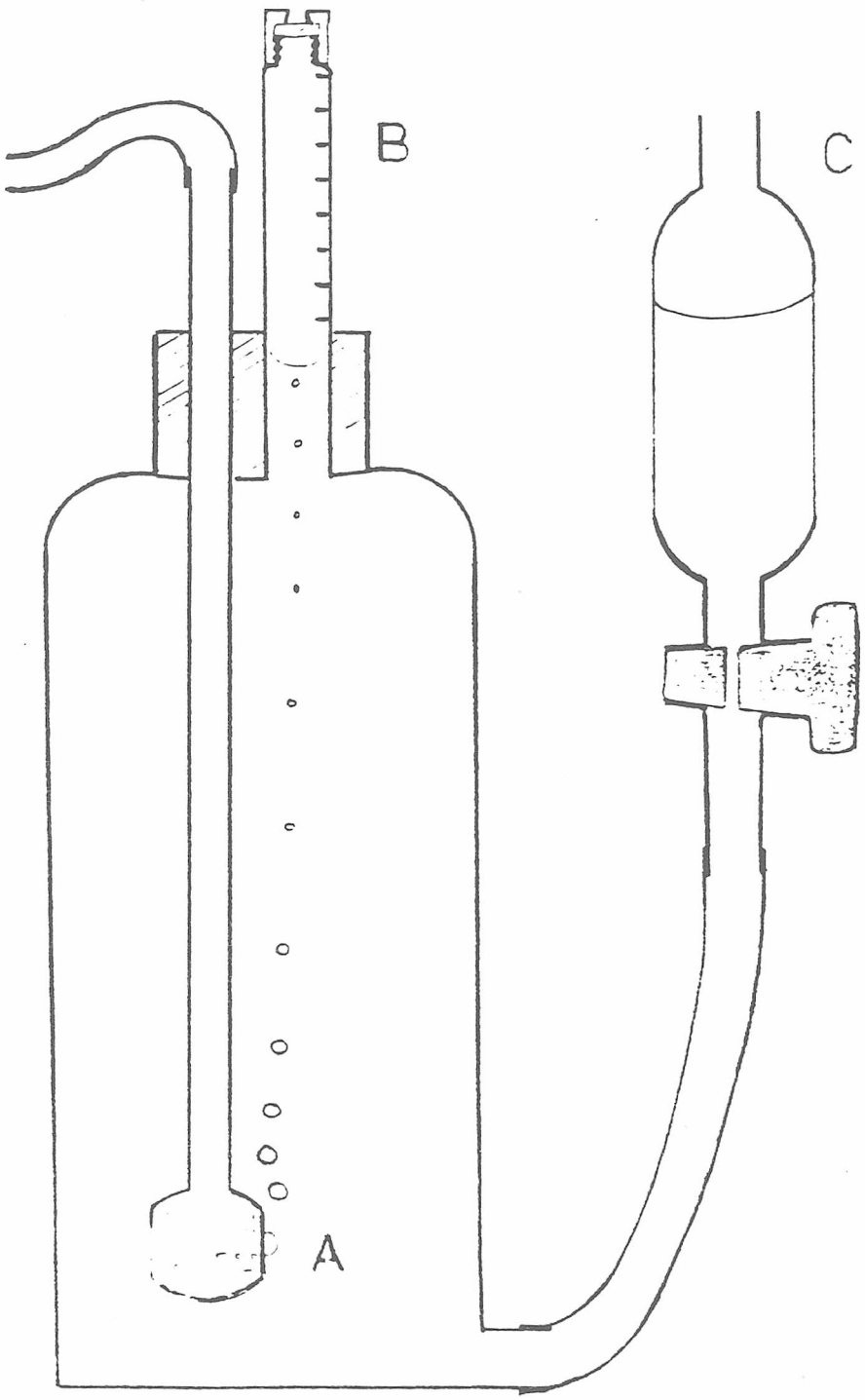
2.12 Preliminary Identification of Bacterial isolates using Antibiotic Sensitivities

Cultures incubated for 3-5 days in the N.S. broths (above) were spread on N-S plates and antibiotic discs positioned 3 cm apart on the surface.

Resistance or sensitivity to individual antibiotics was recorded for each isolate and a preliminary identification to Genus level made using a diagnostic key devised by L. Essers (1982).

For identification of faecal isolates, antibiotic sensitivity testing and end product analysis was supplemented by examination of colony appearance, motility, morphology and Gram reaction.

Figure 2b Gas Collection Apparatus



2.13 Collection; Sampling and Analysis of Hydrogen; Methane and Nitrogen Gases

2.13(1) Collection

Where possible, samples were collected over a 24 hour period to obtain an indication of the range of gas production. Gas was collected while the fermentor was being flushed with O-F CO_2 as normal. The effluent weir was clipped and the gas flow directed from the fermentor into a 1 litre aspirator bottle filled with an aqueous solution of 10N NaOH (see figure 2b). A sintered glass sparge (a) dispersed the gas in the alkali solution enhancing the absorption of CO_2. Unabsorbed gas (H_2, CH_4 and N_2) accumulated at the top of the bottle and was collected in a graduated glass column (b) fitted at the top with a rubber septum through which gas samples could be removed for analyses. Gas collecting at the top of the column displaced alkali from the aspirator bottle into a partially filled separating funnel which acted as a ballast (c).

2.13(2) Sampling

At the end of a collection period the stop-cock on the tube connecting the ballast volume to the aspirator bottle was closed to prevent pressure changes. A 2 ml pressure-lok gas tight syringe was inserted into the septum and the collected gas allowed to expand into the syringe. The total volume of gas was taken to be the volume in the syringe plus the volume remaining in the graduated glass column. Opening the ballast

stop-cock allowed alkali to flow in and replace the gas space. A new septum was carefully secured before beginning another collection period. Gas tight lockable syringes were also used to sample head space gas from tube cultures.

2.13(3) Analysis

Gas samples were analysed on a Pye Unicam G.C.V. chromatograph. The flow rate of the carrier gas Argon was 40 $ml.min^{-1}$. A 3 metre column packed with Poropac Q mesh 80/100 was set at 30°C isothermal. The thermal conductivity detector temperature was 240°C. Peak areas were determined using a Spectra-Physics SP4100 computing integrator/chart recorder. Gas concentrations were calculated by reference to an external standard gas mixture (2 ml) containing hydrogen 10%, nitrogen 35%, methane 10% and carbon dioxide 45%.

2.14 Collection Sampling and Analysis of Hydrogen Sulphide Gas

The effluent weir was clipped and the gas sparged into a 1L aspirator bottle containing 1 litre of 2% (aq) zinc acetate solution. Insoluble zinc sulphide forms a precipitate and therefore, the solution was shaken before samples were removed. The concentrations of sulphide were determined using the method of Trüper and Schlegel (1984).

2.15 Analysis of samples for Ethanol and the Volatile Fatty Acids Acetate, Propionate, Isobutyrate, Butyrate, 2 methylbutyrate, Isovalerate and Valerate

2.15(1) Preparation of Sample

Both fermentor and reservoir samples were prepared so that results would take account of any VFA present in the feedstocks. After defrosting, 12 ml of sample was centrifuged at 2000Xg for 10 minutes. The supernatant was decanted and the fibre pellet stored frozen for later dietary fibre analysis. Samples from anaerobic broth cultures were treated similarly except that any pellet was discarded.

A 0.6 ml aliquot of the supernatant was added to 0.4 ml of 1% H_2SO_4 (v/v) in an autosampler bottle. The rubber septum and screw cap were tightly replaced and the samples stored frozen or analysed immediately.

The remainder of the supernatant was kept for all other biochemical analyses.

2.15(2) Analysis

A Varian model 3700 gas chromatograph equipped with a flame ionization detector was used to quantify volatile fatty acids.

The VFA's were separated on a 2 metre glass column of 2mm I.D. packed with Chromosorb 102 mesh 80/100 and run isothermally at 190°C using Argon as carrier gas at a flow rate of 30 $ml.min^{-1}$. The injector and detector temperatures were 200°C.

Samples of 1 µl volume were injected either manually or automatically using a Varian model 8,000 autosampler. Concentrations of volatile fatty acids and ethanol were calculated from peak areas using a Varian CDS 111 integrator, calibrated with external standard mixtures of ethanol and the relevant acids.

2.15(3) Methanol

Estimations of the amount of methanol present were made by comparing the methanol peak heights on chromatograms of reservoir samples with those of fermentor samples.

2.16 Lactate Analysis

Occasional supernatants were selected for lactate assay using the method described by Conway (1957).

2.17 Formate Analysis

A method described by Marounek and Wallace (1984) was used to monitor periodically fermentor and reservoir samples for formate.

2.18 Sulphate Analysis

A turbidometric technique for the quantitative analysis of sulphate in fermentor and reservoir supernatants was carried out as described in: Standard Methods for Examination of Water and Waste, (1971).

2.19 Estimation of Nitrate- Nitrite- and Ammonia- Nitrogen

Quantitative analysis of nitrogen compounds was made using an automated colorimetric technique with a Technicon Autoanalyser 11 (industrial method 185-75E). This provided a rapid method for the estimation of nitrogen compounds in reservoir and fermentor supernatants over the ranges 0.2 - 1.0 ppm nitrite-N and 10-50 ppm for ammonia-N and nitrate-N.

The supernatant samples prepared for ammonia-N determinations were diluted 50 fold. Reservoir supernatants were analysed along with fermentor supernatants.

2.20 Preparation and Analysis of the Dietary Fibre in Feedstocks and Fermentor Residues

2.20(1) Preparation of Dietary Fibres

The extraction procedure was modified from Southgate et al. (1978).

Eight kilograms each of carrot, turnip, cabbage and cooking apples were prepared along with 340 g of Jordans Natural Country Wheat Bran. The carrot, turnip and cabbage were washed and peeled and then shredded in a catering shredder machine. The apples were cored but not peeled before being comminuted in a food processor to a fine pulp.

The apple and shredded vegetables were then spread onto separate trays and freeze dried.

2.20(2) Extraction Procedure

All procedures were carried out in a fume cupboard. The extractions were designed to remove free sugars, lipids and pigments.

Methanol Extraction

Each fibre including the bran was extracted separately in a 10L round bottomed vessel secured on a heating mantle and fitted with a reflux condenser. Between 4 and 6 litres of 85% (v/v) aqueous methanol was required to immerse about half of a batch of freeze dried fibre. This was refluxed for two hours and the solvent filtered off using a Buchner funnel. The fibre was then refluxed in fresh 85% methanol for a further 2 hours. This was repeated for each of the fibres.

Acetone Extraction

The fibres were individually immersed in acetone and shaken several times over a 12-16 hour period. The acetone was then decanted off and the fibre washed in fresh acetone and left to dry by evaporation in a fume cupboard. Particle size was standardized by brush sieving the dried fibres through an Endecott test sieve mesh size (1.5 mm).

2.20(3) Analysis of Dietary Fibres

Ten milligram amounts of the dietary fibres were analysed for starch and non-starch polysaccharides. Reservoir and fermentor fibre pellets were defrosted and analysed in the same manner.

Dietary fibres were analysed for monosaccharides prepared as alditol acetates using the method of Blakeney et al. (1983) following the digestion procedure of Englyst and Cummings (1984). The α-amylase used was 'Termamyl' - obtained from Novo Industries Copenhagen, the protease 'Alcalase' from the same supplier and the amyloglucosidase was obtained from Boeringer Mannheim Chemicals.

Once prepared the alditol acetates were quantified using a Varian model 3700 gas chromatograph. Alditol acetates were separated on a 2 metre glass column of 2.0 mm I.D. packed with 'Supelcoport' mesh 100/120 coated with 3% SP-2330 and run isothermally at 215^{o}C with argon as carrier gas at a flow rate of 30 ml.min^{-1}. The injector and detector temperatures were 250^{o}C. One microlitre samples were injected manually. The weights of individual sugars were calculated from peak areas using a Varian CDS 111 integrator calibrated with an internal standard, inositol.

The uronic acid content of the digests was assayed using the method described by Blumenkrantz and Asboe-Hanson (1973). An anthrone determination for starch was carried out on digests as described by Scott and Melvin (1953).

2.21 Preparation of Fermentor Media

The overall design of this medium was modified from Miller and Wolin (1981). The vitamin and trace element composition was largely derived from Balch et al. (1979).

Each reservoir was made up to a final volume of 5 litres and contained: NaCl, 14.0g; K_2HPO_4, 1.75g; $NaHCO_3$, 17.5g; KNO_3, 5.0g; $(NH_4)_2SO_4$, 1.0g; $MgSO_4.7H_2O$, 0.175g; $CaCl_2.2H_2O$, 0.29g; NH_4Cl, 6.25g; $FeSO_4.7H_2O$, 0.015g; $Fe(NH_4)_2(SO_4)_2.7H_2O$, 0.1g; Urea, 2.5g; Trypticase-BBL, 7.5g; Yeast extract (oxoid), 2.5g; cholic acid preparation from ox-bile (Sigma), 1.75g; Trace element solution, 50 ml, vitamin solution, 50 ml; Haemin solution, 5.0 ml; faecal extract, 50 ml; Resazurin solution (0.1% w/v), 1 ml; reducing solution, 200 ml.

The trace element solution contained per litre:

Nitrilotriacetic acid, 1.5g; $MgSO_4.7H_2O$, 3.0g; $MnSO_4.2H_2O$, 0.5g; NaCl, 1.0g; $FeSO_4.7H_2O$, 0.1g; $CoCl_2$, 0.1g; $CaCl_2.2H_2O$, 0.1g; $ZnSO_4$, 0.15g; $CuSO_4$, 0.01g; $NH_4(SO_4)_2$, 0.01g; H_3BO_3, 0.01g; Na_2MO_4, 0.01g; NiCl, 0.1g; Na_2SeO_3, 0.1g; and $Na_2WO_4.2H_2O$ 0.1g. The nitrilotriacetic acid was first titrated with 0.5 N KOH to pH 7.0 and the minerals added to this solution.

The vitamin solution contained per litre:

Thiamine hydrochloride, Nicotinamide, Riboflavin, Pyridoxine hydrochloride and Calcium D-pantothenate, 40 mg of each; Cyanocobalamine, 16.0 mg; 4- aminobenzoic acid, Retinol, Ascorbic acid and Calciferol, 8.0 mg of each; Tocopherol acetate, 4.0 mg; Folic acid, 0.8g; Folinic acid, 0.8g; Lipoic acid, 0.8g; Menadione (Vit K2), 0.005g.

The haemin solution was prepared by adding 20 mg of haemin to 50 ml of ethanol: 0.05N NaOH solution (50 : 50).

The faecal extract was prepared as follows:

Freshly voided faeces were made up to 1 litre after being comminuted in a Colworth Stomacher with 200 ml of distilled water. The resultant slurry was filtered through two layers of muslin before being centrifuged at 17,250 xg at 10^{o}C. The supernatant was decanted and dispensed into appropriate volumes and autoclaved for 15 minutes at 15 lbs. in^{-2} before being stored frozen. Before use a residual precipitate was removed by centrifugation at 2,000xg.

Reducing solution contained per 200 ml distilled water: cysteine hydrochloride, 2.5g and $NaHCO_3$, 15.0g.

For all reservoirs a fibre concentration of 10 $mg.ml^{-1}$ was constructed from either 20g of bran plus 7.5g each of cabbage, turnip, carrot and apple per 5 litres or 20g of bran plus 10.0g each of three other fibres per 5 litres depending upon which fibre was deleted.

Before autoclaving the pH was between 7.4-7.5.

The faecal fluid, trace element and vitamin solutions were autoclaved separately at 10lbs. in^{-2} for 20 minutes and added aseptically to the bulk medium which was autoclaved first at 10 lbs. in^{-2} for 15 minutes then 15 lbs. in^{-2} for 30 minutes. The 200 ml of reducing solution was filter sterilized through a cellulose acetate filter pore size 0.45 μm attached to a Buchner flask. This was added to the bulk medium along with

those solutions mentioned above. The final pH was 7.0.

The fermentor medium was stirred and cooled under an atmosphere of O-F CO_2 and anaerobic conditions were indicated by the colourless state of the redox indicator resazurin.

2.22 Procedures for the Preparation of Solid and Liquid Test Tube Media

2.22(1) Agar Roll Tubes

Anaerobic media were prepared and dispensed under O-F CO_2 using the Hungate technique. (Hungate, 1950; 1969). Aliquots of 3-5ml medium were dispensed in a Laminar air flow cabinet and sterilized in sealed Hungate tubes (Bellco) at 15 lbs. in^{-2} for 15 minutes. This method was not suitable for a starch medium described later. Tubes of sterile agar medium were stored at room temperature and melted when required.

2.22(2) Liquid Test-tube Media

These were prepared and dispensed as for solid media, except in the case of anaerobic dilution solution where exactly 9.0 ml volumes were dispensed.

2.23 Preparation of Plate media

2.23(1) Aerobic Plates

The agar medium was sterilized at 15 lbs in^{-2} for 15 minutes except for Violet Red Bile Agar (Oxoid Manual) which was

boiled and then poured aseptically into sterile plates in a Laminar air flow cabinet.

2.23(2) Anaerobic Plates

Media for anaerobic plates were sterilized and cooled under O-F CO_2 in 300 ml screw-capped bottles. The reducing solution and any sugar solutions were filter sterilized and added to the sterilized media. The bottles were then sealed and transferred into an anaerobic chamber at 39°C containing the recommended gas mixture N_2:H_2:CO_2(80:10:10). The plates were poured aseptically and allowed to set. Plates were stored and incubated in anaerobic jars within the chamber to prevent drying-out.

2.24 Inoculation and Cultivation of Test-tube and Plate Cultures

All routine test-tube culture and aerobic plate manipulations were carried out in a Laminar air flow cabinet.

Although Hungate tubes have a rubber septum facility for inoculation and sampling using syringes, greater confidence in the maintenance of anaerobiosis was felt when the tubes were opened under O-F CO_2 or in the anaerobic chamber.

Disposable 1.0 ml syringes were used for agar and liquid transfers. An exception was in cases where the inoculum was fermentor culture or faeces. A sterile 1.0 ml cut-end pipette was reqired to transfer such particulate matter. If

the sample was prepared for a dilution series the first tube was vortexed for 2-3 minutes to dislodge bacteria loosely attached to fibre particles. Bacterial colony transfers were made using sterile disposable plastic inoculating loops. All cultures were incubated at $37^{o}C$-$39^{o}C$.

Contamination of the inside of the anaerobic chamber was minimised by swabbing all glassware and the chamber tray with Savlon solution or methylated spirits.

2.25 Test-Tube and Plate Media

2.25(1) Anaerobic Dilution Solution

For all serial dilutions, medium 1 described by Kurihara et al. (1968) modified by exclusion of lactate and sugars, was used.

2.25(2) Non-Selective Medium (N-S)

This medium contained per litre:

KH_2PO_4, 0.3g; $(NH_4)_2SO_4$, 0.5g; NaCl, 0.6g; $MgSO_4.7H_2O$, 0.165g, $CaCl_2.2H_2O$, 0.066g; NH_4Cl, 0.3g; $NaHCO_3$, 5.0g; KNO_3, 0.5g; Trypticase-BBL, 5.0g Yeast Extract, 2.0g; trace element and vitamin solutions, 10 ml each (see section 2.21); faecal extract, 20 ml (see section 2.21); resazurin solution, 0.2 ml (see section 2.21). 2-methylbutyric, propionic, isobutyric, isovaleric and valeric acids, 0.5 ml of each; combined reducing and carbon substrates solutions, 50 ml and for solid media 25g purified agar.

The reducing and carbon substrate solution contained in 50 ml glucose, cellobiose, maltose, xylose, fructose, lactose, glycerol, mannitol, methanol, ethanol, sodium acetate, sodium formate, lactic acid, 1.0g of each; $NaHCO_3$, 2.0g; cysteine hydrochloride, 0.6g and dithiothreitol 0.2g. The pH was adjusted to 7.0. For preparation of Hungate tubes the medium was first boiled to remove oxygen and then cooled and dispensed under O-F CO_2 following addition of the reducing and carbon substrates solution. The sealed tubes were then sterilized at 15 lbs. in^{-2} for 15 minutes.

For preparation of plates the reducing and carbon substrates solution was filter sterilized and added aseptically to the rest of the medium which had been sterilized at 15 lbs. in^{-2} for 15 minutes and cooled under O-F CO_2. When aerobic plates were prepared, resazurin and reducing agents were omitted.

2.25(3) Minimal Media

Minimal Medium 1.

This was a modification of a medium described by Balch et al (1979).

It contained per litre:

KH_2PO_4, 0.3g; $(NH_4)_2SO_4$, 0.3g; NaCl, 0.6g; $MgSO_4.7H_2O$, 0.13g; $CaCl_2.2H_2O$, 0.08g; $Fe(NH_4)_2(SO_4)_2.7H_2O$, 0.02g; $NaHCO_3$, 4.0g, KNO_3, 0.1g; yeast extract, 0.02g; trace element solution, 10 ml (see 2.21); vitamin solution, 5 ml (see 2.21); faecal extract, 5 ml (see 2.21); resazurin solution 0.2 ml (see

2.21); propionic, isobutyric, isovaleric, 2 methylbutyric and valeric acids 0.1 ml each. The medium was reduced using 20 ml of a reducing solution which contained:

Na_2S, 0.2g; cysteine-HCl, 0.4g and $NaHCO_3$, 2.0g.

The final pH was 7.0.

Several modifications to Min-1 were prepared.

Min-1(a) contained 1.5g.L^{-1} sodium acetate

Min-1(b) contained 1.5g.L^{-1} sodium formate

Min-1(c) contained 2.0 ml.L^{-1} methanol

Min-1(d) contained 2.0g.L^{-1} Na_2SO_4

Min-1(e) VFA's were omitted

Min-1(f) was adjusted to pH 6.5

Min-1(g) contained casein hydrolysate in place of Trypticase - BBL

Min-1(h) in this medium the concentration of amino acids was increased to 2.0g.L^{-1}

Min-1(i) was as for Min 1 except that 500 ml of clarified fermentor effluent replaced 500 ml of the 1L distilled water.

Min + ab This medium contained Penicillin G, Chloramphenicol, Phosphomycin, Colistin sulphate, Metronidazole, Antimycin A, Lysozyme, 0.02g each.

Medium 119 This medium was prepared as described in Deutsche Sammlung von Mikroorganismen Catalogue of Strains (1983) except rumen fluid replaced sewage sludge fluid.

Medium RFH Modified from medium-2 by omission of glucose,

maltose and cellobiose. For Med.2 see: Methods in Microbiology 3b (1969) pp.133-149.

2.25(4) Pectin Medium

The pectin medium contained per litre: KH_2PO_4, 0.3g; $(NH_4)_2SO_4$, 0.3g; NaCl, 0.6g; $MgSO_4.7H_2O$, 0.13g; $CaCl_2.2H_2O$, 0.008g; $NaHCO_3$, 4.0g; $Fe(NH_4)_2(SO_4)_2.7H_2O$, 0.02g; KNO_3, 0.5g; Hemin solution, 1.0 ml (see 2.21); trace element solution, vitamin solution faecal extract, 10 ml of each (see 2.21), Yeast extract, 0.2g; High methoxy citrus pectin, 10.0g; Purified Agar, 17.5g; isobutyric, 2-methylbutyric, valeric, isovaleric, and propionic acids 0.5 ml of each and 20 ml of reducing solution containing: dithiothreitol, 0.2g; Na_2S, 0.1g; Cysteine-HCl, 0.4g and $NaHCO_3$; 2.0g.

On one occasion anaerobic Pectin, Xylan and Starch plates were prepared. In this instance reducing solution was filter sterilized and added (aseptically) to the rest of the medium which had been sterilized and cooled under O-F CO_2 as for N-S anaerobic plates (see 2.25(2)).

2.25(5) Xylan Medium

Xylan medium was prepared as for (2.25(4)) except 10g of Xylan from oat spelts (Sigma) was substituted for pectin.

2.25(6) Cellulose Media

Cellulose medium was prepared as for (2.25(4)) except

cellulose (various forms and sources) was substituted for pectin and for solid media the agar concentration was 1% w/v.

Starch-reduced cabbage dietary fibre was prepared as for (2.20) and then ball-milled to a fine powder. 5.0g of the fibre was incubated at 100°C for 30 minutes in a reaction mixture containing 100 ml of 0.1 M phosphate buffer pH 6.0 and 5.0 ml of the ∝ amylase 'Termamyl'.

The dietary fibre swelled forming a gel-like consistency which could easily be separated from the starch containing supernatant. The gel was then washed twice with 500 ml of distilled water and added to 1 litre of medium. Acid swollen ball-milled filter paper was prepared by soaking the cellulose overnight in 30% v/v HCl and then washing with distilled water. For liquid cultures $20g.L^{-1}$ ball milled cabbage cellulose, untreated ball milled Whatman filter paper or acid swollen filter paper was added to the medium in place of starch reduced cellulose. 1% w/v glucose was incorporated into liquids when cellulose was present as a filter paper strip.

2.25(7) Starch Medium

The composition was as for (2.25(4)) except that 10 ml of a sterilized potato starch solution replaced the pectin.

Starch solution (10g/300 ml) was sterilized by boiling then added to the rest of the medium which had been autoclaved at

15 lbs. in^{-2} for 15 minutes. After addition of the reducing solution the medium was dispensed aseptically into sterile Hungate tubes.

2.26 Antibiotic Sensitivity Tests

Filter paper discs (3mm diameter) were impregnated with Everninomicin-B kindly supplied by Scherring Plough Corp., New Jersey, USA, and Phosphomycin obtained from Sigma at concentrations of 10 µg/disc and 100 µg/disc respectively.

These were dried overnight at 30°C and autoclaved at 10 lbs. in^{-2} for 20 minutes. Metronidazole and Colistin sulphate were available on prepared discs from Oxoid-UK at or near the concentrations used by L. Essers (1982) (5 µg and 10 µg per disc respectively).

SECTION 3

RESULTS

Graphs

Most of the results were derived from fermentor studies carried out during a series of fibre deletion experiments. Graphs of experimental results obtained over a period of days have the x-axis labelled 'days' and each day has 3 sub-divisions representing values immediately before the 9.00 am, 1.00 pm and 5.00 pm feeds respectively. For results recorded within a particular 24 hr period the position of the feed(s) is marked by the symbol △ on the x-axis. Graphs in which individual volatile fatty acid proportions are shown have the following symbols acetate ◇ propionate ○ and butyrate ✳.

3.1 All Fibres (Bran, Cabbage, Carrot, Turnip and Apple)

Two experiments were carried out (3a and 3b) each over a period of 18 days in which the above fibres were included in both feedstocks (for composition of feedstocks see section 2.21). The feedstocks showed no signs of contamination when examined microscopically after 3 days incubation at 37°C and the pH of the fermentor cultures was maintained at between pH 6.8 - 7.1. The experiments were run consecutively and both fermentor cultures were inoculated on day 1. The fermentation was monitored after addition of KNO_3 on days 2, 3, 5 and 8 of experiment 3b and after a single dose of lactulose on day 18. Production of H_2, CH_4 and N_2 gas was measured on day 6 of experiment 3a and microbiological

Figure 3-1-1 Total VFA concentration (mM) during Experiment 3a (All Fibres)

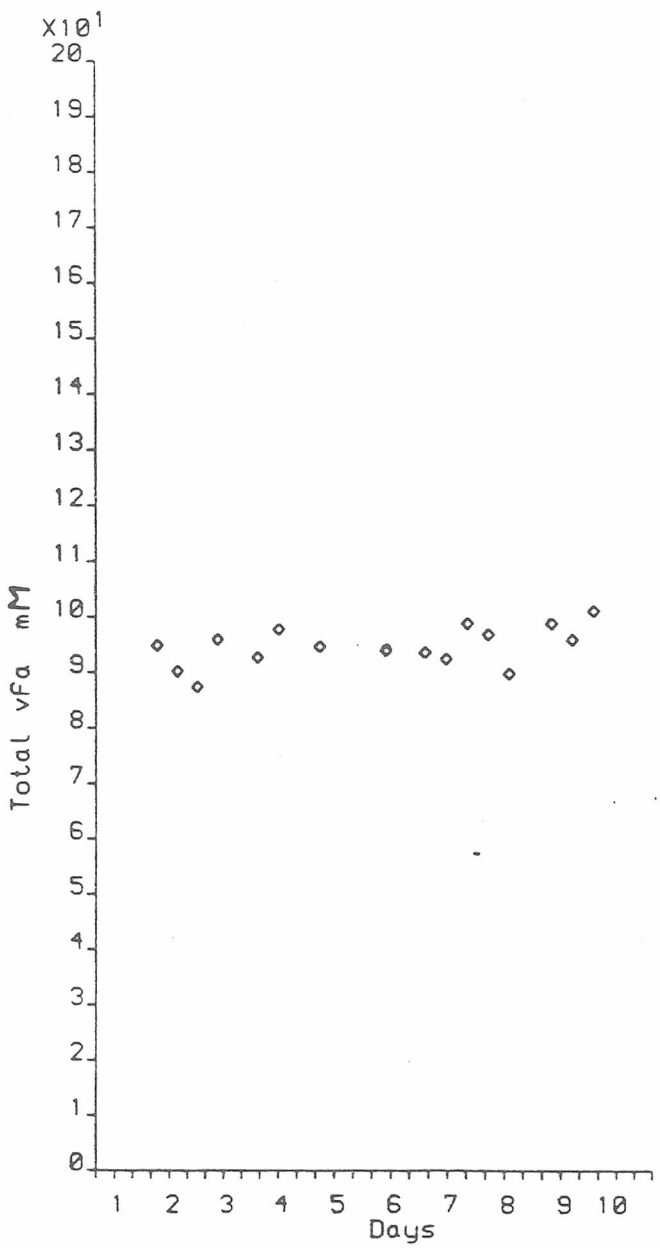


Figure 3-1-2 Total VFA concentration (mM) during Experiment 3b (All Fibres)

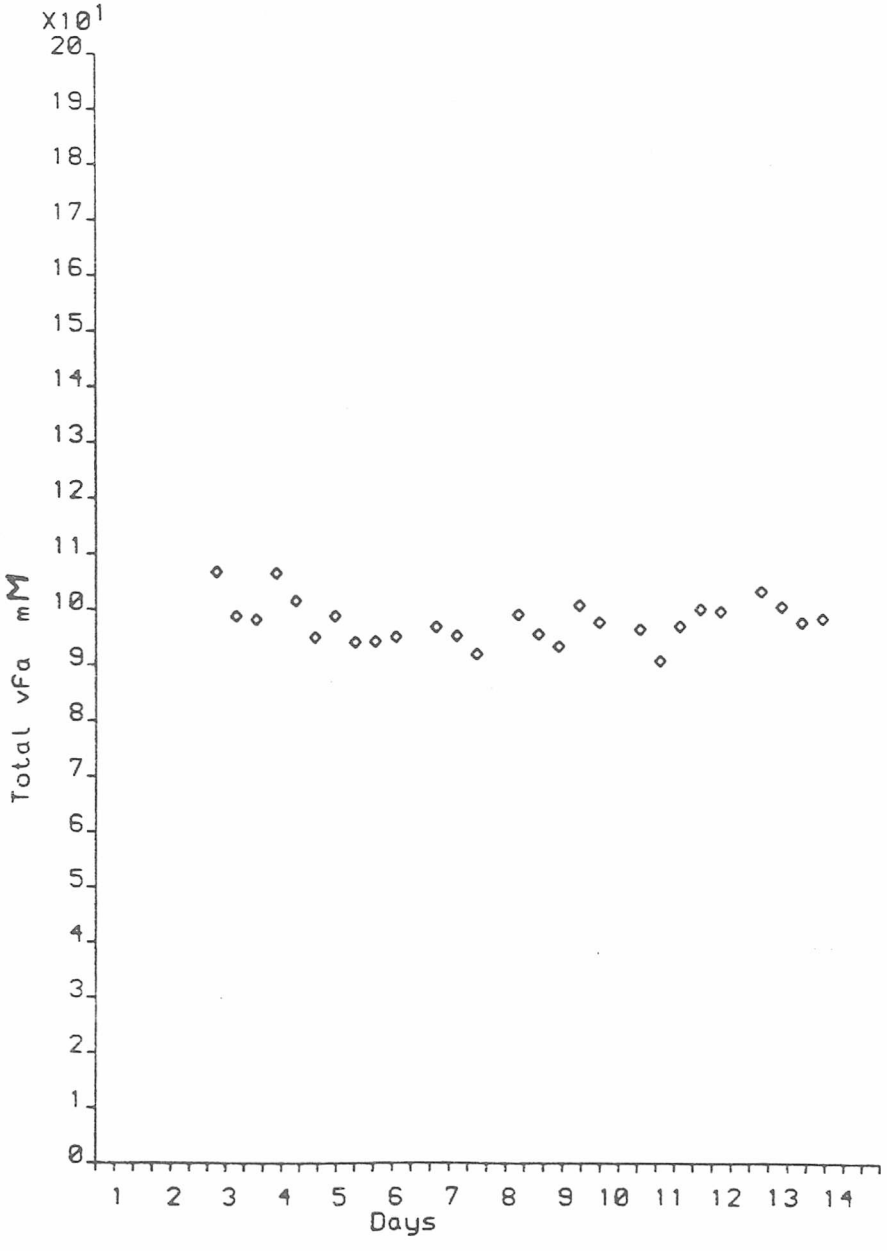


Table 3-1-1: Total VFA concentrations (mM) before the three daily feeds.

	9.00 am	1.00 pm	5.00 pm
Experiment 3a	100.13 ± 3.84[a]	96.84 ± 3.61[a]	96.16 ± 1.7
Experiment 3b	97.82 ± 2.2	95.01 ± 3.3	92.29 ± 2.87

[a] significantly different $P < 0.05$

Table 3-1-2: Proportions of the major VFA (mol/100 mol)

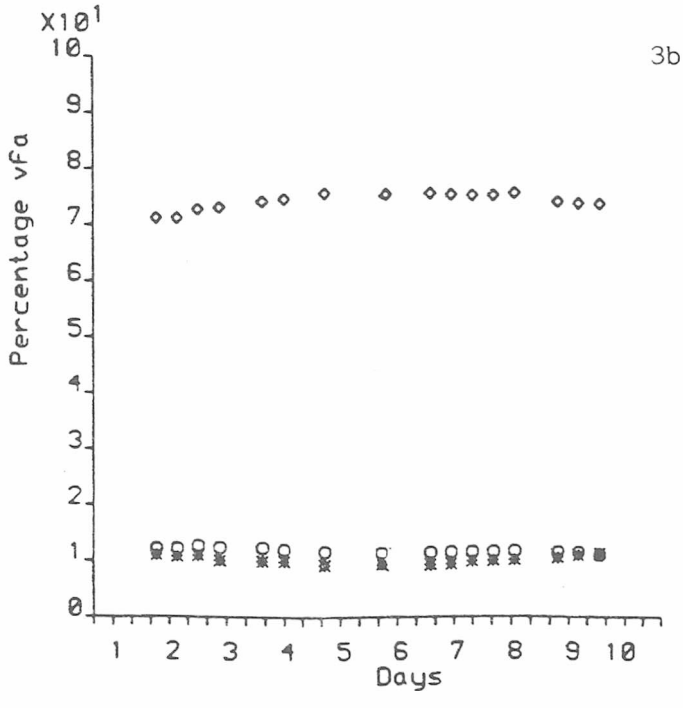
	Experiment 3a	Experiment 3b
Acetate	68.76 ± 0.62	74.12 ± 1.37
Propionate	12.40 ± 0.39	11.89 ± 0.4
Butyrate	12.57 ± 0.24	10.29 ± 0.63

studies were performed on day 11 of experiment 3a. Hydrogen sulphide gas production was measured during the nitrate experiments (days 2, 3, 5 and 8) and on days 10 and 11 of experiment 3b.

3.1(1) Volatile Fatty Acids (VFA)

Figures 3-1-1 and 3-1-2 show the volatile fatty acid concentrations during experiments 3a and 3b respectively. The totals comprise acetate, propionate, butyrate, isobutyrate, 2,methylbutyrate, isovalerate and valerate. The average total VFA concentrations measured immediately before the 9.00 am, 1.00 pm and 5.00 pm feeds are shown in table 3-1-1. Two statistical tests were used throughout the experiments. The Wilcoxon statistical test was used to compare volatile fatty acid concentrations at different times within the same experiment and VFA levels in different experiments were compared using a Mann-Whitney statistical analysis. There were no statistical differences evident when VFA levels in experiment 3a were compared with those of experiment 3b. Statistical comparison between the total VFA concentrations at 9.00 am, 1.00 pm and 5.00 pm within each of the experiments showed that the 9.00 am and 1.00 pm values in experiment 3a were significantly different from each other.

Taking into consideration a dilution factor due to feeding the daily production rates of VFA were calculated to be 36.78 $\pm$ 2.79 µmol/ml/24 hr during experiment 3a and 37.94 $\pm$ 2.09 µmol/ml/24 hr during experiment 3b.



3b

Figure 3-1-3 VFA concentrations (mol/100 mol) during experiments 3a and 3b. (All fibres) ◇ Acetate ○ Propionate ✱ Butyrate

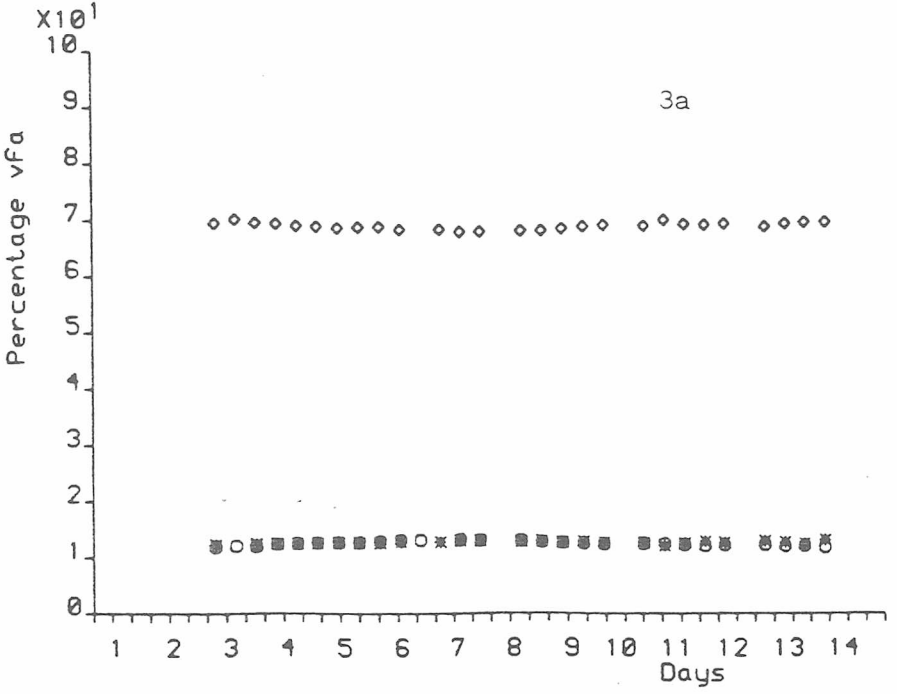
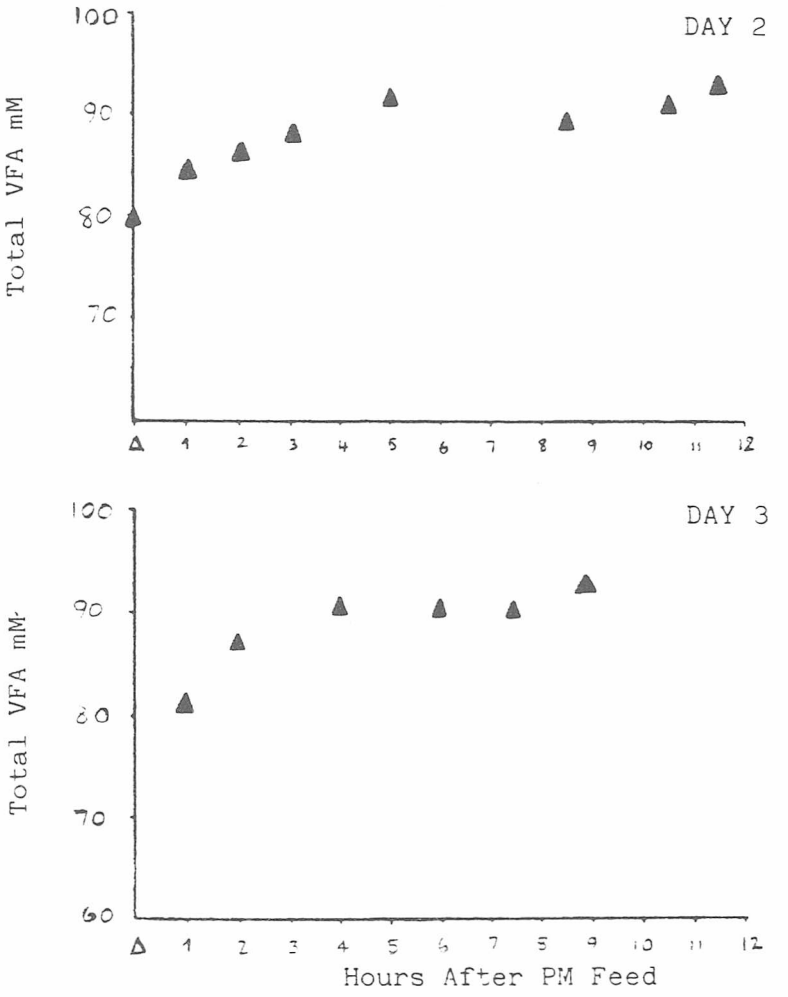


Figure 3-1-4 Total VFA concentrations (mM) in the fermentor following a feed.



The proportions of the total VFA represented by the major acids acetate, propionate and butyrate are shown in figure 3-1-3(a) and (b). Isobutyrate, 2,methylbutyrate, isovalerate and valerate which are not shown accounted for approximately 5% of the total and this was true for all experiments. The average percentages of the total VFA represented by individual VFA are shown in table 3-1-2. The proportions of VFA, total VFA and production rates in both experiments were very similar. From graphs 3-1-1 to 3-1-3 it can be seen that VFA levels stabilised soon after inoculation and that proportions remained constant throughout the experimental periods. The VFA proportions in the faecal samples used to inoculate the fermentor were as follows acetate 66-73%, propionate 10-20% and butyrate 8-21%.

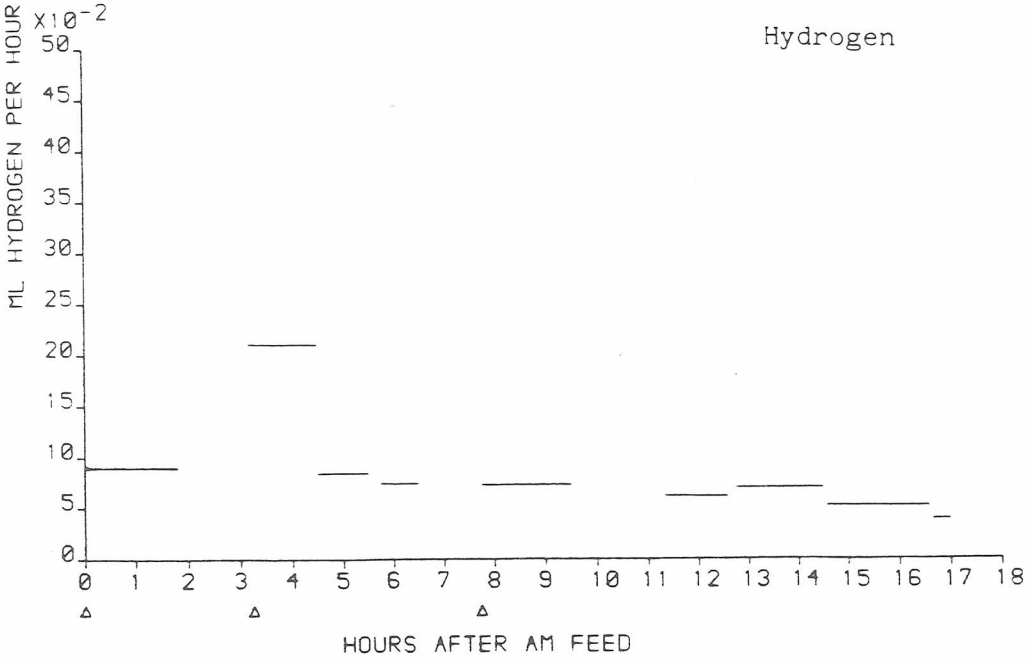
Levels of VFA in the fermentor were measured at intervals between feeds during experiment 3b. Figure 3-1-4 shows the total VFA produced after the 5.00 pm feed on days 2 and 3. Volatile fatty acid production appears to be most rapid in the 4-5 hr after a feed and then continues at a much slower rate for at least 8 hr with most of the increase due to acetate. VFA production was most rapid within the first 2 hr after the 9.00 am and 5.00 pm feeds.

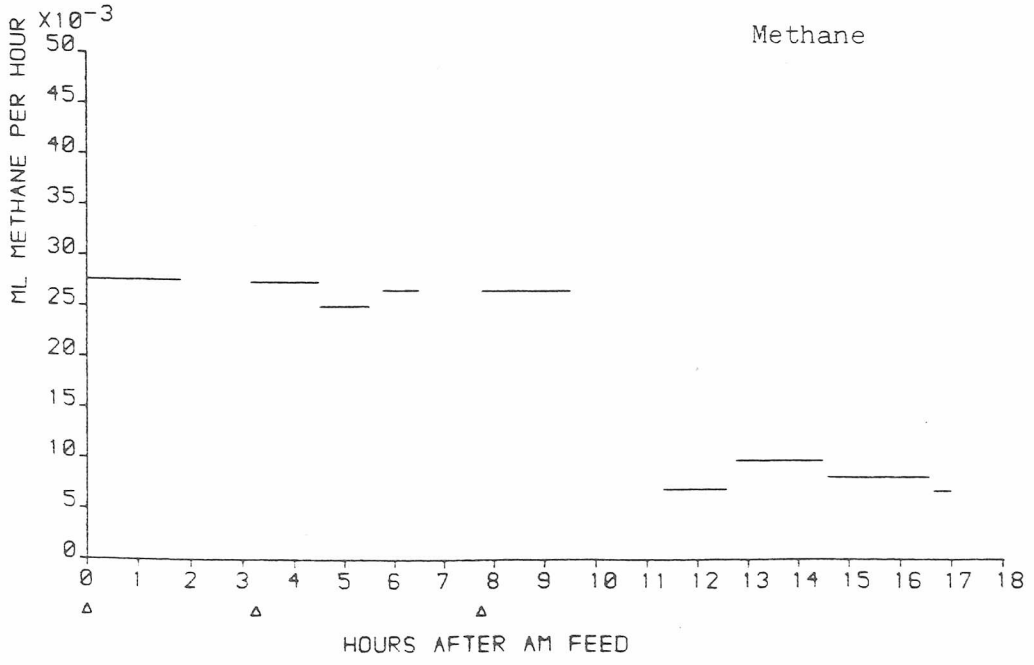
3.1(2) Lactate, Formate, Ethanol and Methanol

Lactate

Lactate did not accumulate in this instance or during any of the deletion experiments.

Figure 3-1-5 Hydrogen and methane production ($ml.hr^{-1}$) during experiment 3a (All Fibres).





Formate

Formate was detected in all feedstocks and at the same concentration in the respective fermentor samples, (Range 0.5 - 1.1 mM) suggesting that it was not used by the bacterial populations. Formate was not included in the feedstocks and was therefore believed to have been produced by a chemical reaction during autoclaving.

Ethanol

Ethanol was always detected in feedstocks at levels between 4 and 9 mM. As with formate, feedstock ethanol must have arisen as a result of a chemical reaction during preparation. In all experiments ethanol decreased rapidly after feeding to levels between 0 - 0.5 mM indicating that it was extensively metabolised by the bacteria.

Methanol

The small amounts of methanol (approx. 2 mM) present in all feedstocks were completely exhausted by the bacterial populations.

3.1(3) Hydrogen and Methane Gases

H_2, CH_4 and N_2 gases were produced during all of the experiments. The amounts of hydrogen ($ml.hr^{-1}$) and methane ($ml.hr^{-1}$) collected over a period of 17 hr after the 9.00 am feed on day 6 of experiment 3a are shown in figure 3-1-5. Hydrogen production was within the range 0.039 - 0.21 $ml.hr^{-1}$ and methane 0.0067 - 0.027 $ml.hr^{-1}$. The production patterns of the two gases were not obviously related to feeds. Hydrogen production appeared to reach a peak only

after the mid-day feed and methane levels were largely independent of feeds. Production of both gases was fairly constant throughout the day but methane levels fell sharply between 10 and 11 hours after the am feed.

The theoretical amount of hydrogen gas produced by the fermentor can be estimated from the total VFA produced and the ratios of individual acids. For experiment 3a, 36.78 μmol/ml/24 hr composed of 68.76% acetate, 12.4% propionate and 12.57% butyrate would result in the formation of 55.27 μmol H_2/ml/24 hr or in a total of 670 ml (fermentor volume) 37.02 mmol H_2/24 hr. This is equivalent to 829.34 ml/H_2/24 hr. In fact, only 2.03 ml of H_2 was collected over a 24 hour period and methane production for the same period was approximately 0.358 ml. If methane was derived from the oxidation of H_2 according to the following reaction.

$4H_2 + CO_2 \text{------>} CH_4 + 2H_2O$

then approximately 1.43 ml of H_2 would have been required. Evidently the production of methane in the fermentor was not a major hydrogenotrophic reaction.

The discrepancy in the hydrogen balance may be explained if the population of bacteria was capable of converting H_2 and CO_2 to acetate. eg

$2\ CO_2 + 4H_2 \text{------->}$ acetate $+ 2H_2O$ (1).

The original hydrogen production was calculated on the basis of the following equation

Hexose -------> 2 acetate $+ 2CO_2 + 4H_2$ (2).

Figure 3-1-6 Ammonia-N, Nitrate-N and Nitrite-N concentrations (ppm) during experiment 3a (All fibres).

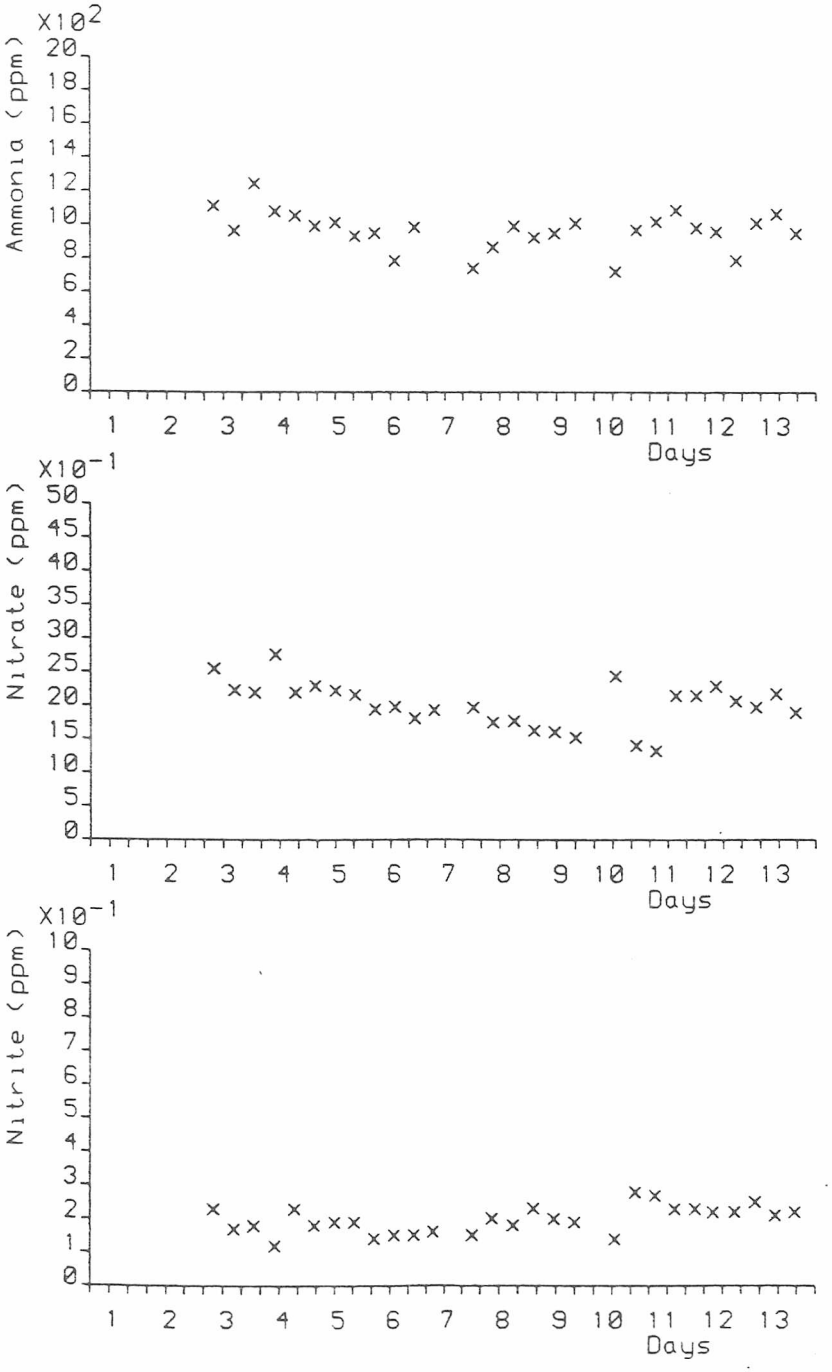


Figure 3-1-7 Ammonia-N, Nitrate-N and Nitrite-N concentrations (ppm) during experiment 3b (All fibres).

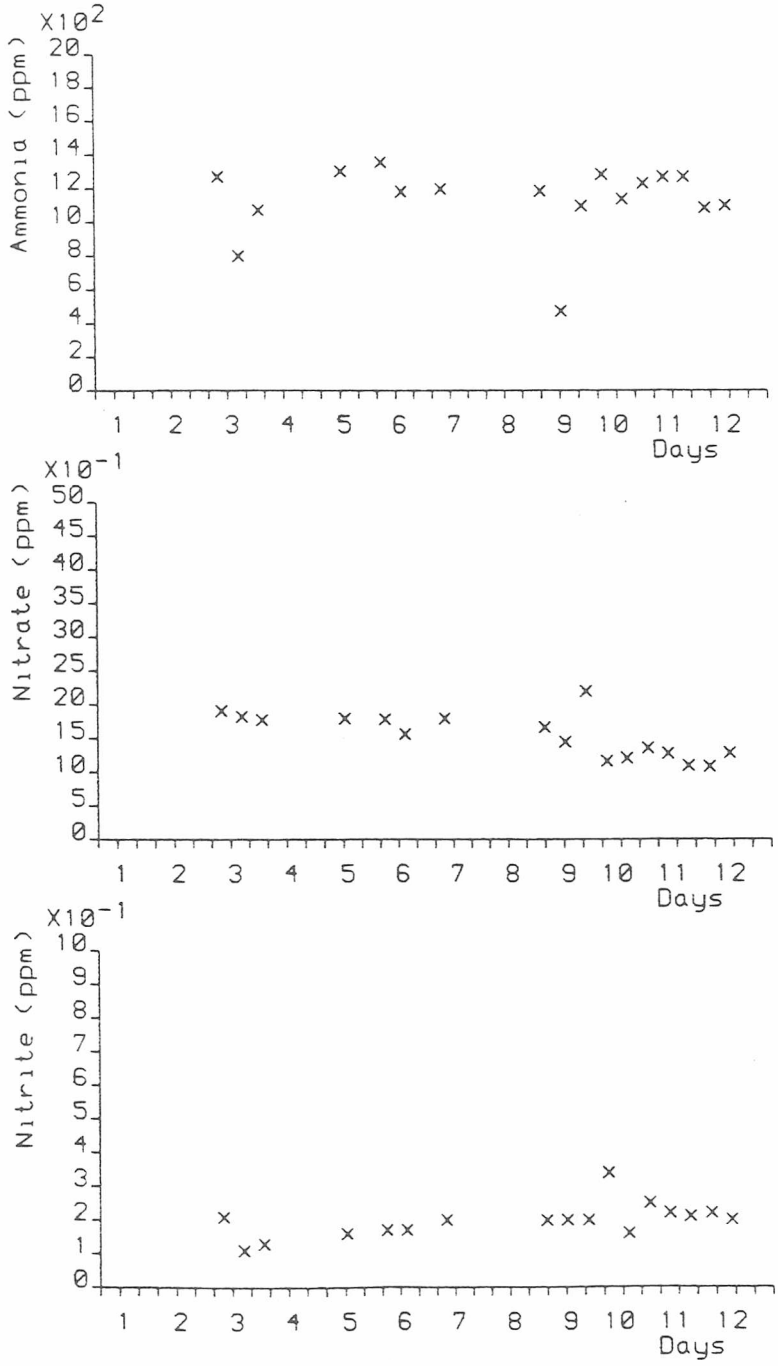


Table 3-1-3: Ammonia-N, Nitrate-N and Nitrite-N concentrations (ppm) in feedstock and fermentor samples.

	Ammonia-N (ppm)	Nitrate-N (ppm)	Nitrite-N (ppm)
Feedstock			
experiment 3a	334	135	0.05
Fermentor samples			
experiment 3a	996 ± 112	2.078 ± 0.31	0.196 ± 0.039
Feedstock			
experiment 3b	352	135	0.05
Fermentor samples			
experiment 3b	1177 ± 128	1.53 ± 0.32	0.20 ± 0.05

If the pathway in equation (1) accounts for part of the acetate production then the hydrogen production calculated by equation (2) will be overestimated by $2H_2$ per acetate formed by pathway (1). The original hydrogen balance may be corrected by deducting 6 moles of hydrogen for every mole of acetate produced by pathway (1). In experiment 3a it can be calculated that 4.82 mmoles of acetate per 24 hr could have been formed by pathway (1) out of a total acetate production of 16.94 mmoles per 24 hr. This calculation also takes account of hydrogen equivalents used in nitrate and sulphate reduction.

3.1(4) Nitrogen

Ammonia-, nitrate- and nitrite-N concentrations (ppm) for experiments 3a and 3b are shown in figures 3-1-6 and 3-1-7. Feedstock and fermentor culture concentrations of ammonia-N, nitrate-N and nitrite-N are shown in table 3-1-3. Ammonia-N was supplied in the feedstock as ammonium sulphate and in faecal fluid. However, urea, amino acids including cysteine hydrochloride and residual protein contained in the dietary fibres are also potential sources. The net increase in ammonia-N concentrations represents an average production of 86 ppm per feed during experiment 3a and 107 ppm per feed during experiment 3b. Feedstock levels of ammonia were higher than expected probably due to the partial hydrolysis of urea during autoclaving. The net increase in ammonia is likely to have been derived from the deamination of proteins, amino acids and possibly nitrate reduction.

Figure 3-1-8 Nitrogen gas production ($ml.hr^{-1}$) during Experiment 3a (All fibres).

Nitrite-N was never detected above 0.196 ± 0.040 ppm (experiment 3a) and 0.197 ± 0.050 (experiment 3b). Calculating from the data in table 3-1-3 an average of 17.26 ppm and 17.33 ppm nitrate-N were metabolised by the bacterial population between each feed in experiment 3a and 3b respectively. If this nitrate was completely reduced to nitrogen gas approximately 9.25 ml would be produced each feed or 27.75 ml N_2 per 24 hr.

$$2NO_3^- + 2[2H] \text{------>} 2NO_2^- + 2H_2O \qquad (3)$$

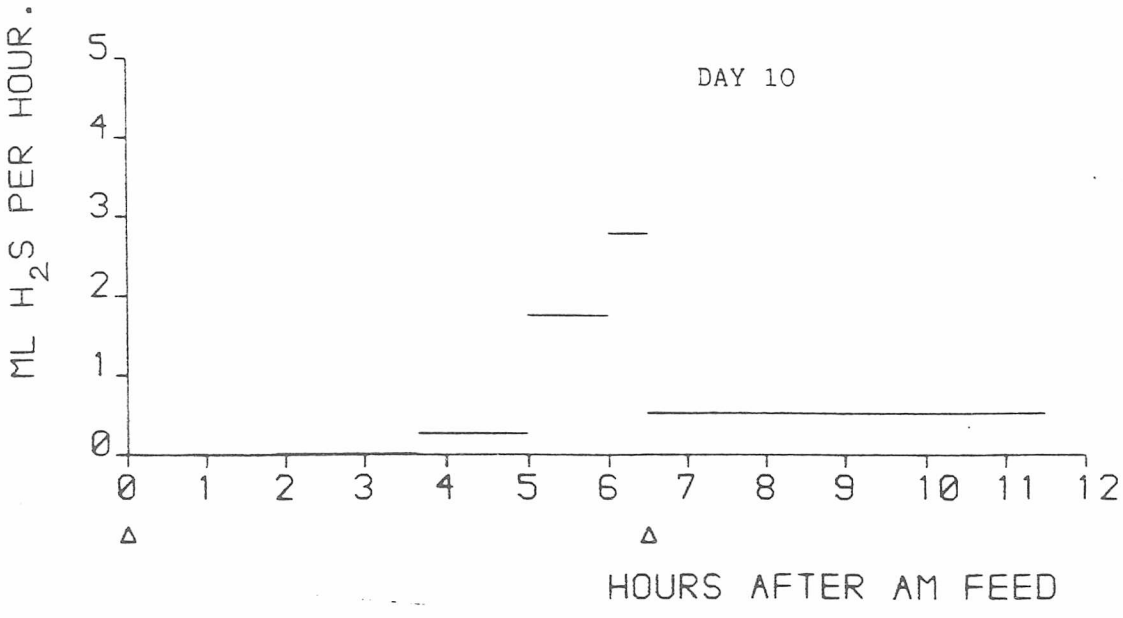
$$2NO_2^- + 3[2H] \text{------>} N_2 + 2H_2O + 2OH^- \qquad (4)$$

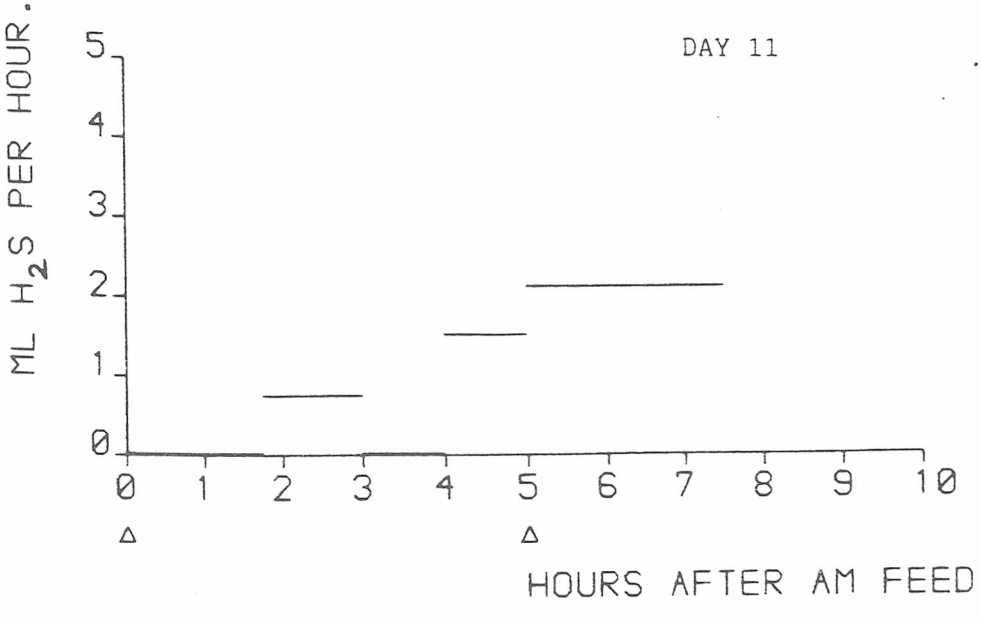
The above equations do not include nitric and nitrous oxides which may be formed during the reduction of nitrite. Analysis of nitrogen gas production on day 6 of experiment 3a is shown in figure 3-1-8. The average production rate was 1.07 $ml.hr^{-1}$ or 25.61 ml per 24 hr, close to the 27.75 ml per 24 hr estimated from the complete reduction of available nitrate to nitrogen gas. Nitrate was almost completely exhausted between feeds and given that nitrite never accumulated it may be expected that if evolution of N_2 gas was the result of nitrate reduction then production would be highest between 4-5 hr after a feed and decline thereafter. From figure 3-1-8 it can be seen that this was the general pattern of N_2 production.

3.1(5) Sulphur

Sulphate concentrations in the fermentor during experiment 3a decreased from 0.296 mM immediately after a feed to 0.080 ± 0.044 mM therefore, an average of 0.216 mM sulphate was

Figure 3-1-9 Hydrogen sulphide production ($ml.hr^{-1}$) during experiment 3b (All fibres).





metabolised between each feed. During experiment 3b sulphate concentrations were 0.315 mM immediately after a feed and 0.097 $\pm$ 0.056 mM before the next feed therefore, an average 0.218 mM sulphate was consumed between each feed. If this sulphate was reduced by bacteria capable of dissimilatory sulphate reduction an average of 3.24 ml and 3.28 ml of H_2S would be produced from each feed in experiment 3a and 3b respectively.

H_2S production was measured on days 10 and 11 of experiment 3b see figure 3-1-9 (10) and (11). On day 10 2.53 ml of H_2S was produced between the morning and mid-day feeds and 5.27 ml after the mid-day feed. On day 11 3.77 ml of H_2S was produced between the morning and mid-day feeds and 2.63 ml after the mid-day feed. In both experiments, H_2S accumulated in an irregular fashion but overall an average of 6.56 ml of H_2S could be expected from the two feeds based on the reduction of available sulphate and in fact 7.8 ml and 6.4 ml were collected on day 10 and 11 respectively. It should be noted that during the first few days of these and other experiments H_2S gas was emitted from the feedstock presumably as a by-product of the breakdown of the reducing agent cysteine hydrochloride. Further, the fermentation of sulphur containing amino acids would also release H_2S.

3.1(6) Fibre Digestion

The starch, uronic acid and sugar composition of feedstock dietary fibre and fermentor residues during experiment 3a are

Table 3-1-4: Composition of dietary fibre (mg.ml

	Starch	Uronic Acids	Arabinose	Xylose
Feedstock experiment 3b	1.7053	1.53	0.824	0.887
Fermentor samples experiment 3b	0.255± 0.063	0.0423 ±0.0076	0.334± 0.236	0.344± 0.175

$^{-1}$) in feedstock and fermentor samples.

Mannose	Galactose	Glucose
0.189	0.656	2.424
0.015	0.030	0.241
±0.01	±0.025	±0.09

Table 3-1-5: Enumeration of bacteria in experiment 3a

	Bacteria/ml of fermentor contents
Total Anaerobic Counts	$1.6 \pm 0.52 \times 10^9$
Total Aerobic Counts	$3.46 \pm 1.59 \times 10^7$
Enterobacteriacea	$3.0 \pm 1.0 \times 10^7$
Xylanolytic bacteria	$1.2 \pm 0.6 \times 10^7$
Pectinolytic bacteria	$3.66 \pm 3.09 \times 10^8$
Starch digesting bacteria	$3.26 \pm 0.9 \times 10^8$

Anaerobic : Aerobic Ratio	46 : 1
Anaerobic : Xylanolytic Ratio	133 : 1
Anaerobic : Pectinolytic Ratio	4 : 1
Anaerobic : Starch digesting ratio	4 : 1

shown in table 3-1-4 an average of 0.914 mg.ml^{-1} or 3.402 mmoles of hexose equivalents/fermentor were metabolised after each feed. From a total of three feeds the amount was 1.84 g/fermentor/24 hr or 10.21 mmoles/fermentor/24 hr.

3.1(7) Microbiology

Table 3-1-5 gives the total numbers of bacteria per ml, those belonging to the various functional groups tested, facultative anaerobes and numbers of Enterobacteriacea. In addition, the ratio of total anaerobes to certain groups was calculated. From table 3-1-5 it can be seen that members of the Enterobacteriacea are the predominant facultative anaerobes and that starch and pectin degrading organisms are approximately ten times greater in number than xylanolytic bacteria.

Total numbers of bacteria present in several faecal samples processed over a period of six months were recorded for comparison with fermentor counts. The counts ranged from 2.94×10^{10} - 1.6×10^{11} bacteria/g wet weight of faeces.

Fifty-eight colonies were isolated from fermentor plate dilutions and grown in N-S broths. Each culture was replica-plated onto starch, xylan, pectin and cellulose plates. None of the isolates were able to digest cellulose. Thirty-three colonies were starch degrading representing approx. 7.11×10^{8}.ml^{-1} fermentor contents, 5 colonies were pectinolytic representing approximately 1.07×10^{8}.ml^{-1} and

Table 3-1-6: Identification of bacteria in experiment 3a

		Bacteria/ml of fermentor contents
1.	Fusobacterium Selenomonas Leptotrichia Vibrio succinogenes	1.18×10^8
2.	Clostridium Eubacterium	8.44×10^7
3.	Bifidobacterium Actinomyces	8.44×10^7
4.	Bacteroides	1.52×10^8
5.	Lactobacillus	3.3×10^7
6.	Propionibacterium	1.52×10^8

24 were xylanolytic representing approx. 5.18 x $10^{8}.ml^{-1}$. The xylanolytic count was unexpectedly high. Three organisms were capable of all three activities, thirteen isolates degraded only starch and xylan and one isolate degraded only starch and pectin. Nine out of the 33 degraded only starch, 1 out of the 5 pectinolytic organisms degraded only pectin and 2 out of the 24 xylanolytic isolates were exclusively xylanolytic. Cellulolytic bacteria were not isolated from the fermentor during any of the experiments using the range of cellulolytic media described in section 2.25(6). On several occasions cabbage dietary fibre broths were inoculated with faeces. The cellulose was completely digested within two days incubation at 37°C and butyrate was the major end-product. Cellulolytic activity was not detected in any of the other cellulose media inoculated with faeces.

Growth was recorded in anaerobic dilution solution up to the $10^{9}/10^{10}$ dilutions in all experiments indicating that bacteria capable of utilising amino acids were amongst the predominant species present in the fermentor.

A preliminary identification of the major groups present in the fermentor was carried out using antibiotic sensitivities and end-product analysis. The results are shown in table 3-1-6. The number of bacteria assigned to the Lactobacilli in this experiment and others is undoubtedly an overestimate. Isolates were identified which conformed to this group

Table 3-1-7: Identification of bacteria in faecal sample.

		Bacteria/g wet weight faeces
1.	Fusobacterium	
	Selenomonas	4.0×10^9
	Leptotrichia	
	Vibrio succinogenes	
2.	Clostridium	
	Eubacterium	1.3×10^9
3.	Bifidobacterium	2.0×10^9
	Actinomyces	0
4.	Bacteroides	8.1×10^9
5.	Lactobacillus	1.5×10^{10}
6.	Propionibacterium	4.0×10^9

according to their antibiotic sensitivity reactions but which produced volatile fatty acids which are not typical of the Lactobacilli.

The major bacterial groups present in a faecal sample were also examined. Antibiotic sensitivity, VFA production, Gram reaction, motility and morphological characteristics were used to identify isolates. The major groups present are recorded in table 3-1-7. Bacteroides spp and Propionibacterium spp are the most numerous as was the case for fermentor samples but the numbers of Lactobacilli were high probably due to the inclusion of species other than Lactobacilli in the same antibiotic sensitivity category.

Hydrogen and carbon dioxide metabolism was detected in Min-1, Min-1(c), Min-1(e) and medium 119. Acetate was the only product in Min 1(e) and medium 119, acetate plus butyrate were produced in Min-1 and Min 1(c).

Comparable amounts of acetate were not detected in cultures incubated under an atmosphere of OF-CO_2 except in the case of Min-1(c) or in minimal medium containing antibiotics (Min + ab) and these results were true for growth of fermentor cultures in all deletion experiments.

Fermentor samples were grown in Min-1(h) to test whether

increasing the concentration of amino acids would increase the amounts of acetate produced. Acetate concentrations did increase in these cultures suggesting that amino acids were fermented to acetate but on a molar basis the fermentation of amino acids alone could not account for the amounts of acetate produced. H_2 - CO_2 metabolism was stimulated in cultures grown in the presence of increased sulphate concentrations Min-1(d) and correspondingly high levels of acetate were recorded.

Methane was never detected in any of the minimal media, N-S medium or anaerobic dilution solution inoculated with samples taken from the fermentor or from faeces. The cellulolytic broths inoculated with faeces were also analysed for methane production but were found to be negative. In order to establish that the anaerobic technique and media were appropriate for growth of methanogenic bacteria, pure cultures of Methanobrevibacter smithii were inoculated into all of the minimal media under H_2:CO_2 80%:20%. Growth and methane production was recorded in all of the media except Min-1(c).

The turnover of bacteria can be used to estimate the amount of hexose equivalents required to sustain the population.

During experi ment 3a, 4.18×10^{11} cells were produced every day in the fermentor. This represents a dry weight of approximately 0.56 g of bacterial cells (Wolin, 1981). Most

Figure 3-1-10 Ammonia-N, Nitrate-N and Nitrite-N concentrations (ppm) on day 2 of experiment 3b (All fibres)

△ Ammonia ✕ Nitrate ● Nitrite

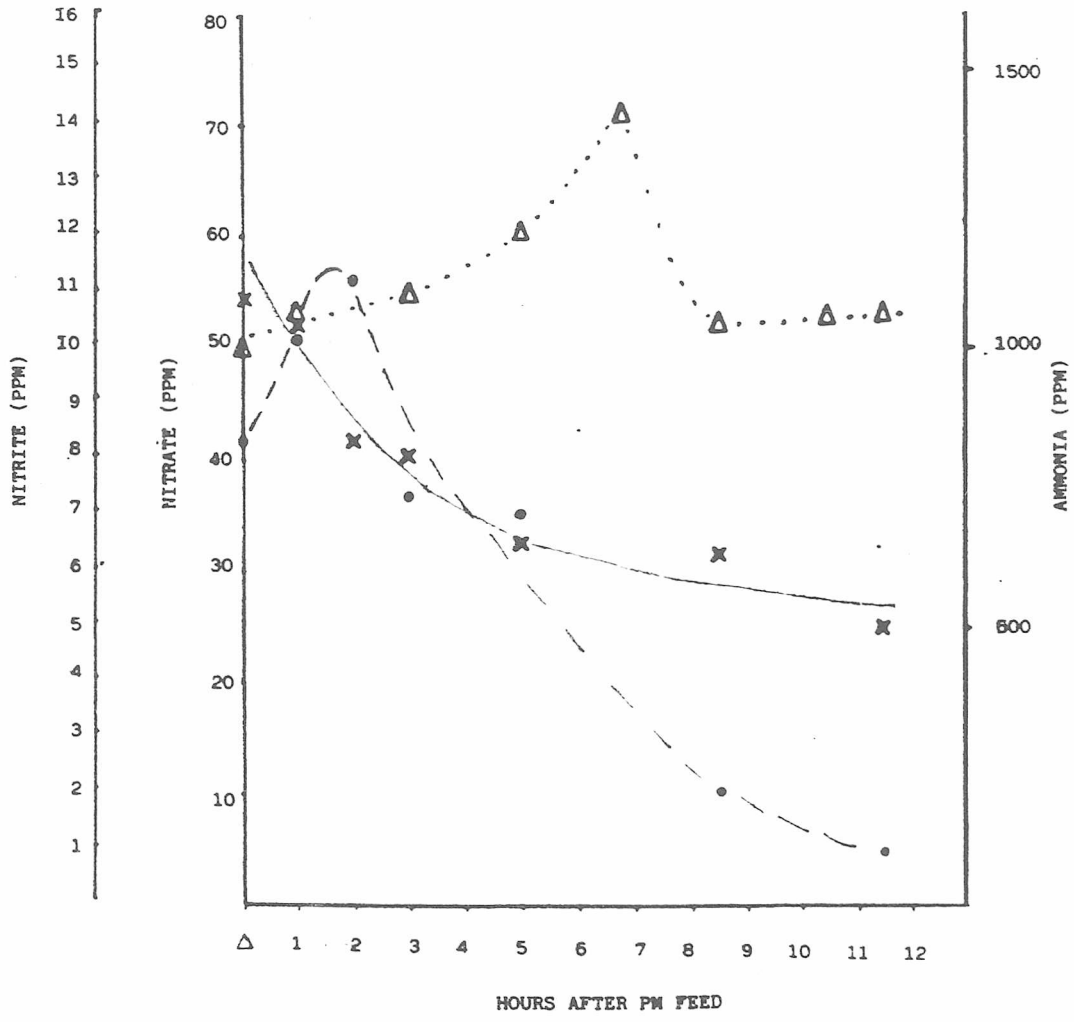
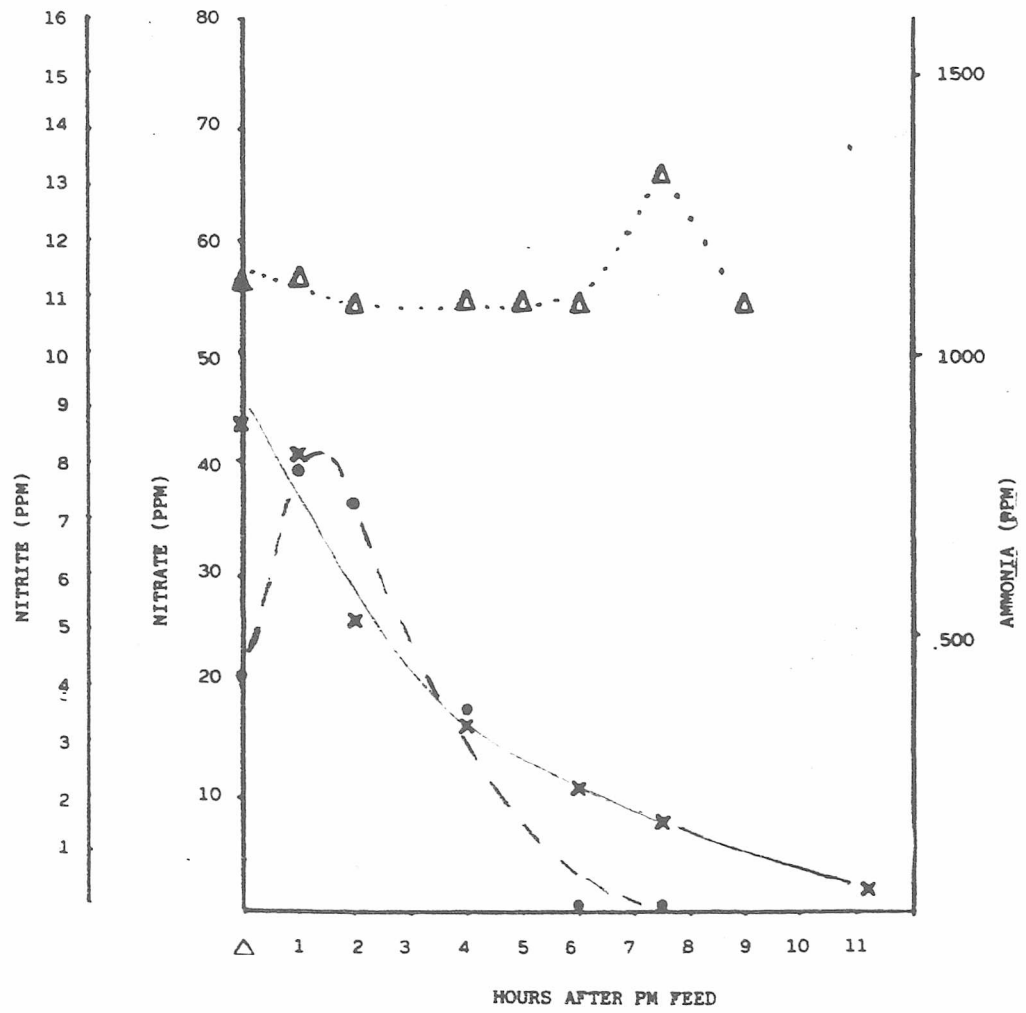


Figure 3-1-11 Ammonia-N, Nitrate-N and Nitrite-N concentrations (ppm) on day 3 of experiment 3b (All fibres)

△ Ammonia ✕ Nitrate ● Nitrite



workers calculate that 3-4 g hexose are required to produce 1 g dry weight of cells therefore, between 1.67 and 2.2g of hexose would be required to sustain the fermentor population each day. An alternative approach (Cummings, 1983) is to assume that the dry weight of bacteria represents between 20 - 30% of the weight of carbohydrate fermented. In this case, 1.85 - 2.78 g hexose per day could have produced the observed turnover in bacterial cells. The actual amount of hexose consumed by the bacterial population was 1.84 g/24 hr. (Section 3.1(6)).

3.1(8) Nitrate Experiment

Nitrate was added to the fermentor in the form of KNO_3 in anaerobic dilution solution. The aims of these experiments were to establish that active nitrate reduction was taking place in the fermentor, to investigate whether or not increased nitrate concentrations would give rise to potentially toxic levels of nitrite and to monitor the effects of increased availability of a terminal electron acceptor on the general fermentation. On day 2 of experiment 3b, 0.3 g of KNO_3 was added to the fermentor immediately after the 5.00 pm feed and the following day (day 3) the experiment was repeated with 0.15 g KNO_3. On days 5 and 8, 0.3 g and 0.5 g KNO_3 were added after the morning feed. Total VFA, VFA proportions, ethanol, methanol and fibre digestion were not affected by these additions. Ammonia-N, nitrate-N, and nitrite-N (ppm) on days 2 and 3 are shown in figures 3-1-10 and 3-1-11 respectively. Nitrite-N levels

Figure 3-1-12 Nitrate-N and Nitrite-N concentrations (ppm) on day 5 of experiment 3b (All fibres)

Nitrate X Nitrite ●

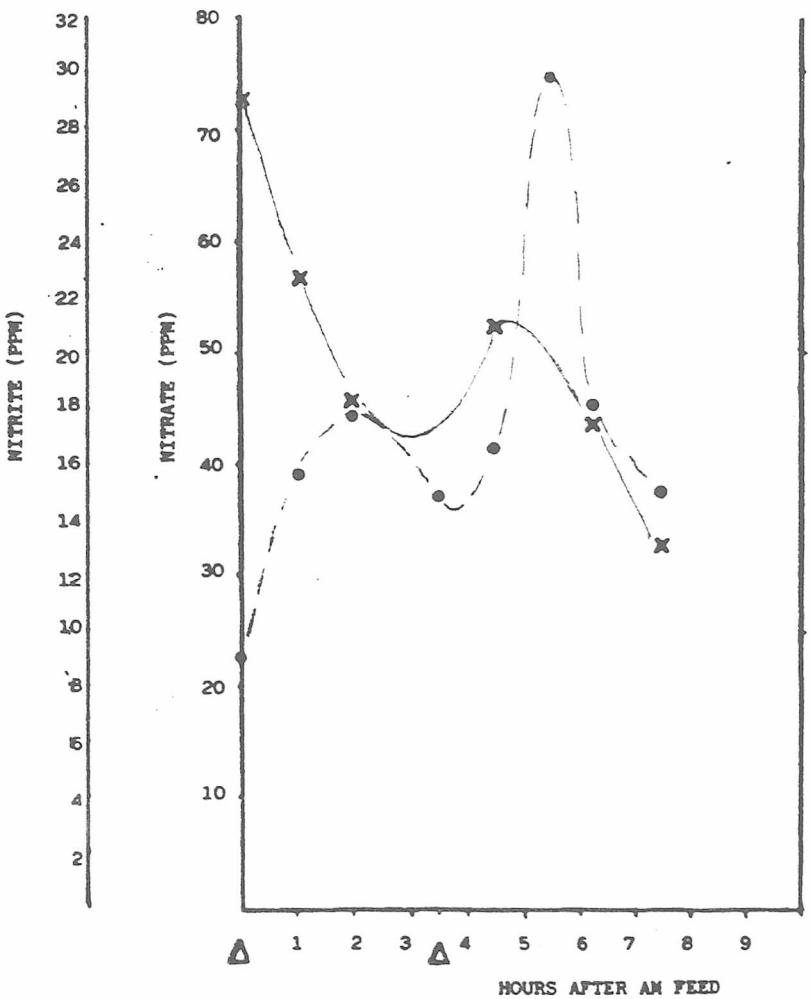
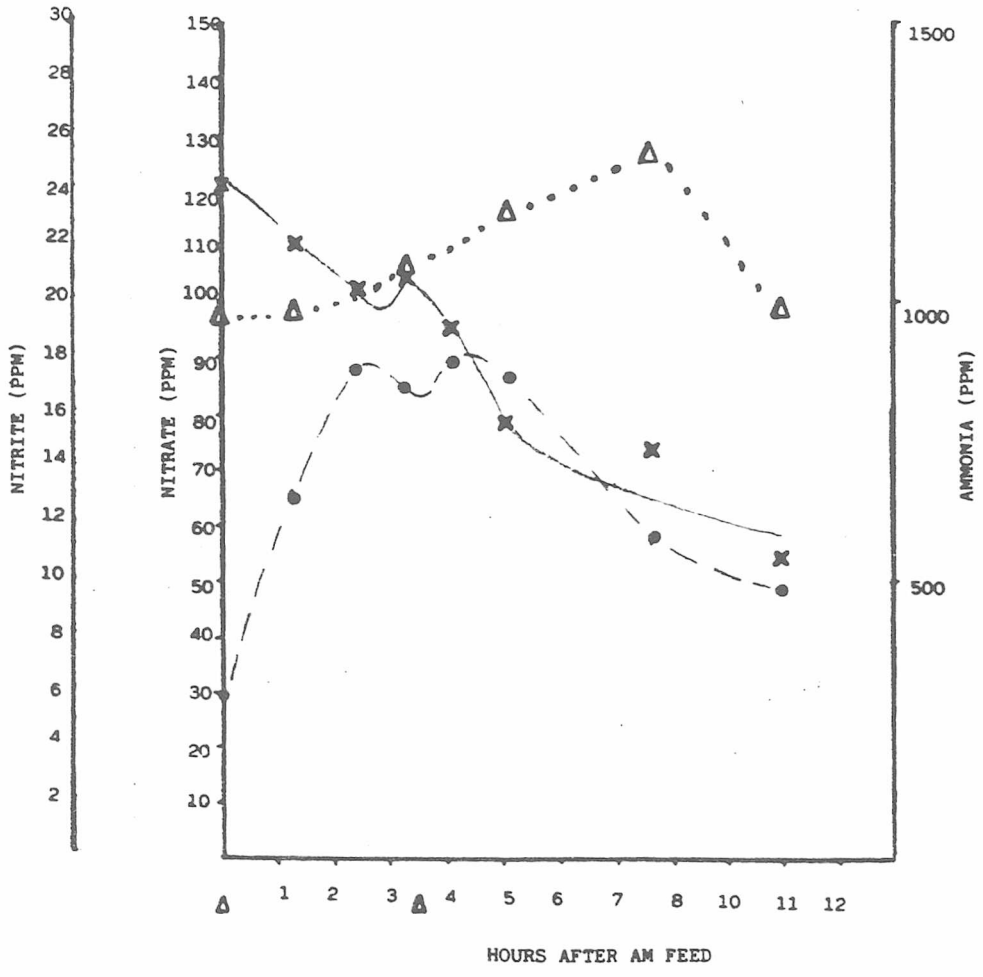


Figure 3-1-13 Ammonia-N, Nitrate-N and Nitrite-N concentrations (ppm) on day 8 of experiment 3b (All fibres)

△ Ammonia ✕ Nitrate ● Nitrite



returned to the baseline within 6 hr and nitrate within 12 hr on day 3. The maximum nitrite-N concentration was 8.0 ppm reached 1 hr after the addition of 43 ppm nitrate-N. On day 2 nitrite-N levels returned to the baseline at between 8.5 and 11.5 hr although nitrate-N was still approx. 10 times higher than normal levels after 11.5 hr. Nitrite-N reached a maximum at 11.2 ppm 2 hr after the addition of 54.5 ppm nitrate-N. The amount of nitrate-N measured after addition of 0.3 g KNO_3 was unexpectedly low. It is likely that the sample was mis-read and that the initial concentration was nearer 80 ppm.

Ammonia-N levels on both days show that levels are constant during the first 6 hr of growth (1000 - 1100 ppm) then increase sharply (1350 - 1450 ppm) between 6 and 8 hr. Ammonia-N concentrations then return to initial levels presumably due to a net consumption by bacteria in the fermentor.

Nitrate-N, nitrite-N and ammonia-N concentrations on days 5 and 8 are shown in figures 3-1-12 and 3-1-13 respectively. On day 5 the fermentor was fed twice during the experimental period of 8 hr. Nitrate concentrations decreased from 73 ppm to 45 ppm within the first 3 - 5 hr. Nitrite levels rose to a maximum of 18 ppm 2 hr after the addition of nitrate. After the mid-day feed (which includes nitrate and ammonia) nitrate-N levels declined further to 33 ppm and nitrite levels rose to 32 ppm 2 hr after the mid-day feed. Neither

nitrate-N nor nitrite-N had returned to 'normal levels' within the 8 hr period. On day 8 the fermentor culture received two feeds. Nitrate-N levels fell from 121 ppm to 105 ppm within the first 3 hr and nitrite-N rose to 17.7 ppm 2.4 hr after the start of the experiment. Nitrate-N decreased to 55 ppm 6.5 hr after the mid-day feed and nitrite rose to a maximum of 17.0 ppm before falling to 9.8 ppm at the end of the experiment. In all cases nitrate and nitrite levels had returned to normal by 9.00 am the following morning.

Ammonia concentrations rose to a maximum of 1280 ppm 4 hr after the mid-day feed and then returned to previous values of approximately 1000 ppm. Overall, levels of ammonia were not significantly higher than those recorded in the absence of added nitrate. Further, the magnitude of the increase in ammonia-N observed in the experiments was such that nitrate reduction could not have been a significant contributor. The results suggest that net ammonia production was mostly derived from organic sources.

Sulphate levels were monitored during and after the nitrate experiments. On each day sulphate reduction was almost completely inhibited as evidenced by the maintenance of constant levels after a feed (day 2 and 3) or accumulating levels after several feeds (day 5 and 8). H_2S was produced but production rates were much lower than usual. Since sulphate reduction was completely inhibited, the H_2S produced

Figure 3-1-14 Hydrogen sulphide production ($ml.hr^{-1}$) in experiment 3b (All fibres) following addition of 0.3 g KNO_3 on day 2.

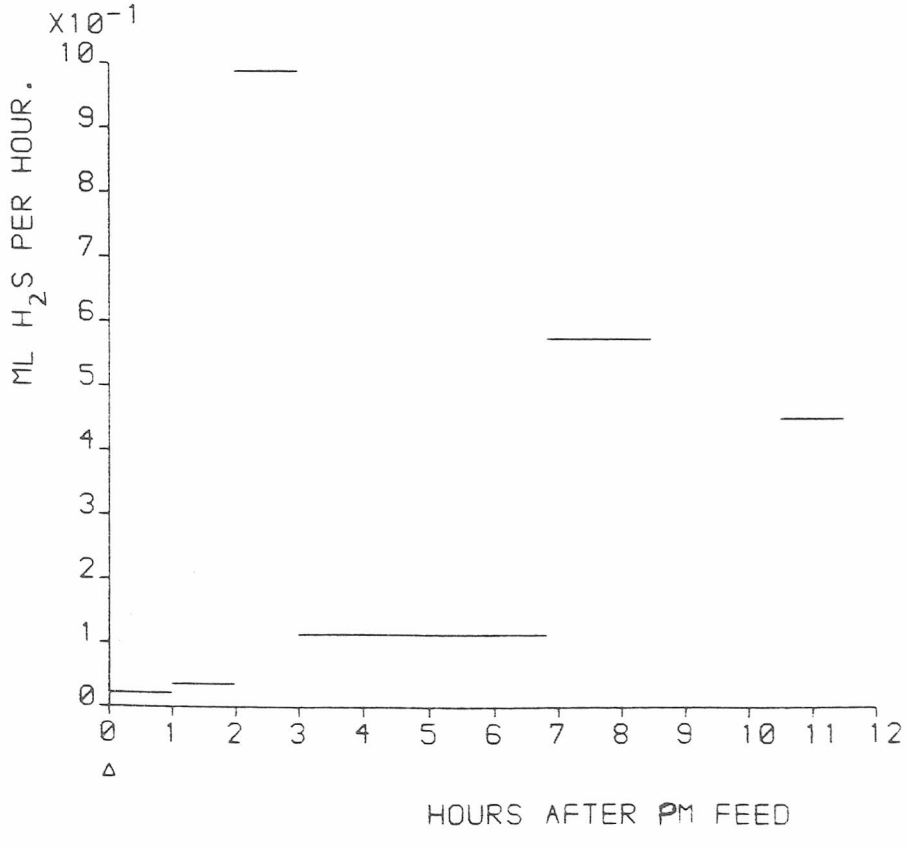


Figure 3-1-15 Hydrogen sulphide production ($ml.hr^{-1}$) in experiment 3b (All fibres) following addition of 0.15 g KNO_3 on day 3.

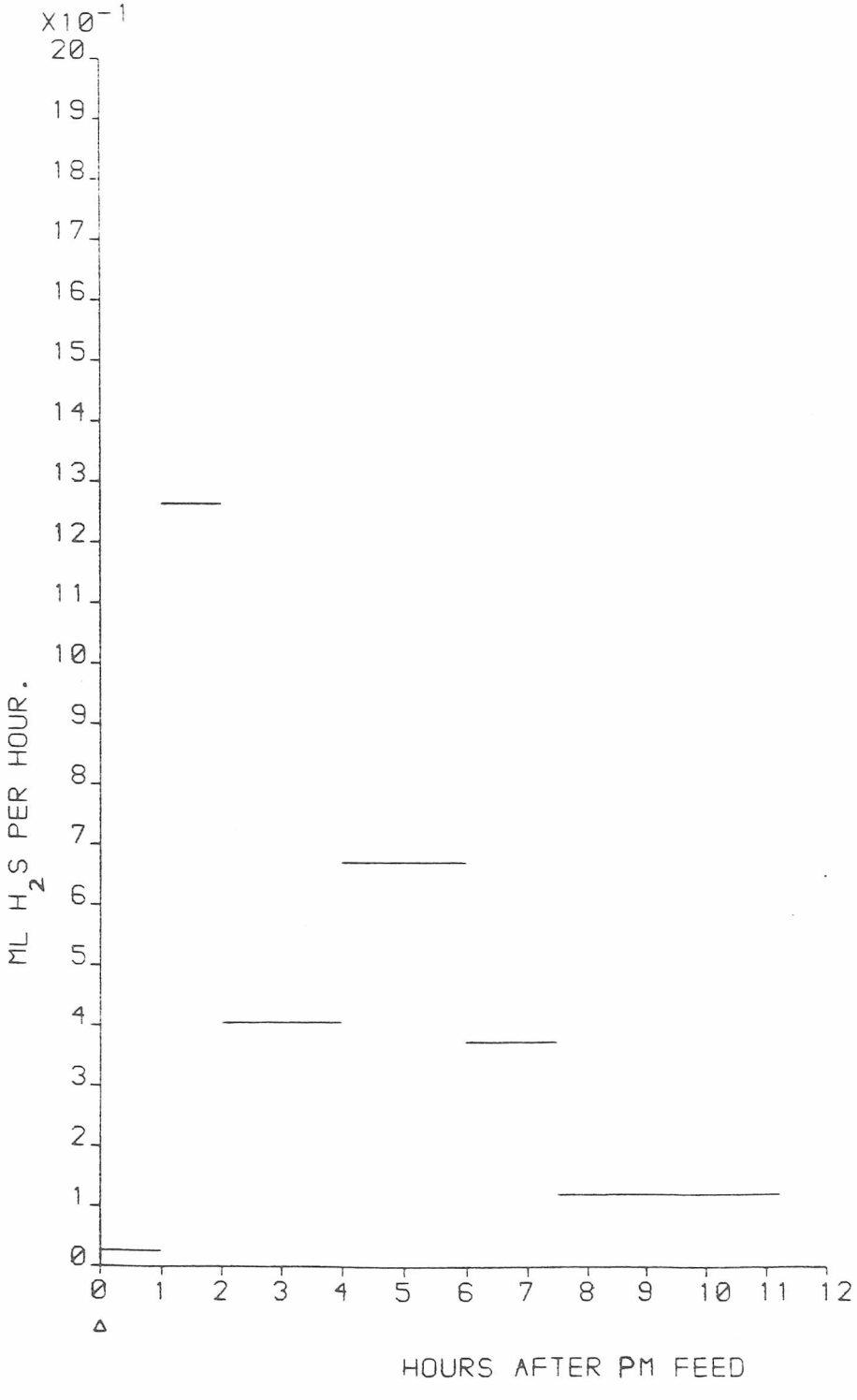


Figure 3-1-16 Hydrogen sulphide production ($ml.hr^{-1}$) in experiment 3b (All fibres) following addition of 0.3 g KNO_3 on day 5.

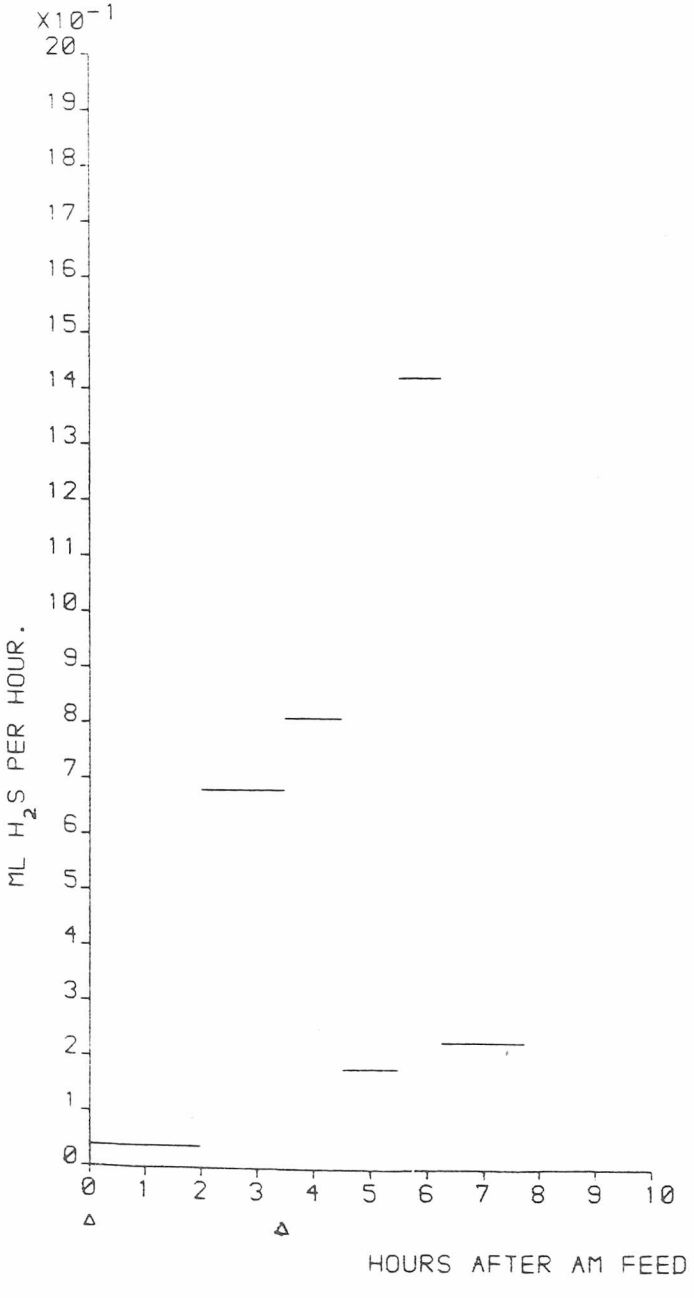
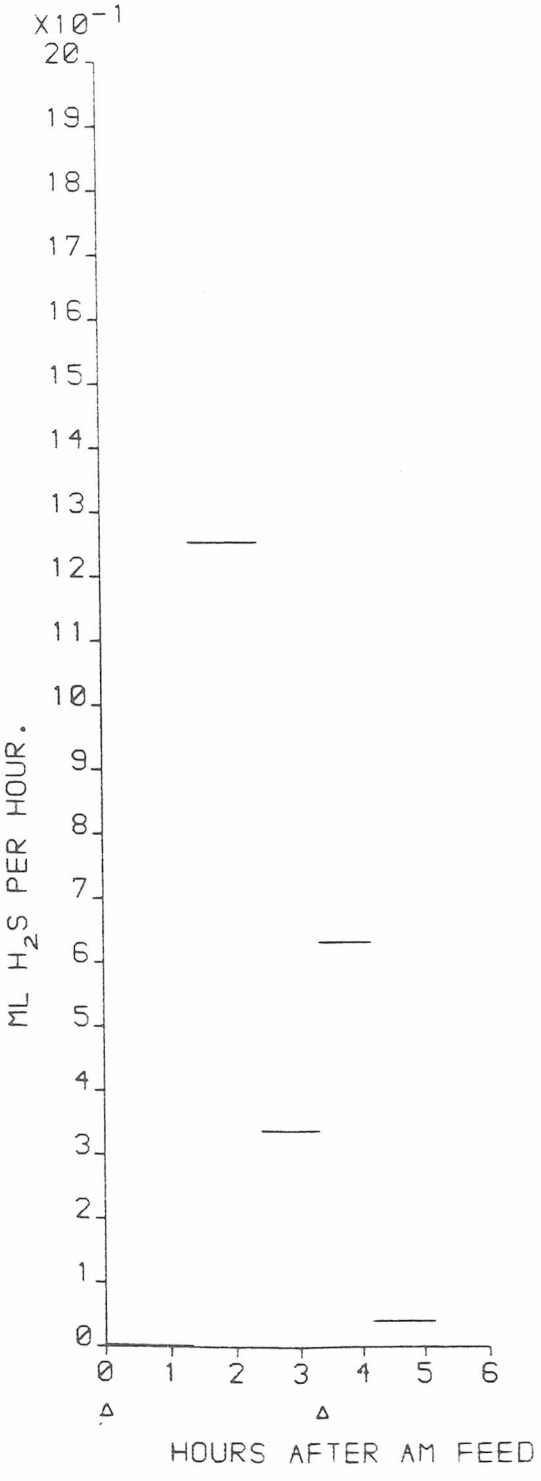


Figure 3-1-17 Hydrogen sulphide production ($ml.hr^{-1}$) in experiment 3b (All fibres) following addition of 0.5 g of KNO_3 on day 8.



is likely to have arisen from the fermentation of sulphur containing amino acids.

The H_2S production on days 2, 3, 5 and 8 are shown in figures 3-1-14 - 3-1-17 respectively. On day 2 a total of only 2.868 ml of H_2S was collected over a period of 11.5 hr and on day 3 a total of 4 ml was produced over a period of 11.25 hr. Under normal circumstances between 3-4 ml of H_2S would have been produced within the first 4 hr. The production of H_2S on days 5 and 8 was markedly inhibited. On day 5, 3.44 ml of H_2S was produced from the two feeds and on day 8, 2.26 ml of H_2S was collected over a period of 6 hr with 2 feeds. It would appear that nitrate reducing bacteria gained a competitive advantage over sulphate reducers when nitrate concentrations were increased.

3.1(9) Lactulose Experiment

The aim of this experiment was to observe the response of the fermentor population to the supply of a readily fermentable substrate. Lactulose was added to the fermentor to a final concentration of 0.5% after the morning feed on day 18.

A total of 23.06 µmol VFA/ml was produced in the 4 hours after addition of lactulose. This contrasts with an average of 9.7 µmol VFA/ml for the same period during the rest of experiment 3b. The relative proportions of individual acids changed such that propionate decreased from 11.62% to 10.73% and butyrate increased from 11.4% to 13.5% within the 4 hr

period. Acetate proportions remained constant at 74%. Ammonia-, nitrate-, nitrite-N, ethanol, methanol, lactate and formate levels were normal and the pH was maintained at 6.9.

H_2 and CH_4 production was several orders of magnitude greater than had been recorded on a normal feed. A total of 16.17 ml of H_2 and 2.124 ml of CH_4 was collected in the first 2 hrs after addition of lactulose. After the mid-day feed which would have diluted bacteria and lactulose, 3.79 ml of H_2 and 0.49 ml of CH_4 were collected over a 4 hr period. Calculating from the VFA results as for section 3-13 an estimated 460 ml of H_2 could have been consumed by homoacetic acid fermentation. Out of a total of 11.43 mmoles acetate/fermentor 3.421 mmoles may have come from homoacetic acid fermentation. A total of 7.45 ml of N_2 gas was collected in the first 2 hrs which is in accord with estimates of between 8 - 9 ml being produced from nitrate supplies. These results demonstrate a rapid response to the introduction of lactulose by the fermentation.

The fact that nitrogen metabolism is largely unaffected tends to suggest that amino acid/protein degrading bacteria are not affected by the inclusion of readily fermentable carbohydrate. Further, the stimulation of methanogenesis by the inclusion of lactulose may suggest that this was mediated by increased concentrations of H_2 although it is possible that some other end-product was responsible.

Figure 3-2-i Total VFA concentration (mM) during experiment 3c (Turnip deletion).

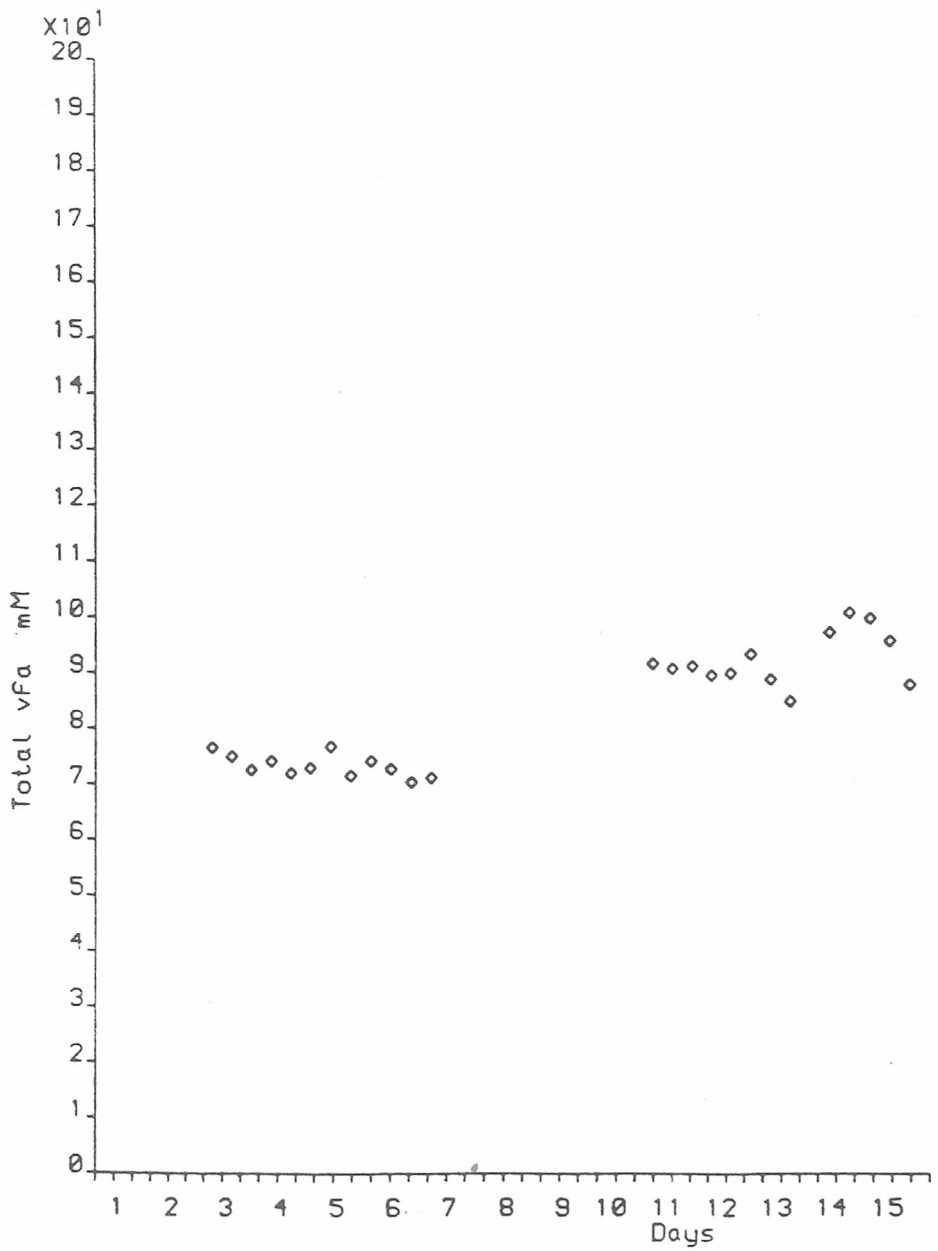


Figure 3-2-2 Total VFA concentration (mM) during experiment 3d (Turnip deletion).

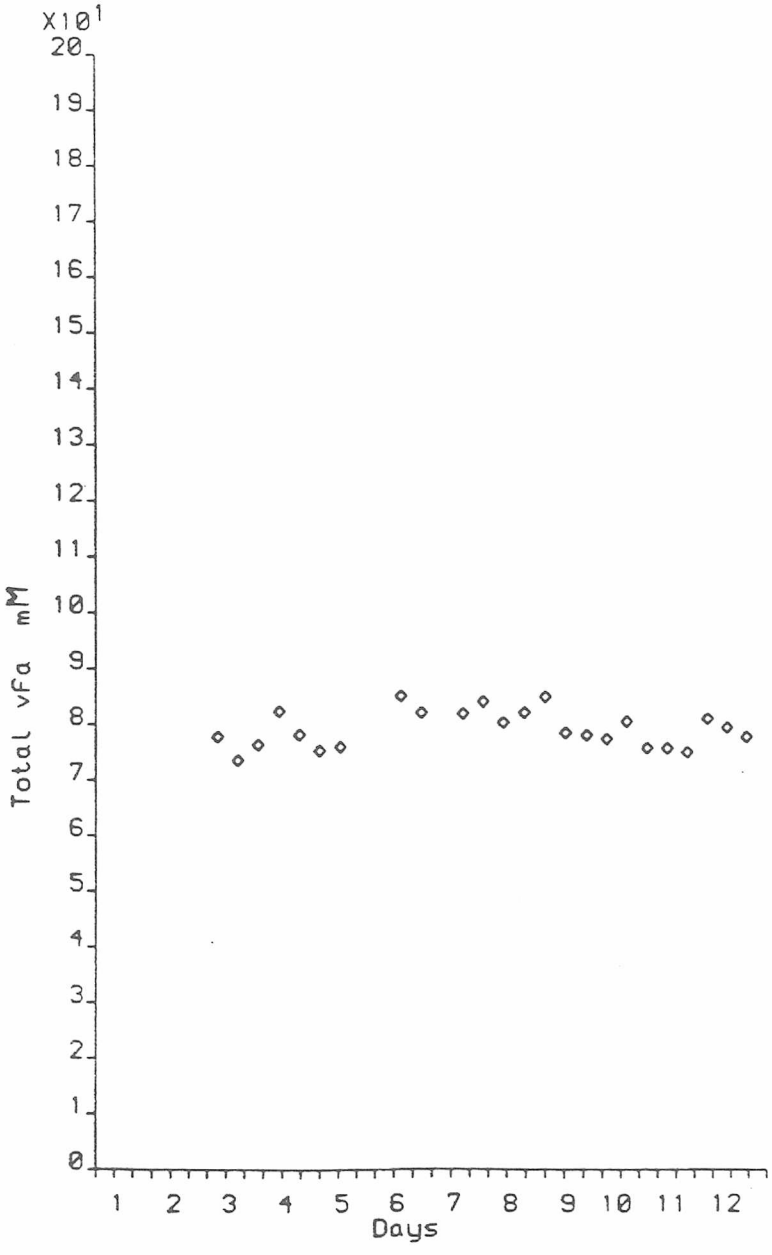


Table 3-2-1: Total VFA concentrations (mM) before the three daily feeds.

	9.00 am	1.00 pm	5.00 pm
Experiment 3c	83.32 ± 10.35^{ab}	83.63 ± 10.5^{a}	82.19 ± 9.73^{b}
Experiment 3d	79.86 ± 2.4^{cd}	79.03 ± 3.86^{c}	77.63 ± 2.1^{d}

[a] significantly different $P < 0.05$

b significantly different $P < 0.05$

c significantly different $P < 0.05$

d significantly different $P < 0.05$

Table 3-2-2: Proportions of the major VFA (mol/100 mol)

	Experiment 3c	Experiment 3d
Acetate	75.5 ± 1.62	61.18 ± 1.03
Propionate	13.57 ± 1.78	15.82 ± 0.68
Butyrate	10.28 ± 1.08	18.99 ± 0.69

3.2 Turnip Fibre Deletion Experiments (Bran, Cabbage, Carrot and Apple)

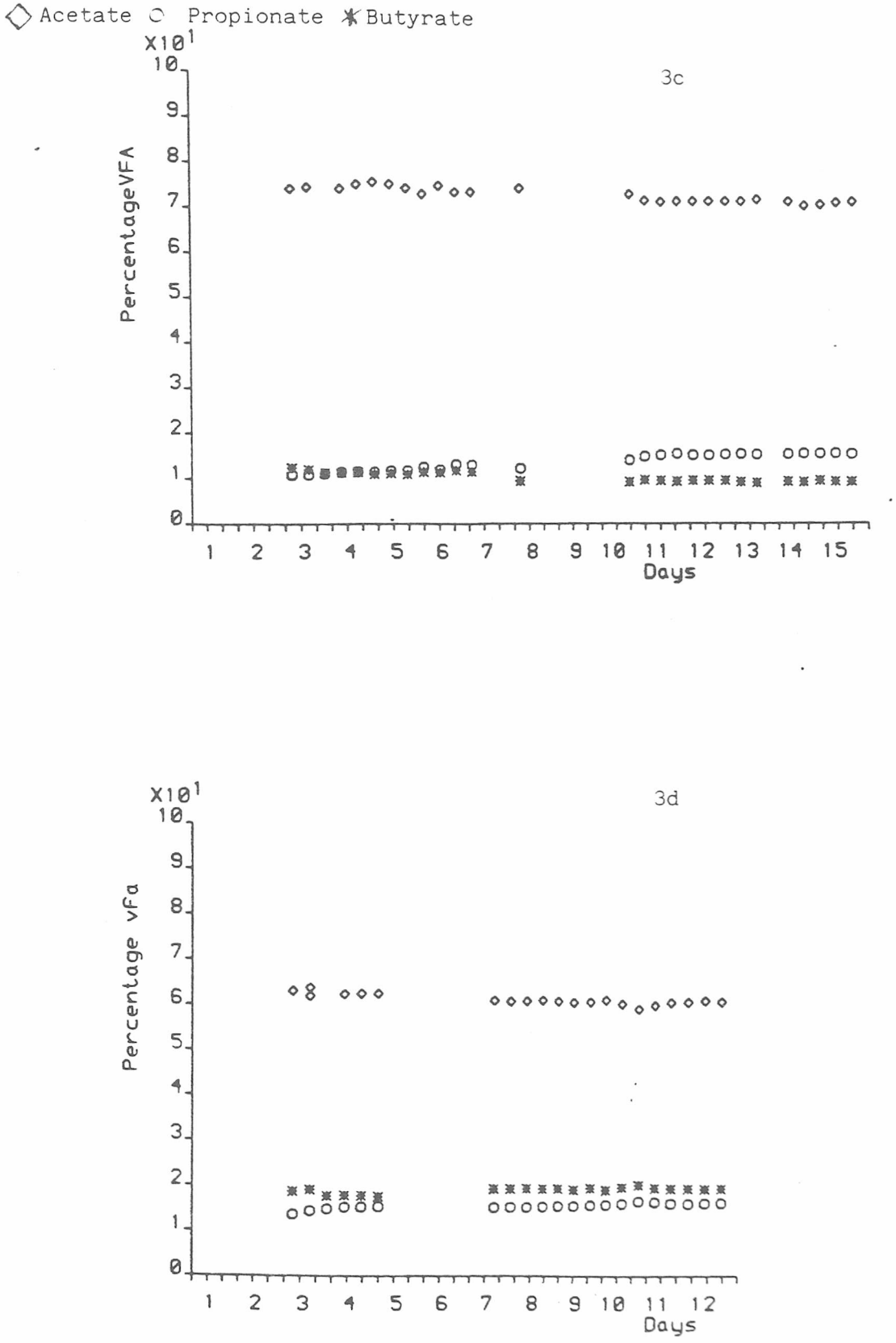
Two turnip fibre deletion experiments were carried out (3c and 3d). Experiment 3d was run 4 months after experiment 3c and both lasted two weeks. The fermentor culture for experiment 3c followed an all fibres experiment and was not reinoculated.

A completely new culture was used for experiment 3d due to accidental washout of the previous culture. There was no evidence of contamination of either feedstock and the pH of fermentor cultures was maintained at between pH 6.8 - 7.1. Quantitative analysis of H_2, CH_4 and N_2 gas was performed on day 8 and H_2S gas production was measured on days 7 and 9 of experiment 3d. Microbiological analysis was done on day 11 of experiment 3c and day 8 of experiment 3d.

3.2(1) Volatile Fatty Acid (VFA)

Figures 3-2-1 and 3-2-2 show the total VFA concentrations recorded during experiment 3c and 3d respectively. The average totals measured immediately before the 9.00 am, 1.00 pm and 5.00 pm feeds during both experiments are shown in table 3-2-1. Although there were no significant differences in the concentrations of total VFA between experiment 3c and 3d it is clear that the culture in experiment 3c did not reach a steady state until day 10. The prolonged period of

Figure 3-2-3 VFA concentrations (mol/100 mol) during experiments 3c and 3d (Turnip deletion)



adjustment probably reflected the time taken by the culture to adapt to a new feedstock in the absence of reinoculation. Statistical comparison between the total VFA concentrations at 9.00 am, 1.00 pm and 5.00 pm within each of the experiments showed that the 9.00 am, 1.00 pm and 5.00 pm values in experiment 3c were significantly different from each other.

Daily production rates were calculated from the average totals allowing for the dilution factor due to feeding. From the data in table 3-2-1 it can be calculated that 28.55 $\pm$ 1.78 µmol VFA/ml/24 hr and 30.11 $\pm$ 2.01 µmol VFA/ml/24 hr were produced in the fermentor during experiment 3c and 3d respectively. The proportions of individual acids recorded during experiments 3c and 3d are shown in figures 3-2-3 (c) and (d) respectively. The steady increase in total VFA recorded throughout experiment 3c was accompanied by an increase in the proportion of propionate. The average proportions of acetate, propionate and butyrate are shown in table 3-2-2. From the information given in table 3-2-2 it can be seen that acetate represented approx. 10% more of the total VFA in experiment 3c compared with 3d. The proportions of propionate were similar in both experiments but butyrate was approx. 9% higher in experiment 3d. In both experiments, VFA production patterns were biphasic. The fermentation was most rapid within the first 2 hr following a feed and slowed down to a constant rate thereafter. There was no lag in the response to feeding at any time of day.

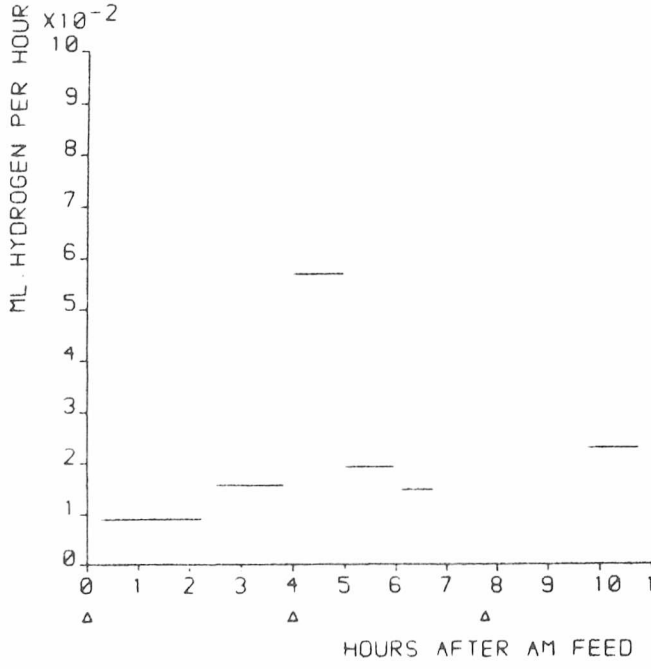
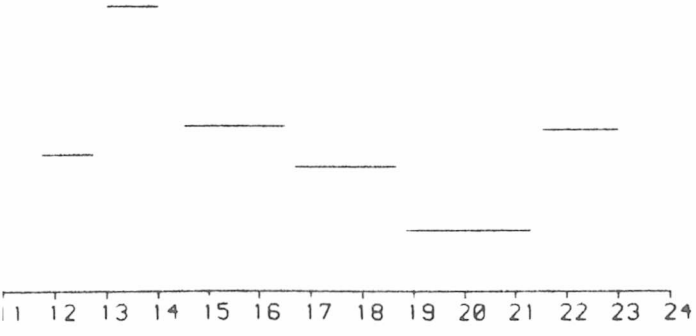


Figure 3-2-4 Hydrogen production ($ml.hr^{-1}$)



during experiment 3d (Turnip deletion)

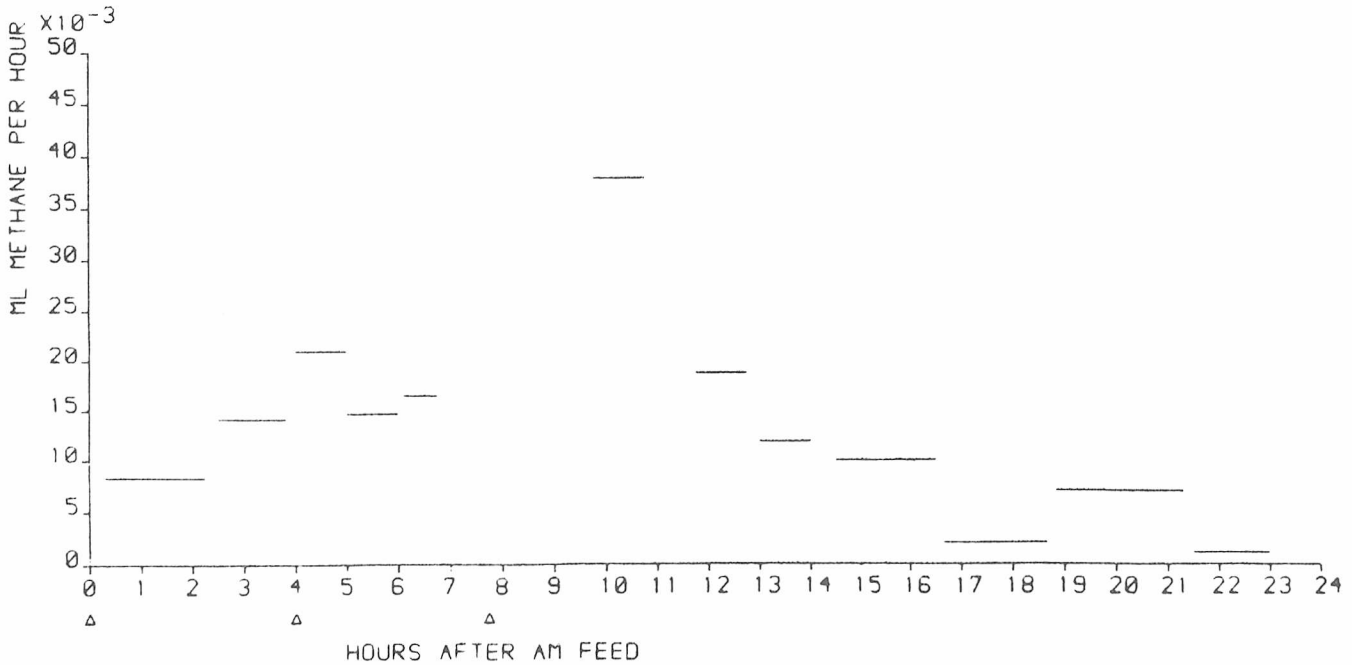


Figure 3-2-5 Methane production ($ml.hr^{-1}$) during experiment 3d (Turnip deletion).

3.2(2) Hydrogen and Methane Gas

The amounts of hydrogen ($ml.hr^{-1}$) and methane ($ml.hr^{-1}$) collected after the 9.00 am feed on day 8 of experiment 3d are shown in figures 3-2-4 and 3-2-5. Hydrogen production was within the range 0.009 - 0.058 $ml.hr^{-1}$ and methane 0.011 - 0.0379 $ml.hr^{-1}$. There were two hydrogen peaks, the first immediately after the mid-day feed and the second 6 hr after the pm feed. Methane production was highest immediately after the mid-day feed and 1.5 hr after the pm feeds. Hydrogen and methane patterns coincide until after the pm feed when methane levels appear to increase before hydrogen.

The amount of hydrogen gas produced by the fermentor per 24 hrs was calculated from the VFA data as described in section 3-1-3. An estimated 676.86 ml/day in experiment 3c and 653 ml/day in experiment 3d could have been produced by fermentation leading to the formation of volatile fatty acids in the proportions recorded for these experiments.

The actual amount of hydrogen and methane gas collected over the 24 hr period studied in experiment 3d was ~ 0.5 ml and 0.15 ml respectively. Together, methane, collected hydrogen, nitrate and sulphate reduction account for only 187.22 ml and an estimated 465.8 ml H_2/day is unaccounted for.

If homoacetic acid fermentation is included in the fermentation balance then approx. 3.46 mmoles acetate out of

Figure 3-2-6 Ammonia-N, Nitrate-N and Nitrite-N concentrations (ppm) during experiment 3c (Turnip deletion).

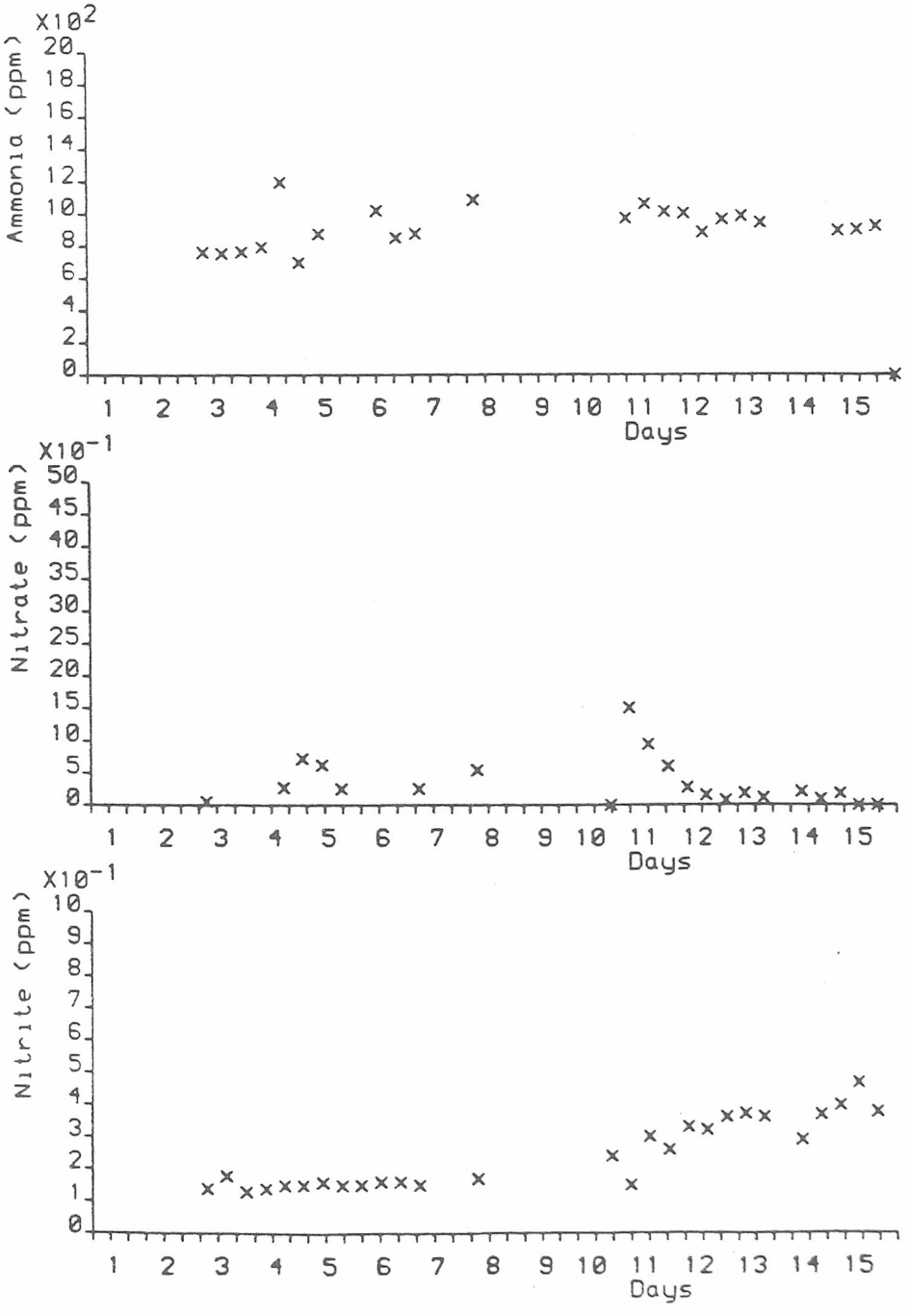


Figure 3-2-7 Ammonia-N, Nitrate-N and Nitrite-N concentrations (ppm) during experiment 3d (Turnip deletion).

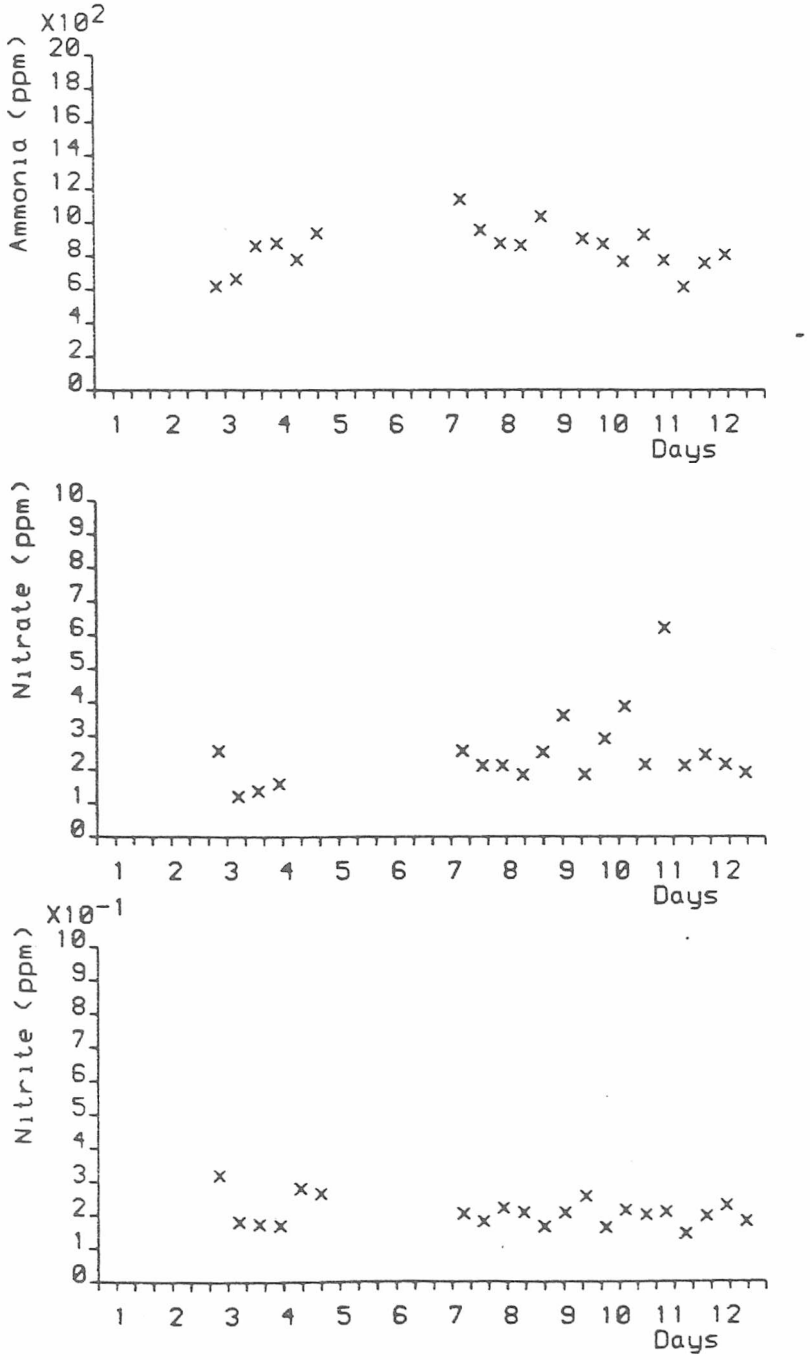
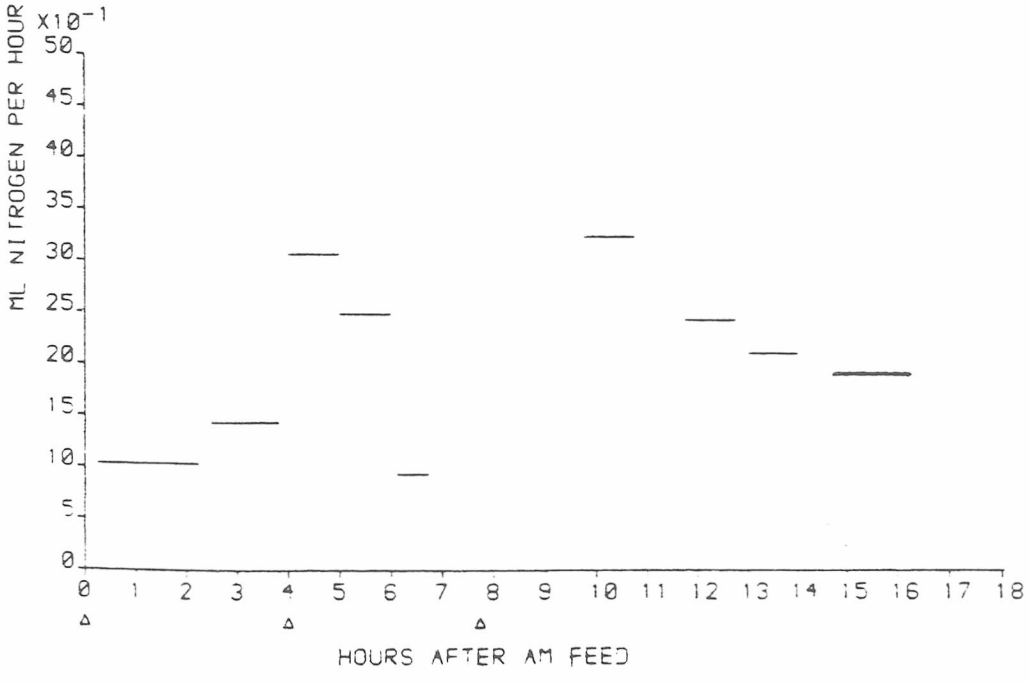


Table 3-2-3: Ammonia-N, Nitrate-N and Nitrite-N concentrations (ppm) in feedstock and fermentor samples.

	Ammonia-N (ppm)	Nitrate-N (ppm)	Nitrite-N (ppm)
Feedstock			
experiment 3c	330	120	0.05
Fermentor samples			
experiment 3c	965 ± 194	0.33 ± 0.36	0.24 ± 0.1
Feedstock			
experiment 3d	310	135	0.06
Fermentor samples			
experiment 3d	842 ± 129	21.48 ± 1.1	0.21 ± 0.5

Figure 3-2-8 Nitrogen gas production ($ml.hr^{-1}$) during experiment 3d (Turnip deletion).



a total of 18.42 mmoles/acetate/day could have been produced from this theoretical surplus of H_2 (see section 3.1(3)).

3.2(3) Nitrogen

Ammonia-, nitrate-, and nitrite-N concentrations measured during experiments 3c and 3d are shown in figures 3-2-6 and 3-2-7 respectively.

Feedstock and fermentor concentrations are shown in table 3-2-3. From the information given in table 3-2-3 the average amount of ammonia-N produced per feed in experiment 3c was calculated to be 82.47 ppm and for experiment 3d 69.1 ppm. An average of 15.54 ppm (experiment 3c) and 17.21 ppm nitrate-N (experiment 3d) were metabolised during each feed and nitrite did not accumulate above 0.31 ppm in either experiment. If the nitrate was reduced to N_2 gas 8.5 ml N_2 could be expected from 15.54 ppm nitrate and 9.22 ml N_2 from 17.21 ppm nitrate. Nitrogen gas was collected on day 8 of experiment 3d and the results shown in figure 3-2-8. A total of 7.59 ml N_2 was collected within 9 hr after the 9.00 am feed and 10.12 ml within 8 hr after the 5.00 pm feed.

3.2(4) Sulphur

Feedstock 3c contained 2.12 mM sulphate from which 0.292 mM was metabolised each feed. During experiment 3d 0.266 mM sulphate was metabolised each feed from a feedstock concentration of 1.75 mM. An average 13.14 ml (experiment

Table 3-2-4: Composition of dietary fibre (mg.ml^{-1}

	Starch	Uronic Acids	Arabinose	Xylose
Feedstock experiment 3d	1.518	1.171	0.917	1.591
Fermentor samples experiment 3d	0.361 $\pm$0.1	0.0923 $\pm$0.058	0.413 $\pm$0.29	0.319 $\pm$0.28

[1]) in feedstock and fermentor samples.

Mannose	Galactose	Glucose
0.202	0.546	1.887
0.016	0.064	0.119
±0.01	±0.027	±0.067

Table 3-2-5: Enumeration of bacteria in experiment 3c and 3d.

	Bacteria/ml of fermentor contents	
	Experiment 3c	Experiment 3d
Total Anaerobic Counts	$2.5 \pm 0.4 \times 10^9$	$1.82 \pm 1.36 \times 10^9$
Total Aerobic Counts	2.0×10^7	NR
Enterobacteriacea	$1.1 \pm 0.08 \times 10^6$	$1.36 \pm 0.36 \times 10^6$
Xylanolytic bacteria	$2.4 \pm 1.2 \times 10^7$	1×10^7
Pectinolytic bacteria	$3.5 \pm 1.5 \times 10^8$	$1.45 \pm 0.5 \times 10^8$
Starch digesting bacteria	$8.1 \pm 0.1 \times 10^8$	$1.56 \pm 1.02 \times 10^8$

Anaerobic : Aerobic Ratio	125 : 1	
Anaerobic : Xylanolytic Ratio	104 : 1	182 : 1
Anaerobic : Pectinolytic Ratio	7 : 1	12 : 1
Anaerobic : Starch digesting ratio	3 : 1	11 : 1

3c) and 11.97 ml (experiment 3d) of H_2S would be produced if this sulphate was being reduced by the process of dissimilatory sulphate reduction. The amounts of H_2S collected between the morning and mid-day feeds on days 7 and 9 of experiment 3d were 7.9 ml on day 7 and 11.29 ml on day 9

3.2(5) Fibre Digestion

The starch, uronic acid and sugar composition of feedstock dietary fibre and fermentor sample residues during experiment 3d are shown in table 3-2-4. It was calculated that an average total of 0.879 mg/ml carbohydrate was fermented between each feed. In a fermentor volume of 670 ml the total amount fermented from 3 daily feeds was 1.78 g/day or 9.81 mmoles hexose equivalents/fermentor.day.

3.2(6) Microbiology

Table 3-2-5 shows the microbiological data collected for experiments 3c and 3d. The numbers of bacteria in the various groups in experiment 3c are very close to those for experiment 3d.

Aerobic plate counts could not be read in experiment 3d due to the presence of a green spreading organism later identified as Pseudomonas aeruginosa.

The organism was isolated repeatedly from faecal samples and remained in the fermentor during experiments 3a, 3b, 3d, 3f, 3h and 3i.

Table 3-2-6: Identification of bacteria in experiment 3d

		Bacteria/ml of fermentor contents
1.	Fusobacterium Selenomonas Leptotrichia Vibrio succinogenes	1×10^8
2.	Clostridium Eubacterium	5×10^7
3.	Bifidobacterium Actinomyces	5×10^7
4.	Bacteroides	7.0×10^8
5.	Lactobacillus	0
6.	Propionibacterium	9.0×10^8

The ratios calculated for both turnip deletion experiments were close to those recorded for experiment 3a (all fibres). The numbers of organisms belonging to the major groups present during experiment 3d are shown in table 3-2-6. The numbers in group 5 were low by comparison with experiment 3a (all fibres).

Acetate was the major product in Min-1(f) Min-1(g) and medium 119 cultures incubated under H_2:CO_2 80%:20%. During experiment 3d a total of 4.17×10^{11} cells were produced every day in the fermentor. This represents a dry wt of 0.55 g/day. If 3-4 g of carbohydrate are required to produce 1 g dry weight cells then between 1.67 - 2.22 g carbohydrate would have been necessary to sustain the daily turnover. Alternatively, if the dry weight of cells produced represents 20-30% of the weight of carbohydrate fermented then 1.85 - 2.78 g of carbohydrate would have been consumed each day.

The actual amount of carbohydrate consumed by the bacterial population in the fermentor was 1.78 g/fermentor/day.

Figure 3-3-1 Total VFA concentration (mM) during experiment 3e (Carrot deletion).

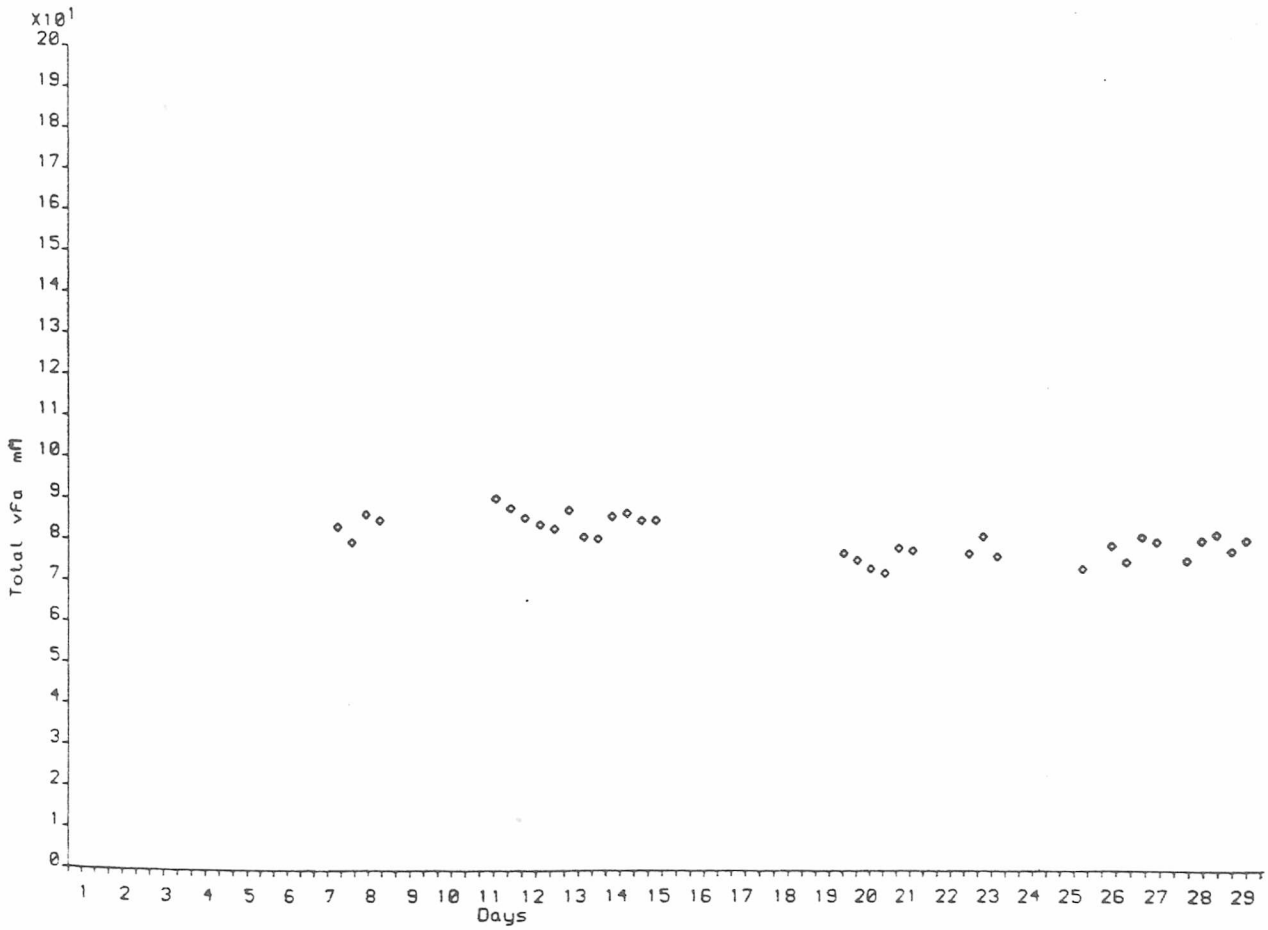


Figure 3-3-2 Total VFA concentration (mM) during experiment 3f (Carrot deletion).

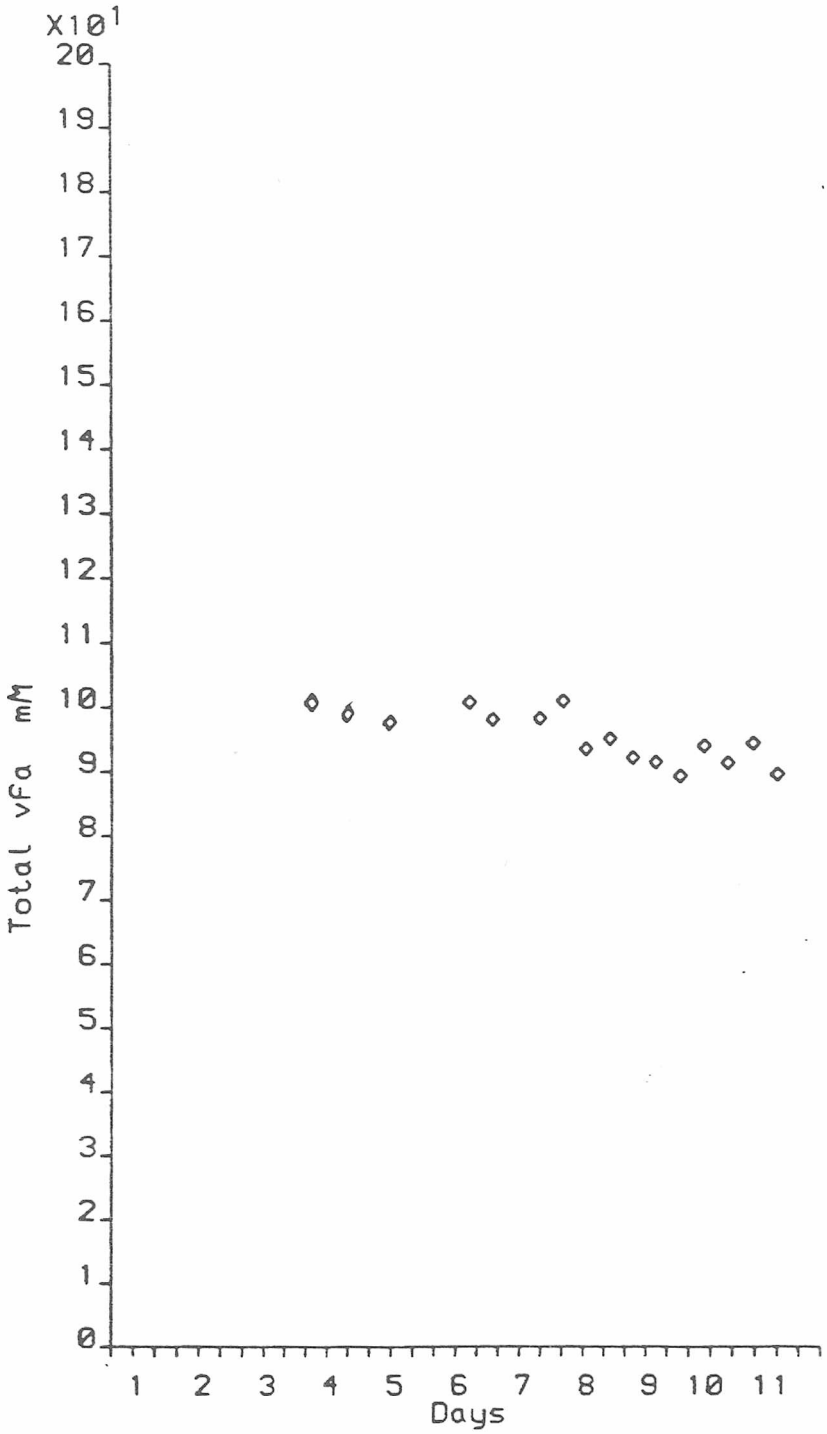


Table 3-3-1: Total VFA concentrations (mM) before the three daily feeds.

	9.00 am	1.00 pm	5.00 pm
Experiment 3e	81.64 ± 4.8^{x}	81.95 ± 2.8^{y}	80.73 ± 4.8^{z}
Experiment 3f	95.85 ± 3.8^{x}	94.64 ± 4.5^{y}	92.41 ± 1.0^{z}

x significantly different $P < 0.05$

y significantly different $P < 0.05$

z significantly different $P < 0.05$

Table 3-3-2: Proportions of the major VFA (mol/100 mol)

	Experiment 3e	Experiment 3f
Acetate	68.79 ± 0.86	59.88 ± 1.9
Propionate	16.68 ± 0.57	19.2 ± 2.0
Butyrate	9.79 ± 0.45	14.59 ± 1.06

3.3 Carrot Fibre Deletion Experiments (Bran, Cabbage, Turnip and Apple)

Two carrot deletion experiments were carried out 4 months apart. Experiment 3e was run over a period of 30 days and experiment 3f for 14 days. In both cases pre-existing fermentor cultures were reinoculated on day 1.

Both feedstocks were examined and found to be free from contamination and the pH of the fermentor cultures was maintained between 6.9 and 7.1 throughout. The microbiological analyses were performed on day 21 of experiment 3e and day 6 of experiment 3f. Quantitative analysis of H_2, CH_4 and N_2 was carried out on day 7 of experiment 3f. H_2S results were obtained on day 10 of experiment 3f.

3.3(1) Volatile Fatty Acids (VFA)

The total VFA concentrations recorded during 3e and 3f are shown in figures 3-3-1 and 3-3-2. Average total VFA at 9.00 am, 1.00 pm and 5.00 pm in experiments 3e and 3f are shown in table 3-3-1. There were no significant differences evident when VFA levels at 9.00 am, 1.00 pm and 5.00 pm were compared within each experiment.

Statistical comparison between 9.00 am, 1.00 pm and 5.00 pm values in experiment 3e and the corresponding values in experiment 3f showed that the VFA levels in experiment 3e

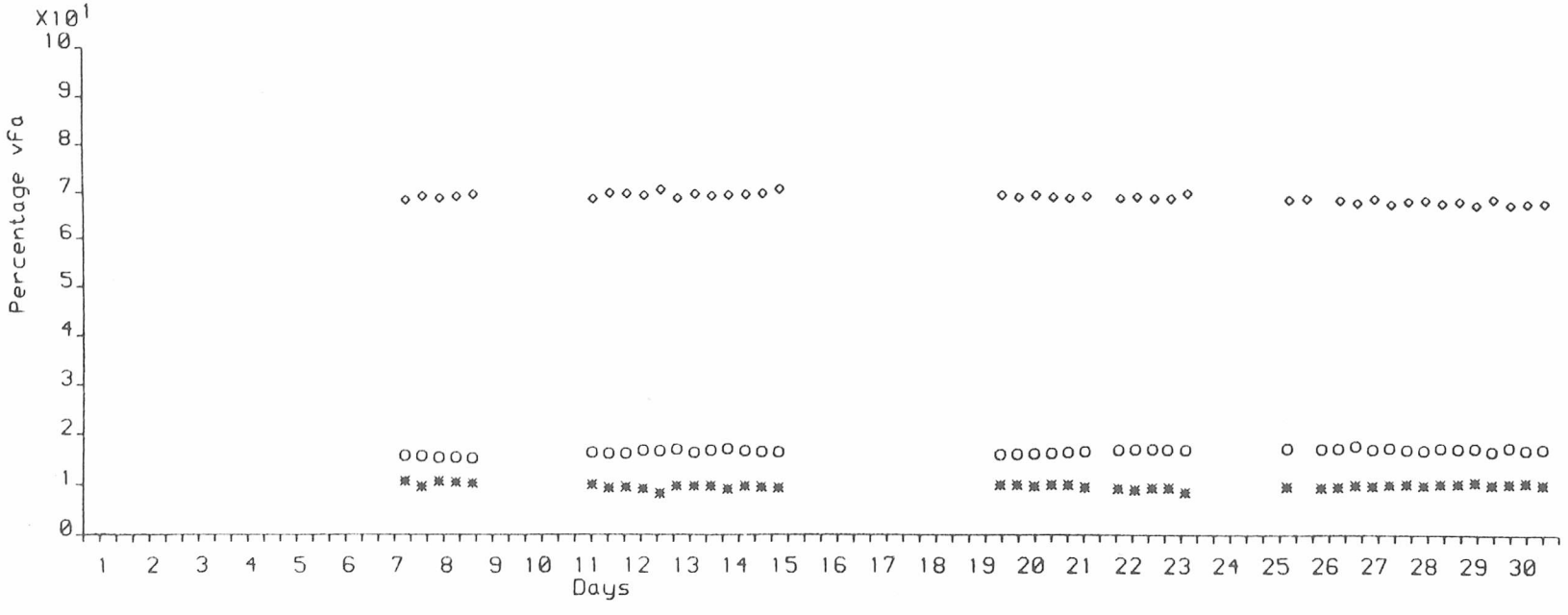


Figure 3-3-3 VFA concentrations (mol/100 mol) during experiment 3e (Carrot deletion)

◇ Acetate ○ Propionate ✳ Butyrate

Figure 3-3-4 VFA concentrations (mol/100 mol) during experiment 3f (Carrot deletion)

◇ Acetate ○ Propionate ✳ Butyrate

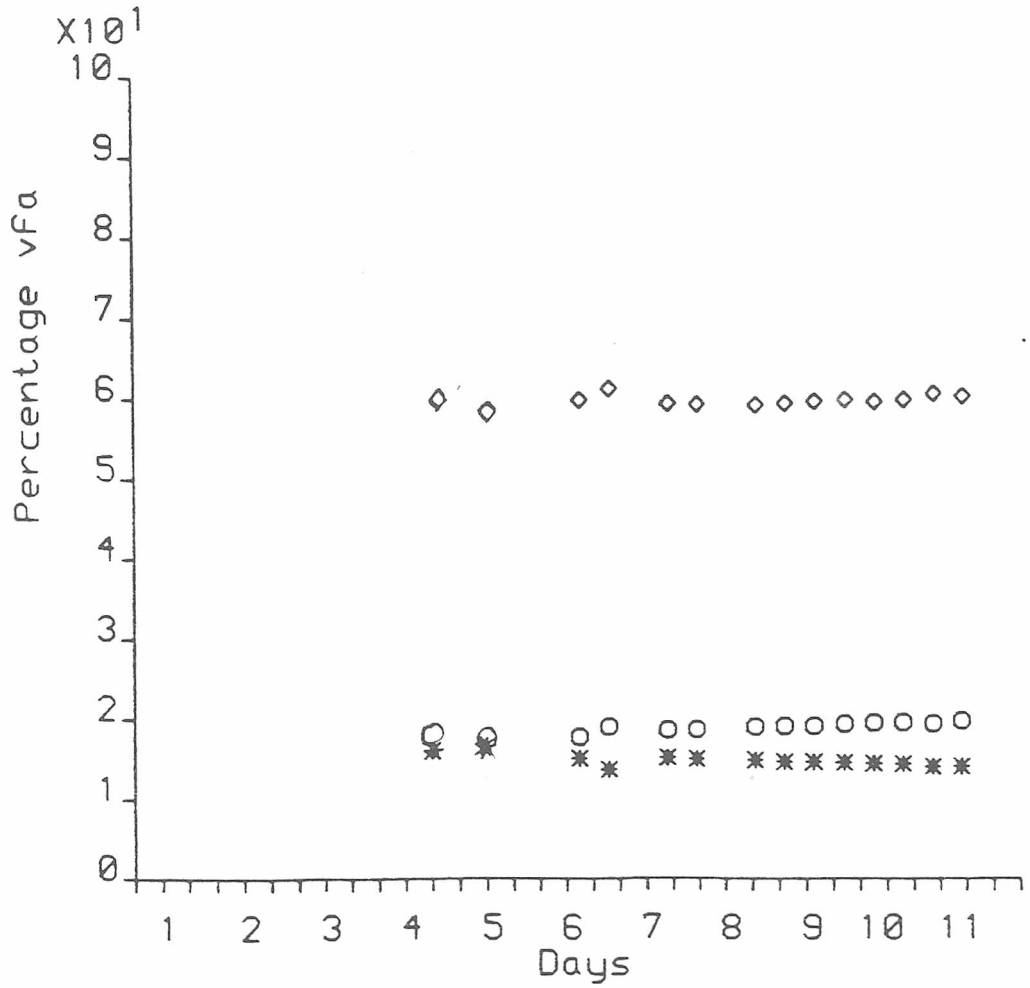


Figure 3-3-5 Hydrogen production ($ml.hr^{-1}$) during experiment 3f (Carrot deletion)

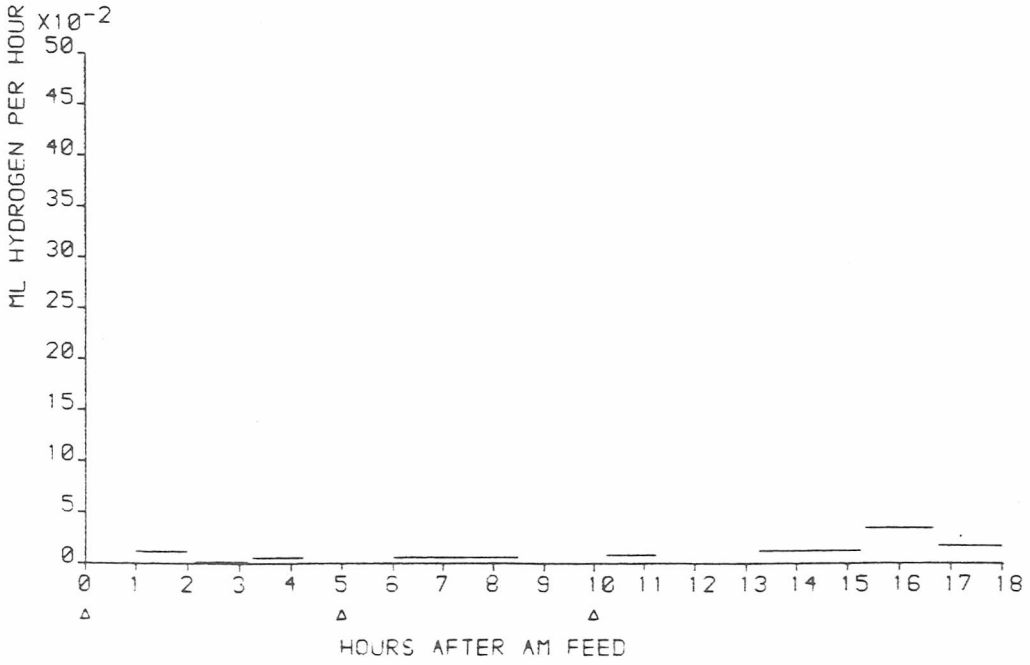
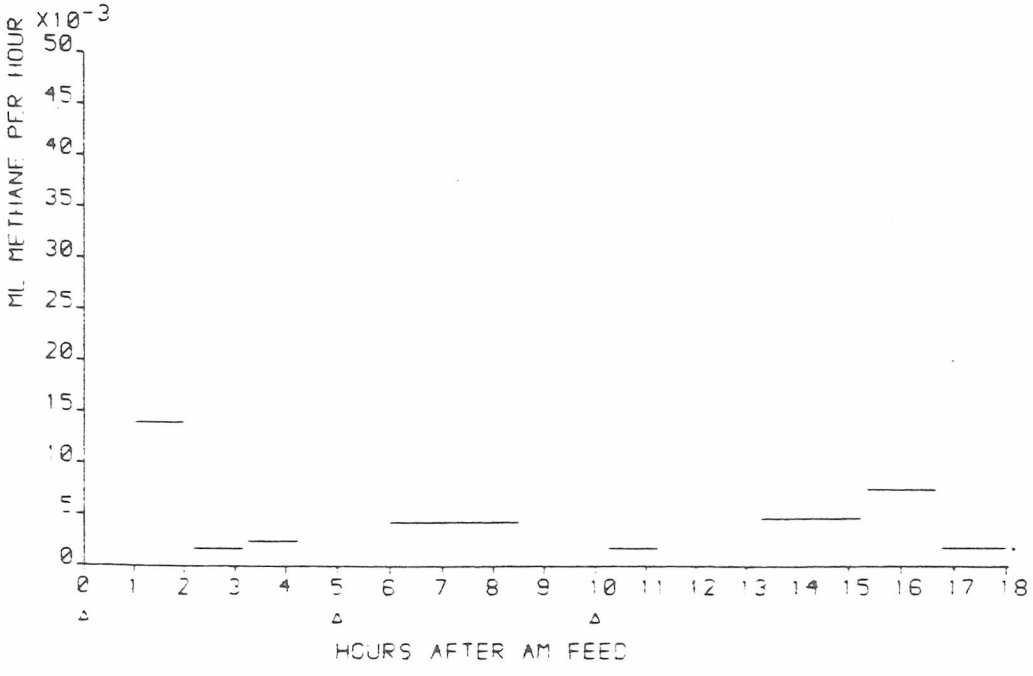


Figure 3-3-6 Methane production ml.hr^{-1}) during experiment 3f (Carrot deletion).



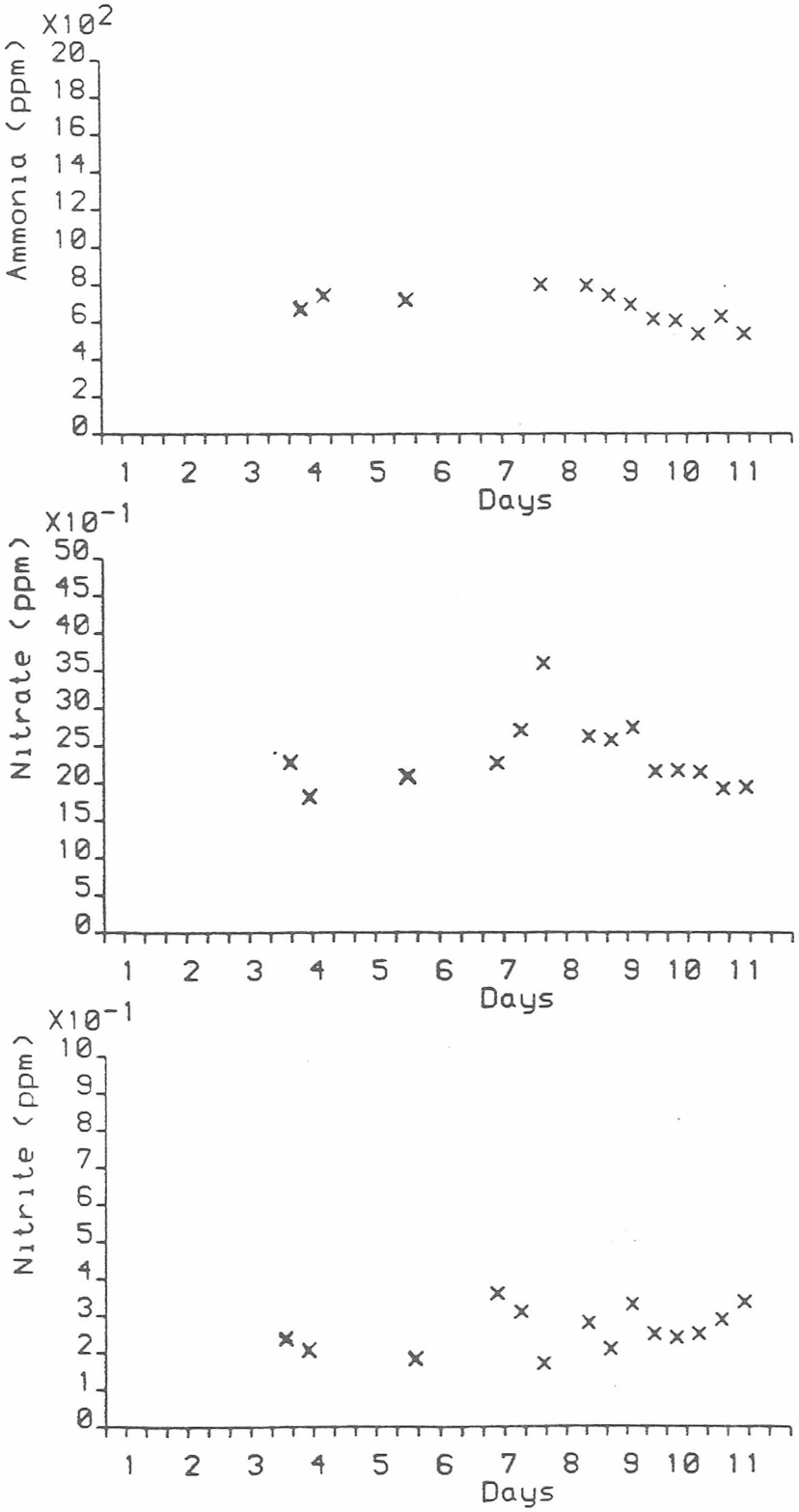
were significantly different from those in experiment 3f at 9.00 am, 1.00 pm and 5.00 pm. Allowing for a dilution factor due to feeding the average daily production rates were 29.49± 2.2 µmol/ml/24 hr during experiment 3e and 36.48±2.4 µmol/ml/24 hr during experiment 3f. The proportions of acetate propionate and butyrate are shown in figures 3-3-3 and 3-3-4. The average proportions of the individual acids in experiments 3e and 3f are shown in table 3-3-2. From the data in table 3-3-2 it can be seen that as a percentage of the total, acetate was approx 10% less in experiment 3f compared with 3e and butyrate approx 5% more in experiment 3f.

The proportions stabilised soon after inoculation and remained constant throughout the experiments. The fermentation in both experiments was rapid with 60% - 78% of VFA production within the first 4 hrs after a feed.

3.3(2) Hydrogen and Methane Gases

Hydrogen (ml/hr) and methane (ml/hr) collected over a period of 18 hrs after the 9.00 am feed on day 7 (experiment 3f) is shown on figures 3-3-5 and 3-3-6. Hydrogen was within the range 0.0058 - 0.035 $ml.hr^{-1}$ and methane 0.0017 - 0.014 $ml.hr^{-1}$. Hydrogen levels were very low and did not appear to respond to feeds. Methane levels rose within the first hour after the morning and mid-day feeds and 4 hrs after the pm feed but were also generally low. In total approx. 0.2 ml of H_2 and 0.06 ml of CH_4 were collected.

Figure 3-3-7 Ammonia-N, Nitrate-N and Nitrite-N concentrations (ppm) during experiment 3e (Carrot deletion)



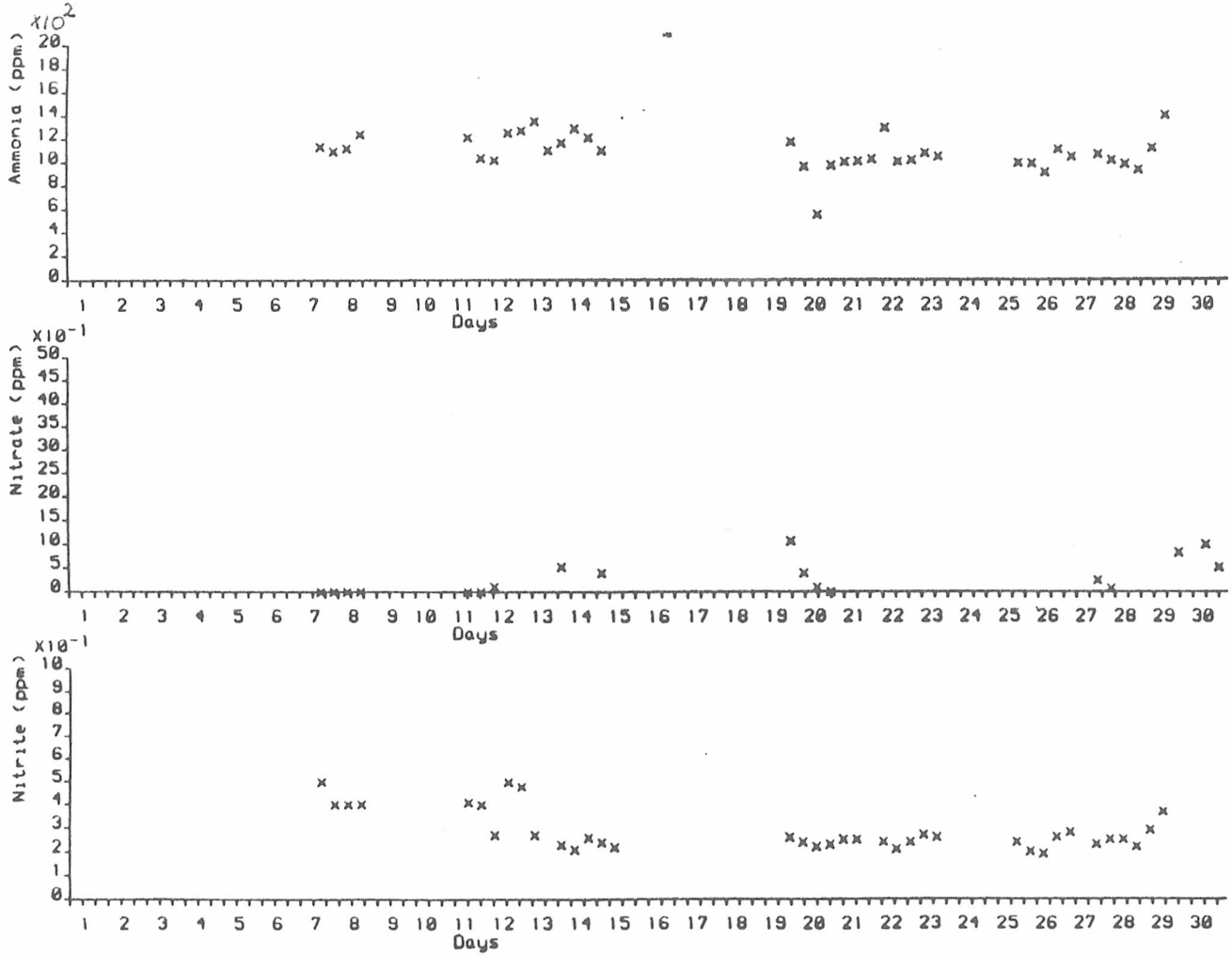
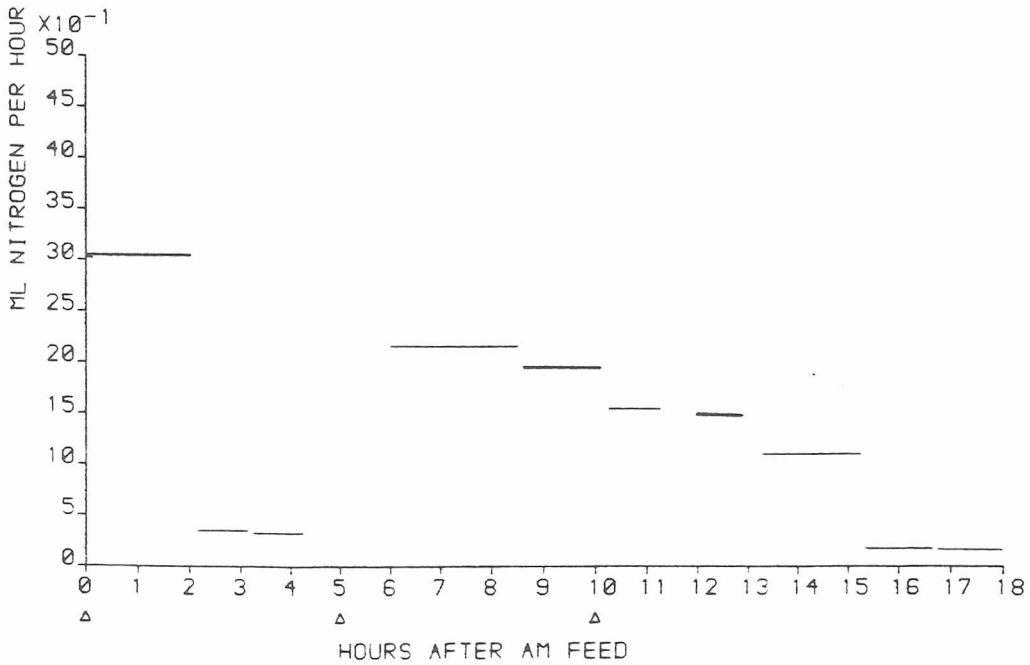


Figure 3-3-8 Ammonia-N, Nitrate-N and Nitrite-N concentrations (ppm) during experiment 3f (Carrot deletion).

Table 3-3-3: Ammonia-N, Nitrate-N and Nitrite-N concentrations (ppm) in feedstock and fermentor samples.

	Ammonia-N (ppm)	Nitrate-N (ppm)	Nitrite-N (ppm)
Feedstock			
experiment 3e	370	140	0.05
Fermentor samples			
experiment 3e	1102 ± 119	0.29 ± 0.35	0.28 ± 0.08
Feedstock			
experiment 3f	320	135	0.1
Fermentor samples			
experiment 3f	656 ± 91	2.44 ± 0.46	0.27 ± 0.05

Figure 3-3-9 Nitrogen gas production ($ml.hr^{-1}$) during experiment 3f (Carrot deletion)



From the volatile fatty acid data (section 3.3(1)) an estimated 621.63 ml H_2 and 710.24 ml H_2 would have been produced by the fermentations in experiments 3e and 3f. Evidently metabolic reactions other than those of methanogenic bacteria were responsible for the disappearance of such amounts of hydrogen. Based on the calculations contained in section 3.1(3) the amount of hydrogen unaccounted for during experiment 3f (535.2 ml) could have provided an estimated 3.98 mmol acetate/day by homoacetic acid fermentation.

3.3(3) Nitrogen

Ammonia-, nitrate- and nitrite-N concentrations are shown in figures 3-3-7 (experiment 3e) and 3-3-8 (experiment 3f). Feedstock and fermentor concentrations are shown in table 3-3-3. From the data presented in table 3-3-3 it was calculated that an average of 95 ppm ammonia-N and 43.6 ppm ammonia-N were produced during experiment 3e and 3f respectively. In experiments 3e and 3f an average of 18.14 ppm nitrate-N and 17.20 ppm nitrate nitrogen were consumed between feeds. Nitrite levels were maintained below 0.3 ppm in both experiments.

Assuming the nitrate was reduced to nitrogen gas, 9.87 ml N_2 per feed and 9.22 ml N_2 per feed would be produced in experiments 3e and 3f. Nitrogen gas production was measured on day 7 of experiment 3f (see figure 3-3-9). A total of 7 ml were collected between the morning and mid-day feeds 9.15

Table 3-3-4: Composition of dietary fibre ($mg.ml^{-1}$) in feedstock and fermentor samples.

	Starch	Uronic Acids	Arabinose	Xylose	Mannose	Galactose	Glucose
Feedstock experiment 3f	1.43	1.26	0.835	0.902	0.199	0.47	1.92
Fermentor samples experiment 3f	0.104 ±0.054	0.072 ±0.02	0.590 ±148	0.373 ±0.29	0.016 ±0.009	0.065 ±0.05	0.151 ±0.074

Table 3-3-5: Enumeration of bacteria in experiment 3e and 3f.

	Bacteria/ml of fermentor contents	
	Experiment 3e	Experiment 3f
Total Anaerobic Counts	$4.38 \pm 2.86 \times 10^9$	$4.41 \pm 1.36 \times 10^9$
Total Aerobic Counts	$1.38 \pm 0.22 \times 10^7$	$1.66 \pm 0.5 \times 10^7$
Enterobacteriacea	$1.3 \pm 0.3 \times 10^7$	$1.55 \pm 0.15 \times 10^6$
Xylanolytic bacteria	$5.0 \pm 1.0 \times 10^6$	$1.12 \pm 0.5 \times 10^7$
Pectinolytic bacteria	$3.73 \pm 1.32 \times 10^8$	$1.52 \pm 0.5 \times 10^8$
Starch digesting bacteria	8.4×10^8	$2.85 \pm 0.8 \times 10^8$

Anaerobic : Aerobic Ratio	317 : 1	265 : 1
Anaerobic : Xylanolytic Ratio	876 : 1	394 : 1
Anaerobic : Pectinolytic Ratio	12 : 1	29 : 1
Anaerobic : Starch digesting ratio	5 : 1	15 : 1

ml between mid-day and pm feeds and 5.5 ml after the pm feed.

3.3(4) Sulphur

The feedstock in experiment 3f contained 1.64 mM sulphate from which an average of 0.202 mM sulphate was reduced each feed. If this sulphate was reduced to H_2S then 3.03 ml of the gas would be produced each feed. A total of 4.6 ml of H_2S was collected between morning and mid-day feeds on day 10 of experiment 3f.

3.3(5) Fibre Digestion

The starch, uronic acid and sugar composition of feedstock dietary fibre and fermentor residues for experiment 3f are shown in table 3-3-4. A total of 0.777 mg/ml carbohydrate was fermented from each feed. The total amount fermented from the 3 daily feeds was calculated to be 8.67 mmoles hexose equivalents/fermentor/day or 1.56 g carbohydrate/fermentor/day.

3.3(6) Microbiology

Microbiological data collected during experiments 3e and 3f are presented in table 3-3-5. The numbers of bacteria in both experiments were within the same range for all groups and in general compared well with those of experiment 3a (all fibres).

The ratio of anaerobes : aerobes was considerably higher in experiments 3e or 3f compared with experiment 3a. Table

Table 3-3-6: Identification of bacteria in experiment 3f

		Bacteria/ml of fermentor contents
1.	Fusobacterium Selenomonas Leptotrichia Vibrio succinogenes	1.56×10^8
2.	Clostridium Eubacterium	6.25×10^8
3.	Bifidobacterium Actinomyces	3.12×10^8
4.	Bacteroides	1.87×10^9
5.	Lactobacillus	1.56×10^8
6.	Propionibacterium	9.3×10^8

3-3-6 shows the numbers of organisms assigned to the major groups present during experiment 3f. The numbers of bacteria in group 2 and 4 were higher in experiment 3f compared with 3a.

Acetate was the major product in Min-1, medium 119 and Min-1 (i) cultures incubated under H_2:CO_2 80%:20%.

During experiment 3f a total of 1.01×10^{12} cells were produced every day in the fermentor. This represents a dry weight of 1.34 g/day. If 3-4 g of carbohydrate are required to produce 1 g dry weight of cells then between 4.03 - 5.38 g carbohydrate would have been necessary to sustain the daily turnover. Alternatively, if the dry weight represents 20-30% of the weight of carbohydrate fermented then 4.48 - 6.72 g of carbohydrate would have been consumed each day.

The actual amount of carbohydrate consumed by the bacterial population in the fermentor was 1.56 g/fermentor/day.

Figure 3-4-1 Total VFA concentration (mM) during experiment 3g (Apple deletion).

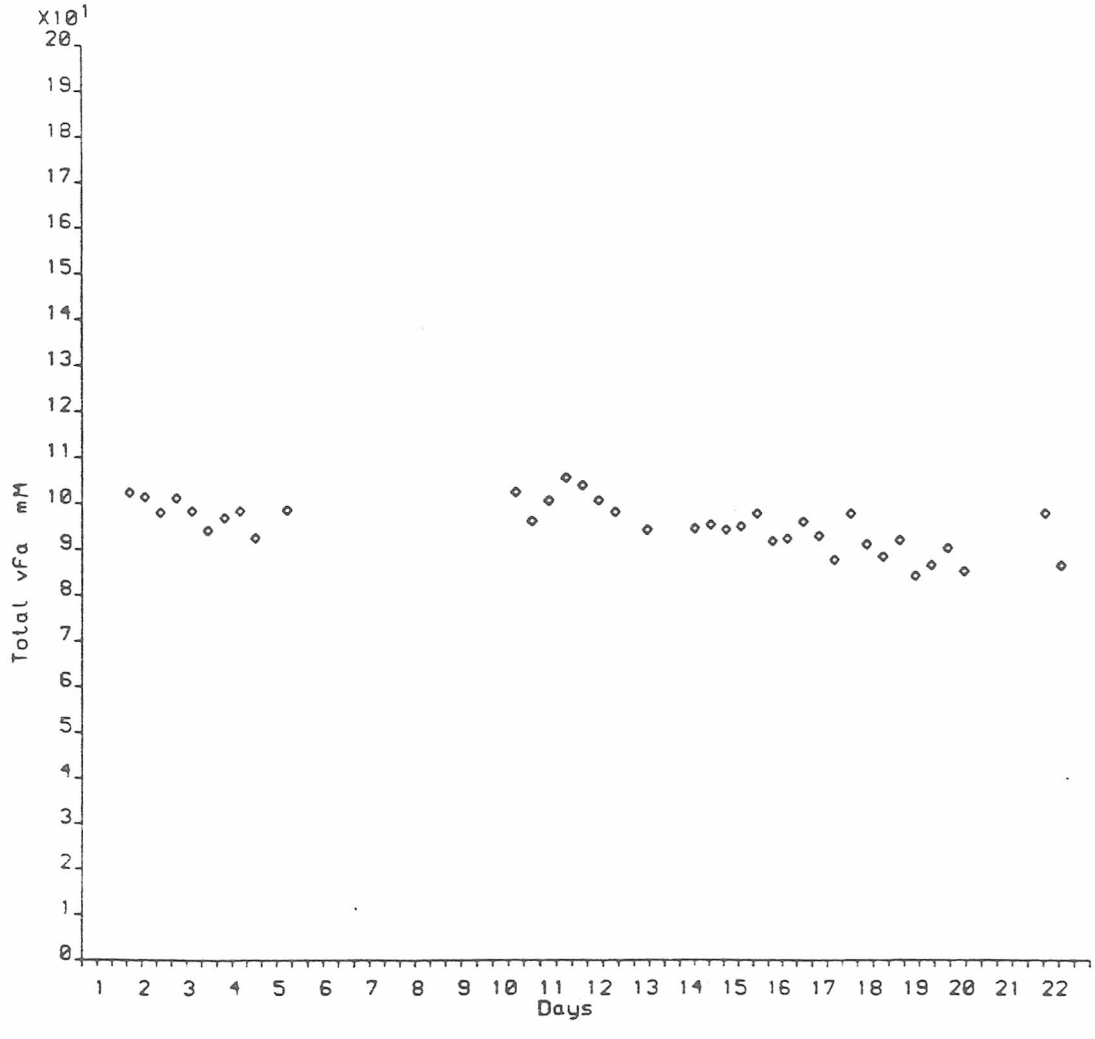


Figure 3-4-2 Total VFA concentration (mM) during experiment 3h (Apple deletion).

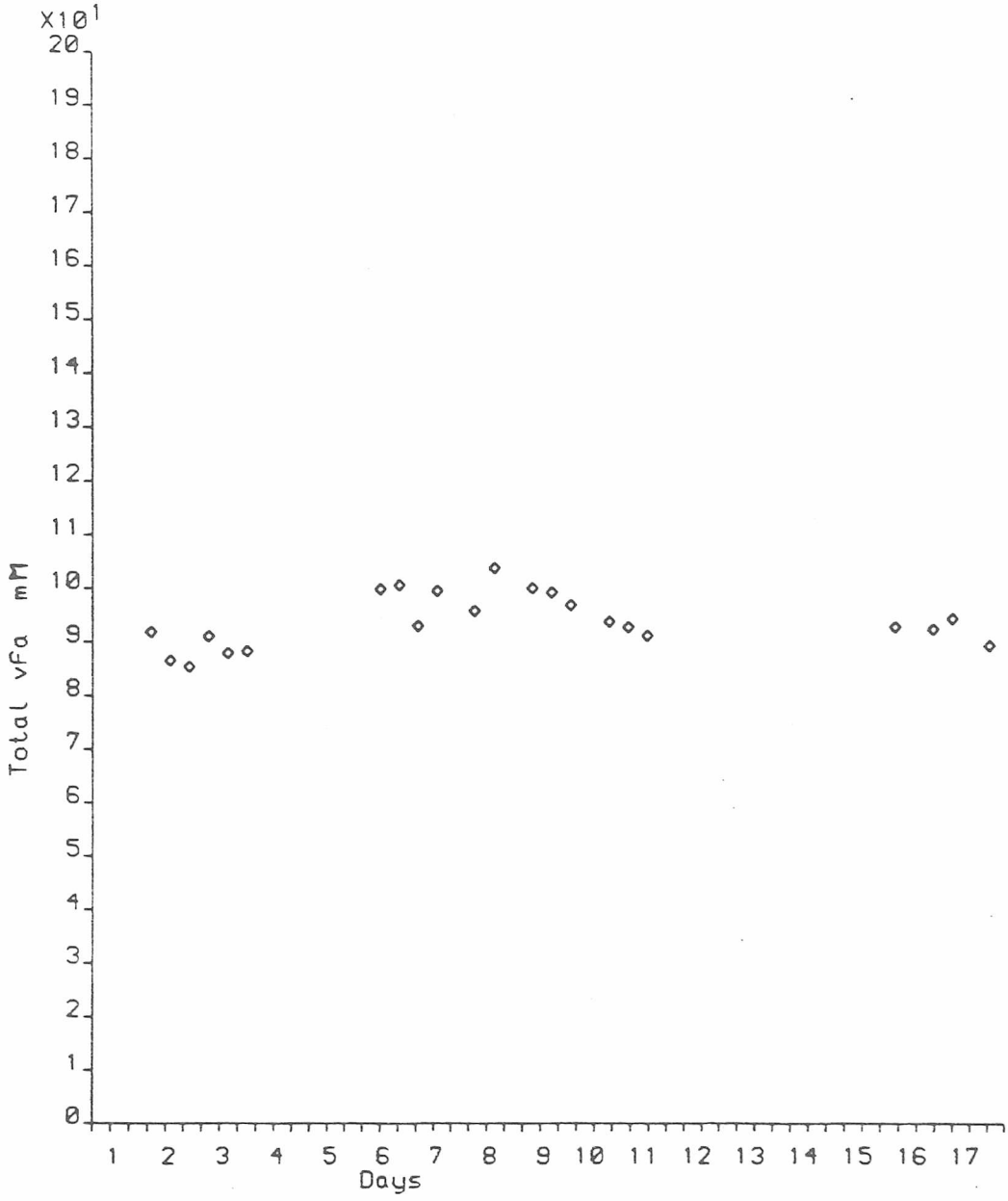


Table 3-4-1: Total VFA concentrations (mM) before the three daily feeds.

	9.00 am	1.00 pm	5.00 pm
Experiment 3g	98.11 ± 4.01[ab]	94.19 ± 5.7[a]	93.85 ± 4.38[b]
Experiment 3h	96.49 ± 4.2	93.15 ± 5.3	92.17 ± 4.3

a significantly different $P < 0.05$

b significantly different $P < 0.05$

Table 3-4-2: Proportions of the major VFA (mol/100 mol)

	Experiment 3g	Experiment 3h
Acetate	60.35 ± 1.65	66.52 ± 2.4
Propionate	17.19 ± 1.39	17.57 ± 0.4
Butyrate	18.11 ± 2.4	8.48 ± 2.0

3.4 Apple Fibre Deletion Experiments (Bran, Cabbage, Carrot and Turnip)

Two apple deletion experiments were carried out 2 months apart. Experiment 3g which followed a cabbage deletion was inoculated on day 1 and ran for 22 days. The culture used for experiment 3h was not reinoculated. Experiment 3h followed a carrot deletion experiment and was run for 16 days. The feedstocks showed no signs of contamination when examined microscopically after 3 days incubation at 37°C and the pH of the fermentor cultures was maintained at between pH 6.8 and 7.2. Microbiological analyses was performed on day 16 of experiment 3g and day 7 of experiment 3h. Hydrogen and methane gas production was measured on day 16 of experiment 3g and analysis of H_2, CH_4 and N_2 production was carried out on day 8 of experiment 3h. H_2S was measured on days 11 and 13 of experiment 3h.

3.4(1) Volatile Fatty Acids (VFA)

Figures 3-4-1 and 3-4-2 show the total volatile fatty acid concentrations recorded throughout experiments 3g and 3h respectively. The average total VFA at 9.00 am, 1.00 pm and 5.00 pm are shown in table 3-4-1. There were no significant differences apparent when total VFA concentrations at 9.00 am, 1.00 pm and 5.00 pm in experiment 3g were compared with corresponding VFA concentrations in experiment 3h. Statistical comparison between the total VFA concentrations at 9.00 am, 1.00 pm and 5.00 pm within each of the

Figure 3-4-3 VFA concentrations (mol/100 mol) during experiment 3g (Apple deletion)

◇ Acetate ○ Propionate ✳ Butyrate

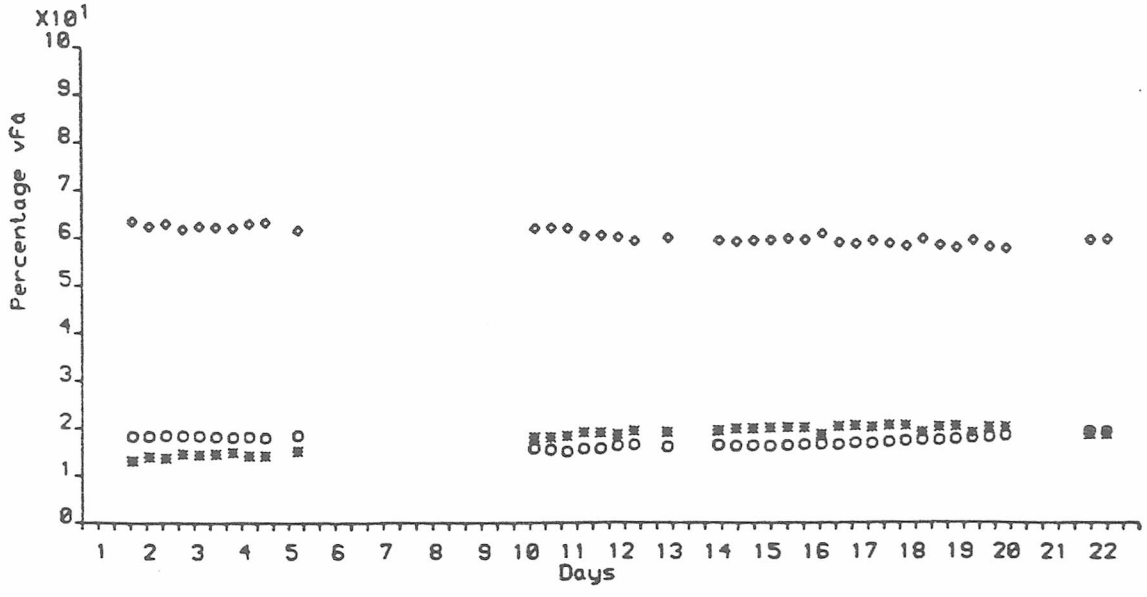
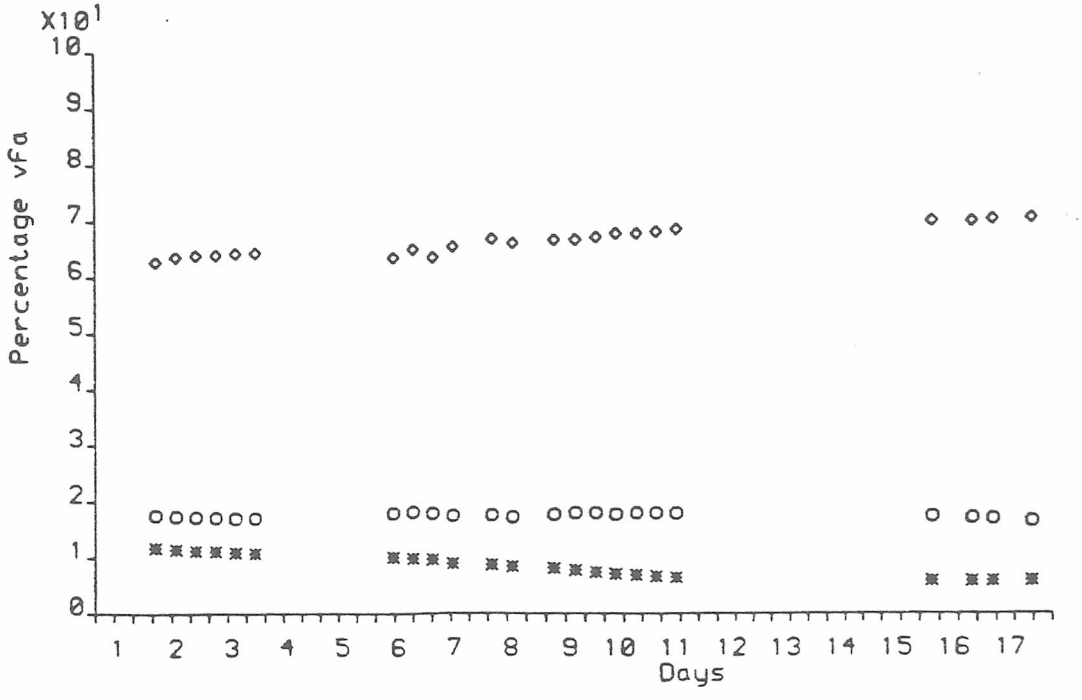


Figure 3-4-4 VFA concentrations (mol/100 mol) during experiment 3h (Apple deletion)

◇ Acetate ○ Propionate ✱ Butyrate



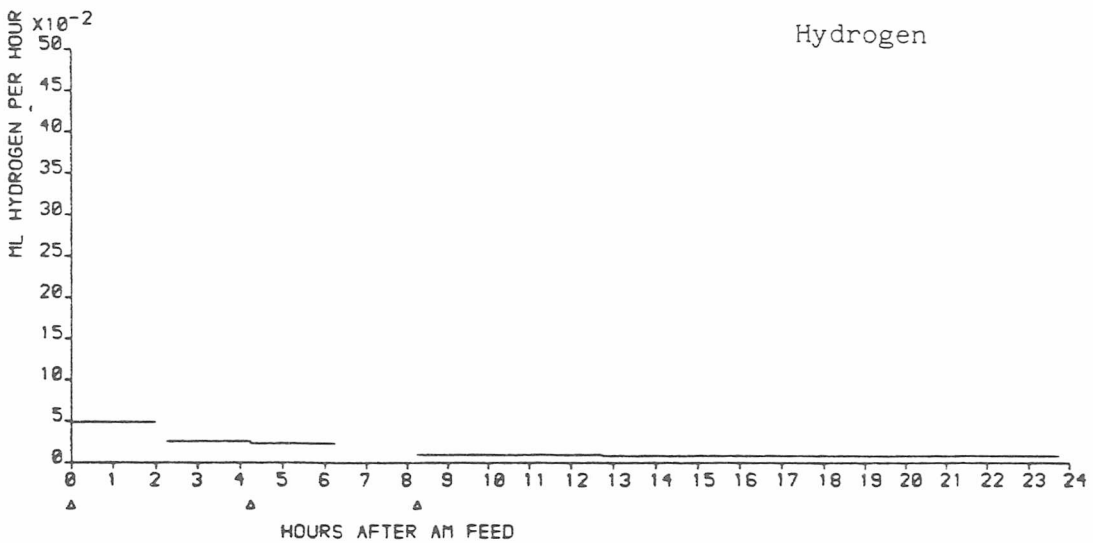
experiments showed that the morning values in experiment 3h were significantly different from those at 1.00 pm and 5.00 pm.

From the data in table 3-4-1 the average daily production rates were calculated to be 37.28 μmoles/ml/24 hr in experiment 3g and 36.33 μmoles/ml/24 hr in experiment 3h.

The proportions of the total VFA represented by acetate, propionate and butyrate are shown in figures 3-4-3 (experiment 3g) and 3-4-4 (experiment 3h). The average percentages of the total VFA represented by the three acids are shown in table 3-4-2. From figure 3-4-4 it is obvious the fermentation did not reach a steady state within the experimental period. Butyrate appeared to decrease as proportions of acetate increased. The apparent instability may have resulted from the fact that the culture had adapted to the previous feedstock and was not reinoculated.

In contrast, VFA production during experiment 3g stabilised within the first 24 hr after reinoculation. The variation in the proportions of VFA's in experiment 3h was not reflected in the total amounts of VFA produced and hence the production rates in experiments 3g and 3h were very similar. The fermentation in both experiments was most rapid within the first 2-4 hr with 70% - 72% of the VFA produced during this period. Thereafter most of the increase in VFA was due to acetate.

Figure 3-4-5 Hydrogen and methane production ($ml.hr^{-1}$) during experiment 3g (Apple deletion)



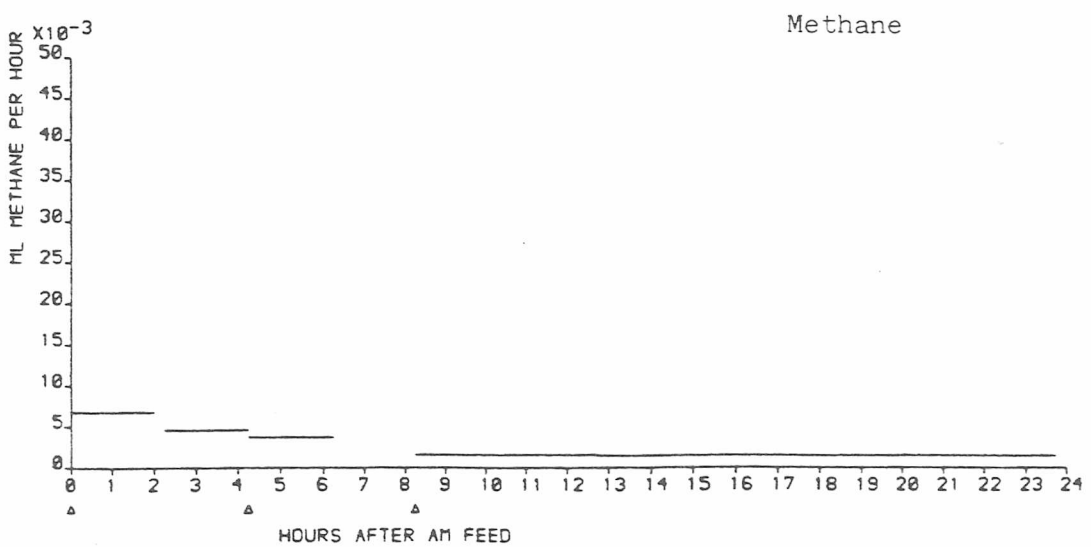


Figure 3-4-6 Hydrogen production ($ml.hr^{-1}$) during experiment 3h (Apple deletion).

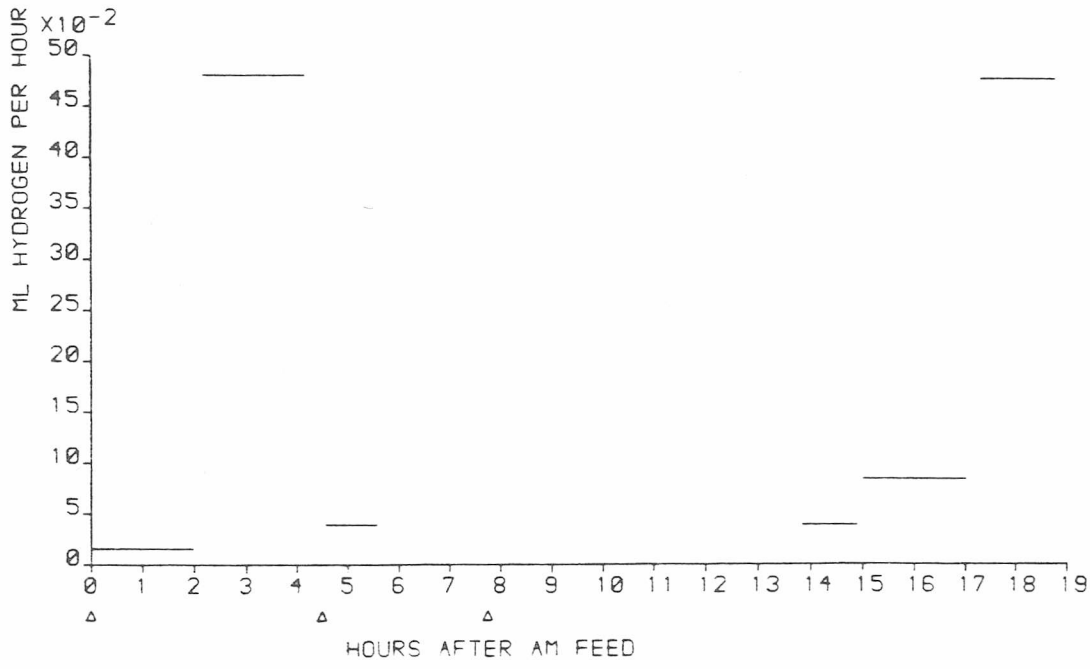


Figure 3-4-7 Methane production ($ml.hr^{-1}$) during experiment 3h (Apple deletion)

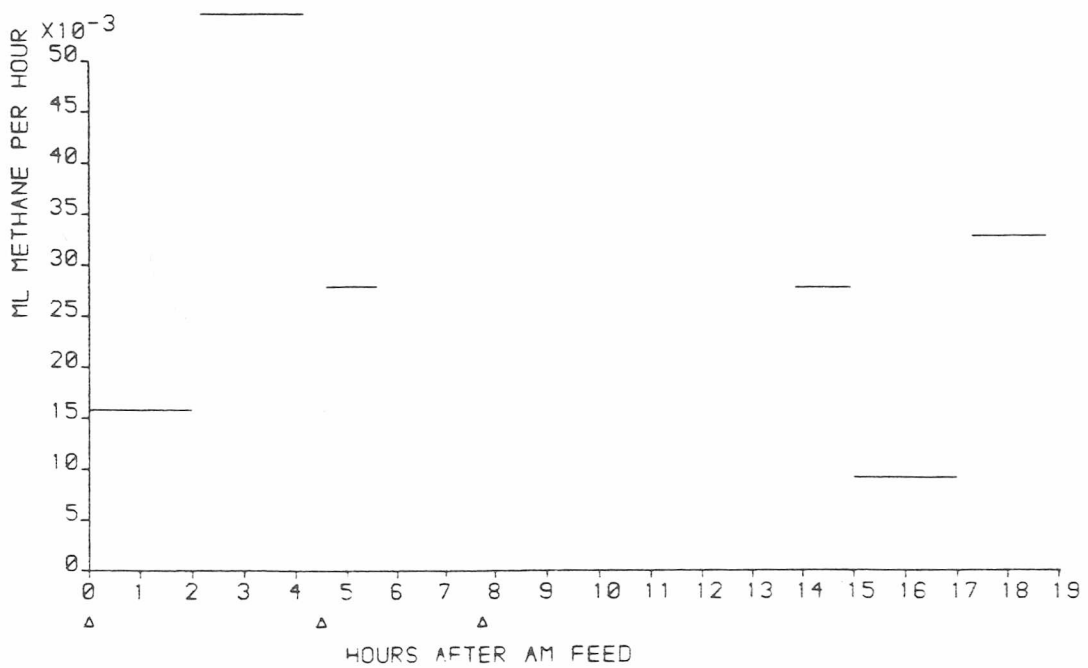


Figure 3-4-8 Ammonia-N, Nitrate-N and Nitrite-N concentrations (ppm) during experiment 3g (Apple deletion)

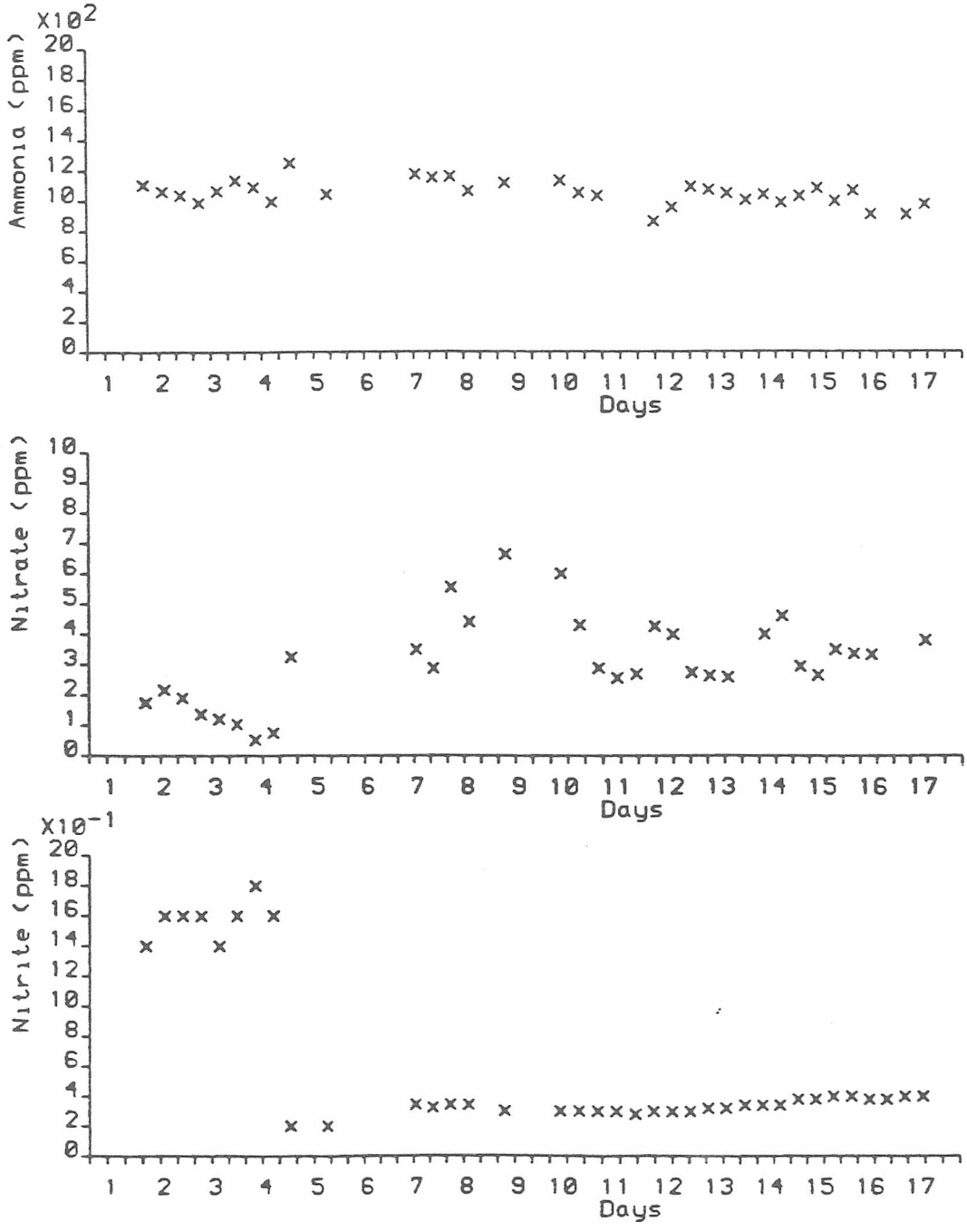


Figure 3-4-9 Ammonia-N, Nitrate-N and Nitrite-N concentrations (ppm) during experiment 3h (Apple deletion)

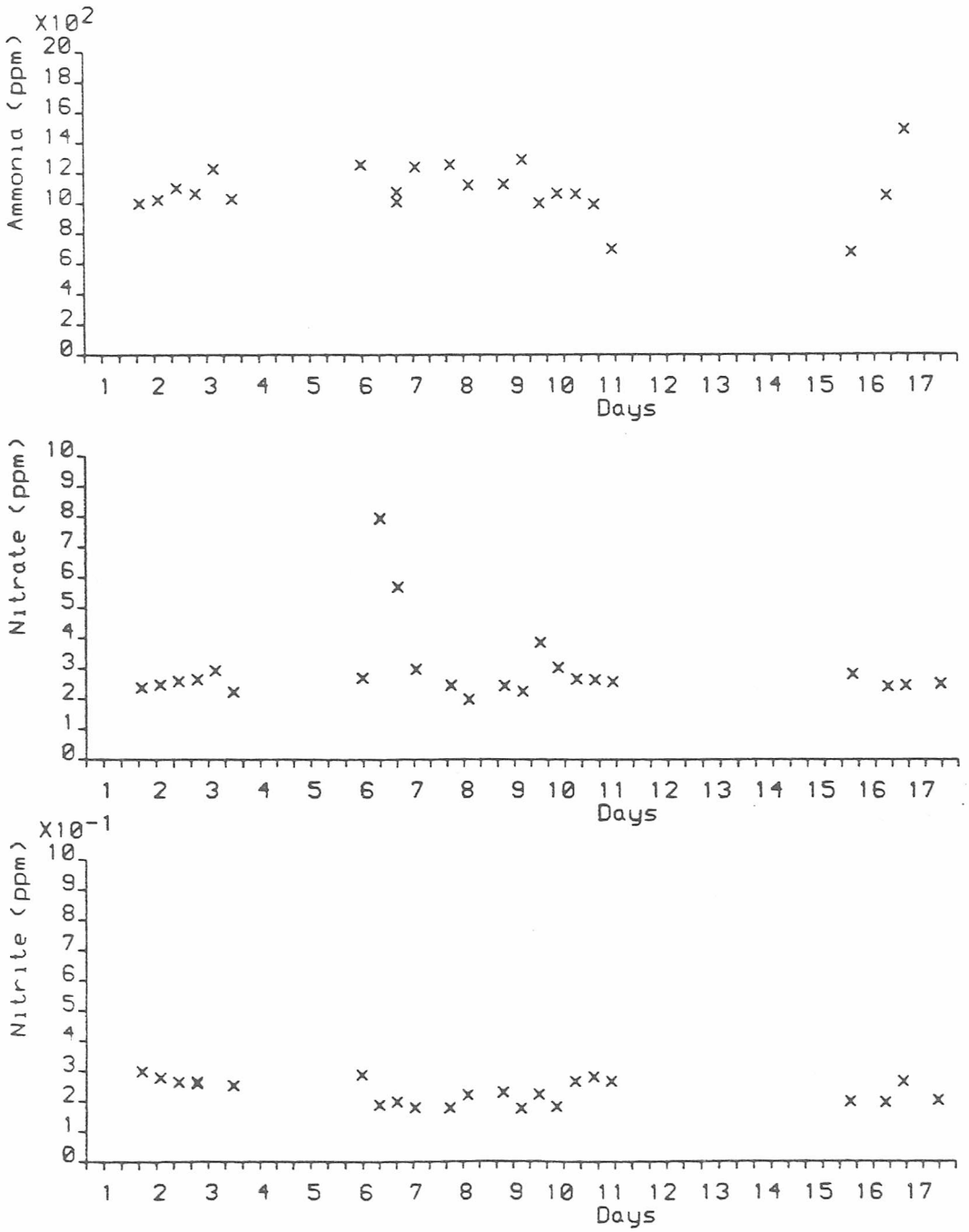


Table 3-4-3: Ammonia-N, Nitrate-N and Nitrite-N concentrations (ppm) in feedstock and fermentor samples.

	Ammonia-N (ppm)	Nitrate-N (ppm)	Nitrite-N (ppm)
Feedstock			
experiment 3g	386	132	0.05
Fermentor samples			
experiment 3g	1040 ± 83	3.12 ± 1.42	0.33 ± 0.04
Feedstock			
experiment 3h	389	135	0.1
Fermentor samples			
experiment 3h	1118.7 ± 125	2.61 ± 0.37	0.13 ± 0.04

3.4(2) Hydrogen and Methane Gases

The amounts of H_2 ($ml.hr^{-1}$) and CH_4 ($ml.hr^{-1}$) collected on day 16 of experiment 3g and day 8 of experiment h are shown in figures 3-4-5 (experiment 3g), and 3-4-6 and 3-4-7 (experiment 3h). Hydrogen production was within the range 0.0098 - 0.049 $ml.hr^{-1}$ and 0.009 and 0.48 $ml.hr^{-1}$ in experiments 3g and 3h and methane 0.0016 - 0.0068 $ml.hr^{-1}$. and 0.00913 - 0.0 $ml.hr^{-1}$. The patterns of production of hydrogen and methane appear to parallel one another in both experiments but total production of both gases was considerably higher in experiment 3h compared with experiment 3g.

In experiment 3g approximately 0.65 ml H_2 and 0.1 ml CH_4 were produced per day and in experiment 3h 4.0 ml H_2 and 0.63 ml CH_4. In total 1.059652 ml H_2/day would be accounted for by hydrogen collected and methane produced in experiments 3g and 3h respectively. From the amounts of volatile fatty acids produced, an estimated 765.26 ml/day and 722.18 ml/day of H_2 were produced in experiments 3g and 3h. If homoacetic acid fermentation was taking place 4.01 mmoles acetate could have been produced from the theoretical surplus in experiment 3h.

3.4(3) Nitrogen

Ammonia-, nitrate- and nitrite-N concentrations are shown in figures 3-4-8 and 3-4-9. Feedstock and fermentor concentrations of ammonia-N, nitrate-N and nitrite-N are shown in table 3-4-3. The net increase in ammonia

Figure 3-4-10 Nitrogen gas production ($ml.hr^{-1}$) during experiment 3h (Apple deletion).

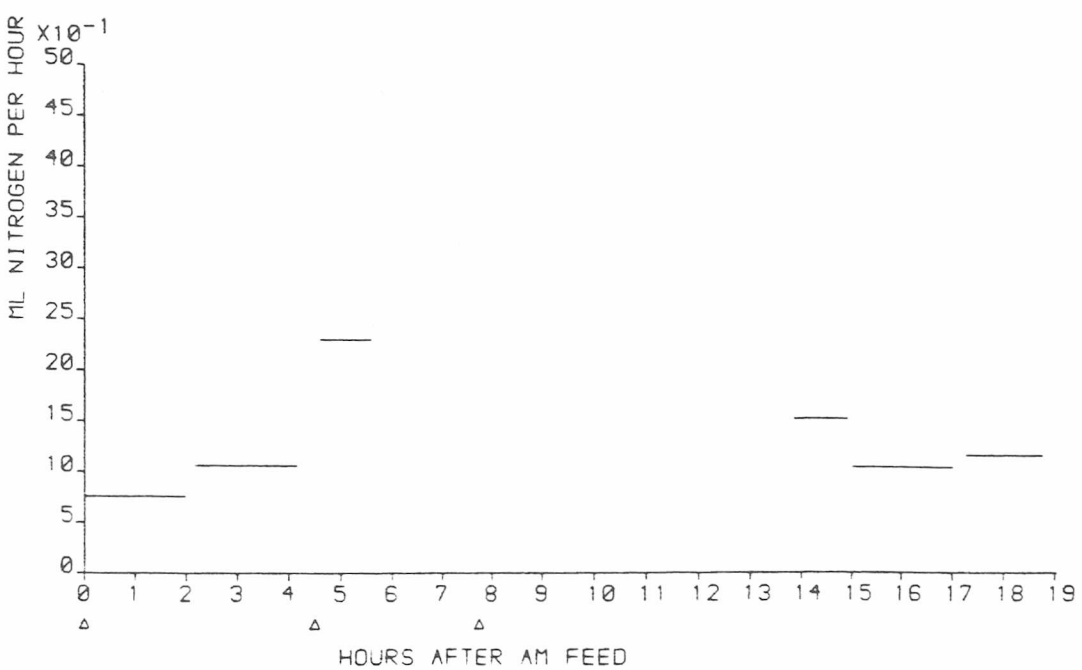
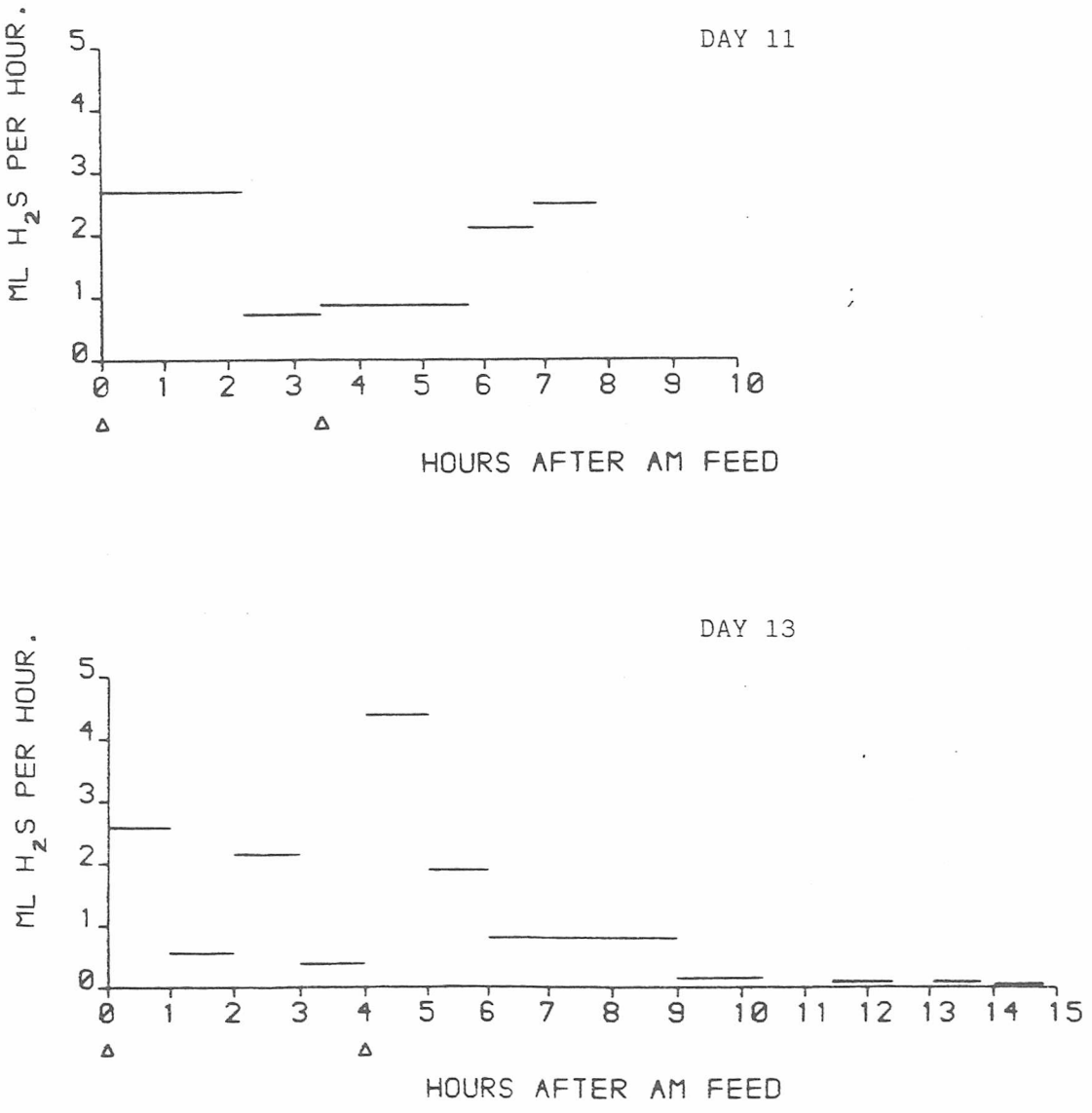


Figure 3-4-11 Hydrogen sulphide production ($ml.hr^{-1}$) during experiment 3h (Apple deletion)



concentrations above feedstock levels represents on average of 84.93 ppm in experiment 3g and 94.76 ppm during experiment 3h. Nitrite concentrations in the fermentor never accumulated above 0.36 ppm.

From the data in table 3-4-3 it can be calculated that an average of 16.73 ppm and 17.19 ppm nitrate-N were reduced each feed in experiments 3g and 3h respectively. If the nitrate was completely reduced to nitrogen gas approximately 8.97 ml and 9.21 ml N_2 per feed would be produced.

Figure 3-4-10 shows the results of the gas analysis performed on day 8 of experiment 3h. An estimated 31.33 ml N_2 was produced over a 24 hr period or 10.44 ml per feed.

3.4(4) Sulphur

During experiment 3h sulphate concentrations in the fermentor decreased from 0.265 mM immediately after a feed to 0.059 $\pm$ 0.018 mM therefore an average of 0.212 mM sulphate was consumed each feed.

If this sulphate was reduced during dissimilatory sulphate reduction an average of 3.18 ml H_2S/feed could be expected. The results for H_2S collected on days 11 and 13 are shown in figure 3-4-11. A total of 13 ml (day 11) and 14 ml (day 13) were collected. A maximum of 7.0 ml could be accounted for from the 2 feeds on each day which suggests an additional source of H_2S must have been present.

Table 3-4-4: Composition of dietary fibre ($mg.ml^{-1}$) in feedstock and fermentor samples.

	Starch	Uronic Acids	Arabinose	Xylose	Mannose	Galactose	Glucose
Feedstock experiment 3h	1.542	1.26	0.754	0.880	0.172	0.635	2.101
Fermentor samples experiment 3h	0.081 ±0.039	0.057 ±0.015	0.226 ±0.142	0.222 ±0.124	0.022 ±0.0037	0.020 ±0.011	0.084 ±0.03

Table 3-4-5: Enumeration of bacteria in experiment 3g and 3h.

	Bacteria/ml of fermentor contents	
	Experiment 3g	Experiment 3h
Total Anaerobic Counts	$1.49 \pm 0.57 \times 10^9$	$2.25 \pm 1.31 \times 10^9$
Total Aerobic Counts	4.0×10^7	$6.56 \pm 1.25 \times 10^7$
Enterobacteriacea	$2.6 \pm 0.4 \times 10^6$	$5.00 \pm 1.22 \times 10^6$
Xylanolytic bacteria	$1.6 \pm 0.4 \times 10^7$	$4.4 \pm 2.73 \times 10^7$
Pectinolytic bacteria	$6.6 \pm 2.56 \times 10^8$	$8.8 \pm 3.7 \times 10^8$
Starch digesting bacteria	$4.33 \pm 1.65 \times 10^8$	$9.7 \pm 4.01 \times 10^8$

Anaerobic : Aerobic Ratio	37 : 1	34 : 1
Anaerobic : Xylanolytic Ratio	93 : 1	51 : 1
Anaerobic : Pectinolytic Ratio	22 : 1	25 : 1
Anaerobic : Starch digesting ratio	3.4 : 1	2.32 : 1

3.4(5) Fibre Digestion

The starch, uronic acid and sugar composition of the dietary fibre in feedstock and fermentor residues during experiment 3h are shown in table 3-4-4. From the data given in table 3-4-4 it can be calculated that 0.899 $mg.ml^{-1}$ carbohydrate was metabolised each feed (1.81 g/fermentor/day) or 10.03 mmoles of hexose equivalents per fermentor per day.

3.4(6) Microbiology

Some of the microbiological data collected for experiment 3g and 3h are shown in table 3-4-5.

The numbers of bacteria in the various groups in experiment 3g were very close to those recorded for experiment 3h.

Acetate was the major product in Min-1, Min 1(d), Min-1(i) and in medium 119 and RFH incubated under H_2:CO_2 80%:20%. Small amounts of butyrate were detected in cultures grown in M-1 and Min-1(c).

The numbers of bacteria present in the fermentor represent a daily bacterial cell turnover of 5.14×10^{11}/fermentor/24 hr which is equivalent to a dry wt of 0.69 g/24 hr. If 3-4 g of carbohydrate were required to produce 1 g dry weight of bacteria then between 2.06 - 2.74 g of carbohydrate would be required per 24 hr. Alternatively if the dry weight represented 20 - 30% of the weight of carbohydrate fermented

Table 3-4-6: Identification of bacteria in experiment 3h

		Bacteria/ml of fermentor contents
1.	Fusobacterium Selenomonas Leptotrichia Vibrio succinogenes	3.94×10^8
2.	Clostridium Eubacterium	7.89×10^7
3.	Bifidobacterium Actinomyces	1.58×10^8
4.	Bacteroides	3.16×10^8
5.	Lactobacillus	0
6.	Propionibacterium	2.63×10^7

then between 1.71 g and 2.29 g of carbohydrate would have been fermented each day. The amount of carbohydrate metabolised each day was calculated to be 1.81 g/24 hr (section 3-4-5).

The major groups of bacteria present are shown in table 3-4-6. The numbers of Propionibacterium and Lactobacilli were low when compared with experiment 3a.

Figure 3-5-1 Total VFA concentration (mM) during experiment 3i (Cabbage deletion)

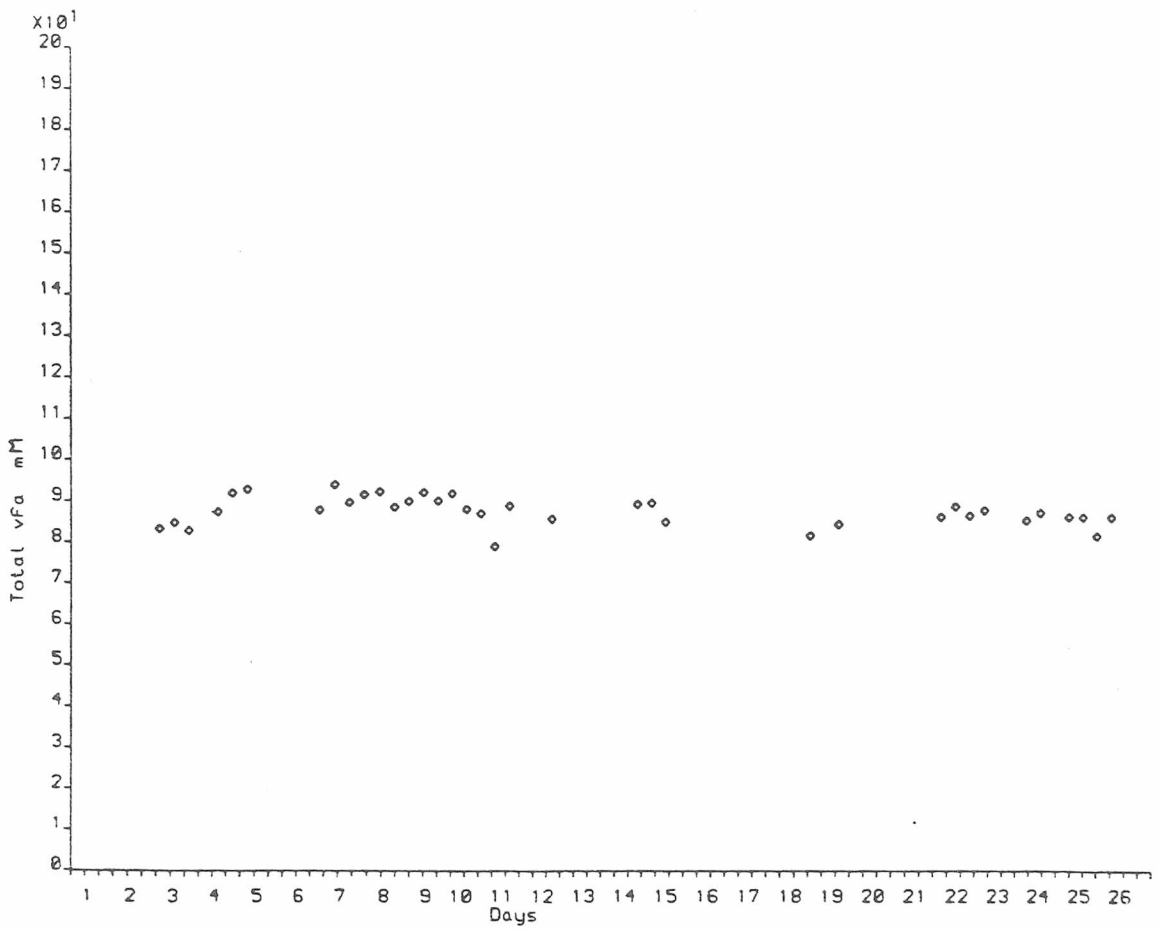


Figure 3-5-2 Total VFA concentration (mM) during experiment 3j (Cabbage deletion)

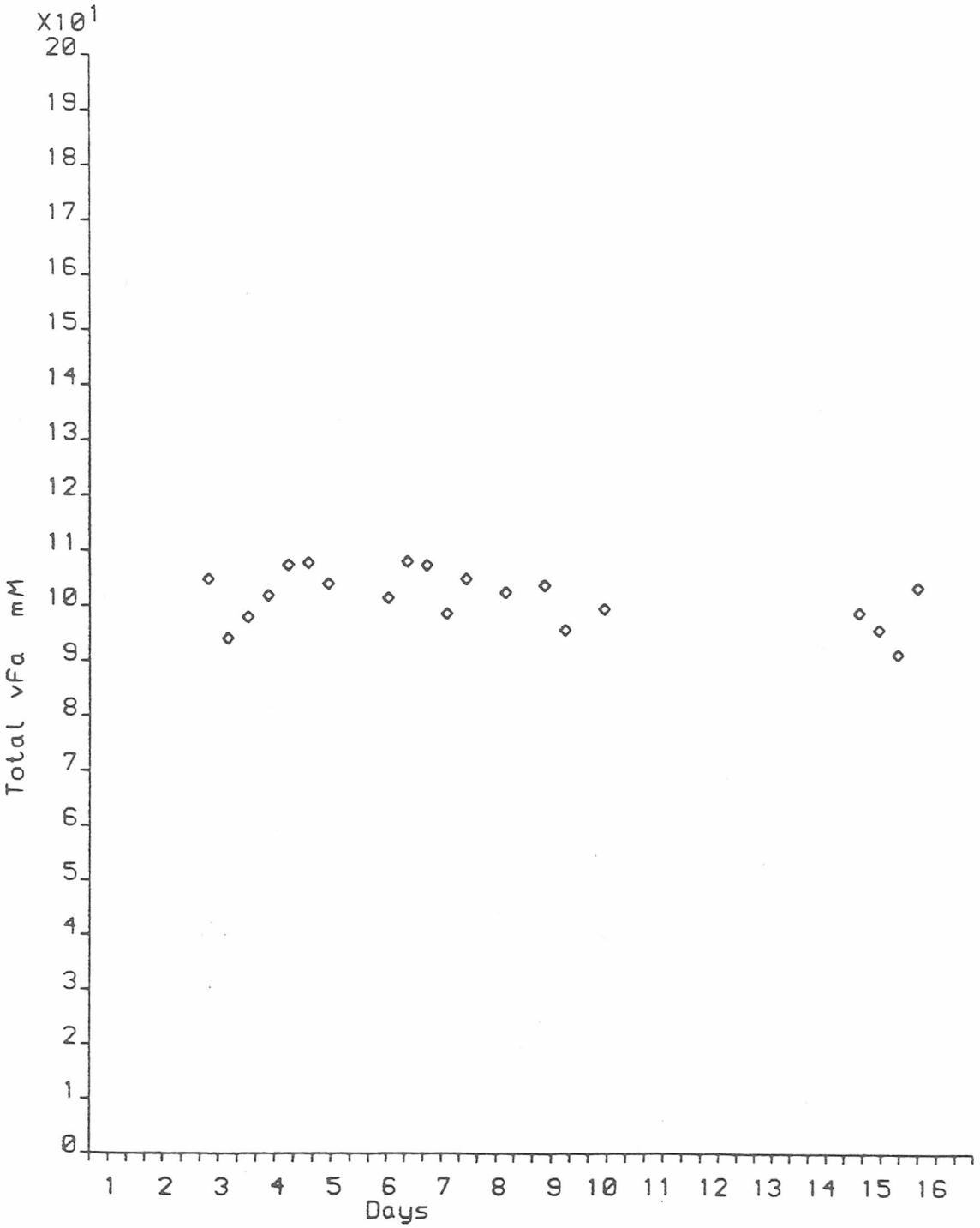


Table 3-5-1: Total VFA concentrations (mM) before the three daily feeds.

	9.00 am	1.00 pm	5.00 pm
Experiment 3i	87.83 ± 3.53^{x}	87.9 ± 1.58^{y}	86.5 ± 4.26^{z}
Experiment 3j	101.35 ± 2.8^{x}	102.16 ± 5.9^{y}	101.4 ± 5.7^{z}

x significantly different $P < 0.05$

y significantly different $P < 0.05$

z significantly different $P < 0.05$

Table 3-5-2: Proportions of the major VFA (mol/100 mol)

	Experiment 3i	Experiment 3j
Acetate	67.26	72.34
Propionate	16.72	13.73
Butyrate	11.39	8.12

3.5 Cabbage Fibre Deletion Experiment (Bran, Carrot, Turnip and Apple)

Two cabbage deletion experiments 3i and 3j were carried out over a period of 26 days and 18 days respectively. The experiments were run 4 months apart. Experiment 3i followed a carrot deletion experiment and experiment 3j followed an apple deletion experiment. Both were reinoculated with a faecal sample on day 1. The feedstocks showed no signs of contamination when microscopically examined after 3days incubation at 37°C and the pH was maintained at pH 6.9 - 7.1 throughout. Hydrogen, methane and nitrogen gas production was measured on day 18 of experiment 3i and day 10 of experiment 3j. H_2S was collected and assayed on days 4 and 11 of experiment 3j. Microbiological analyses were performed on day 7 of experiment 3i and day 6 of experiment 3j.

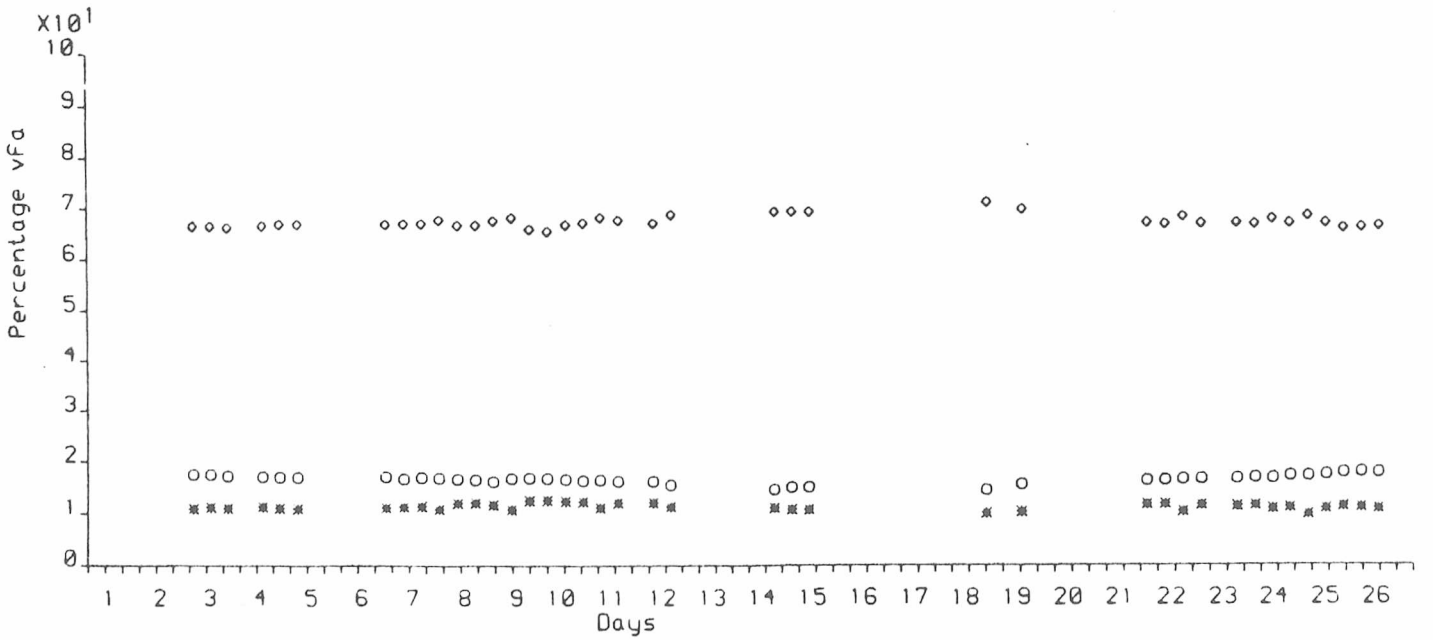
3.5(1) Volatile Fatty Acids VFA

Total volatile fatty acid concentrations during experiments 3i and 3j are shown in figures 3-5-1 and 3-5-2. The average total VFA at 9.00 am, 1.00 pm and 5.00 pm are shown in table 3-5-1. There were no significant differences in VFA levels within experiment 3i and 3j at 9.00 am, 1.00 pm and 5.00 pm but experiment 3i and 3j were significantly different from each other at 9.00 am, 1.00 pm and 5.00 pm.

From the data in table 3-5-1 it was calculated that an average of 33·64±2·61 µmoles VFA/ml/day and 39·87±2·7 µmoles/ml/day

Figure 3-5-3 VFA concentrations (mol/100 mol) during experiment 3i (Cabbage deletion)

◇ Acetate ○ Propionate ✱ Butyrate



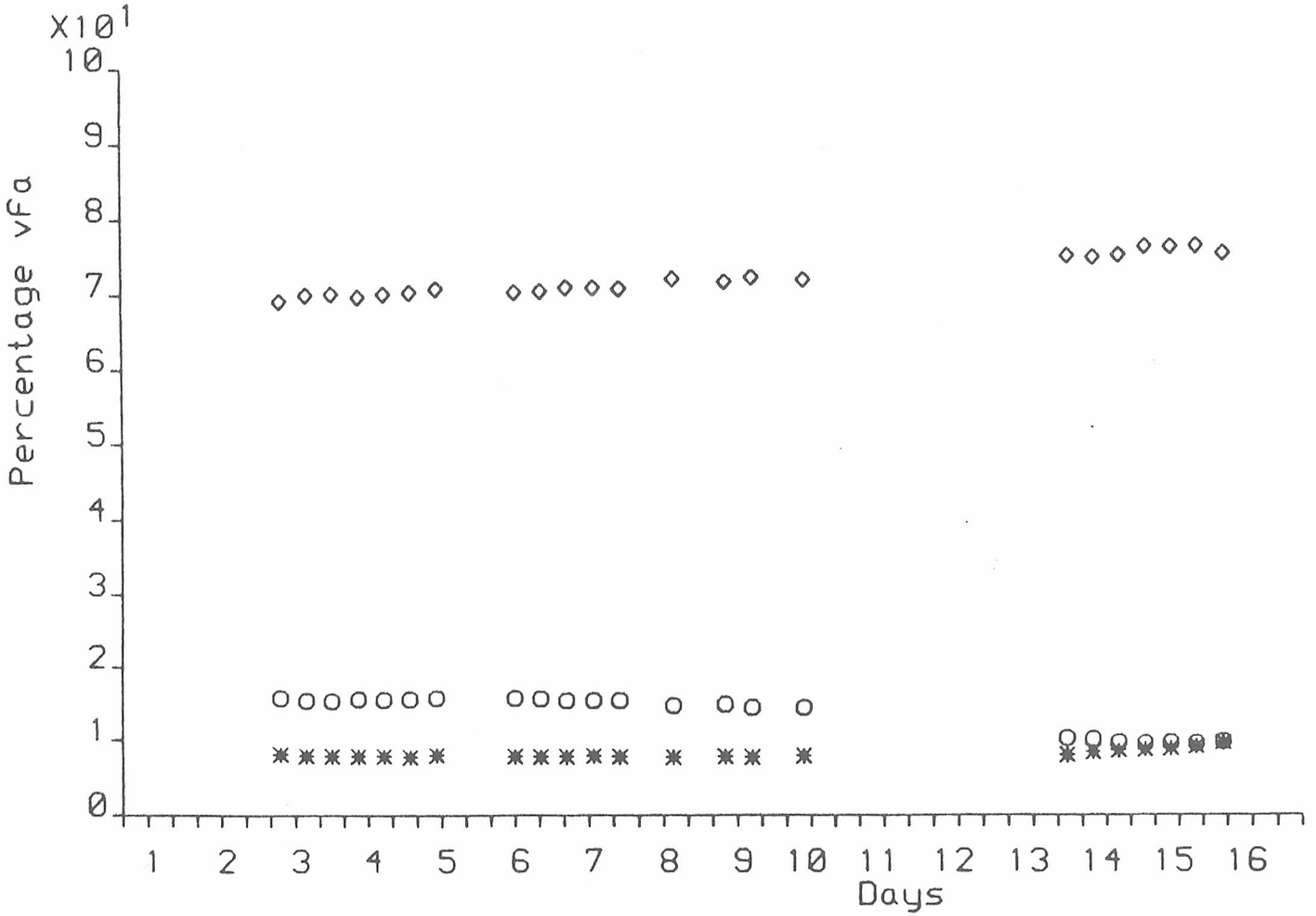
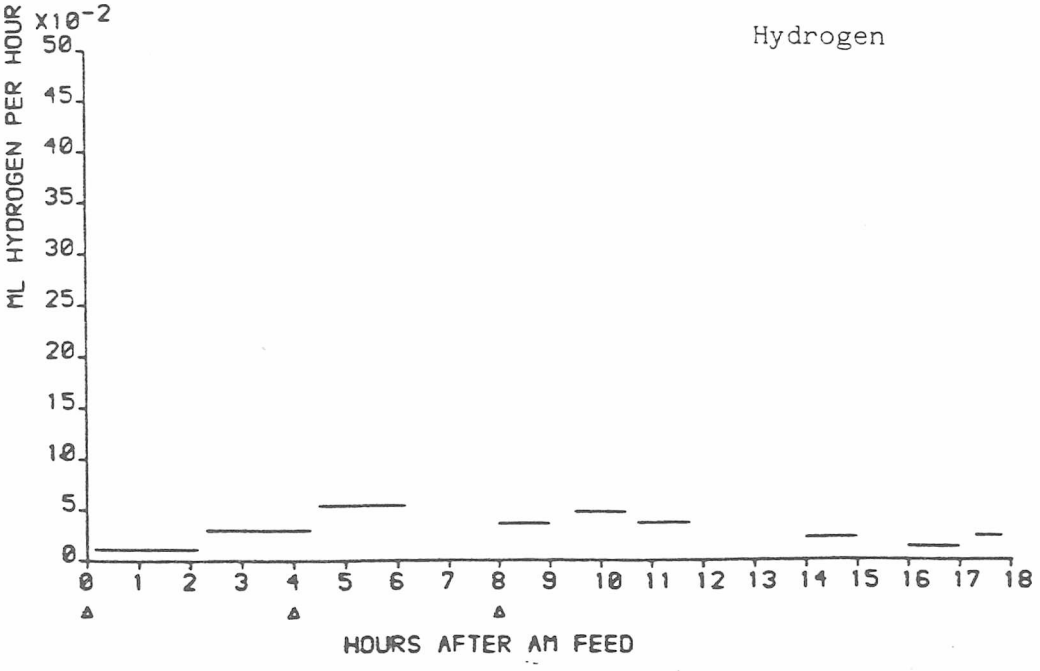


Figure 3-5-4 VFA concentrations (mol/100 mol) during experiment 3j (Cabbage deletion)

◇ Acetate o Propionate * Butyrate

Figure 3-5-5 Hydrogen and methane production ($ml.hr^{-1}$) during experiment 3i (Cabbage deletion)



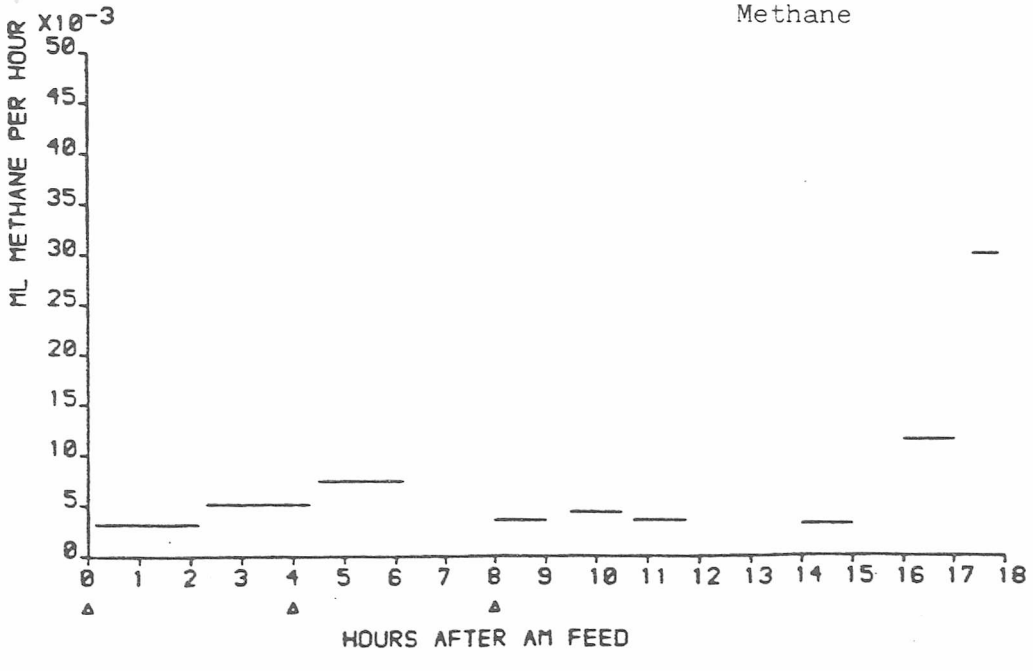
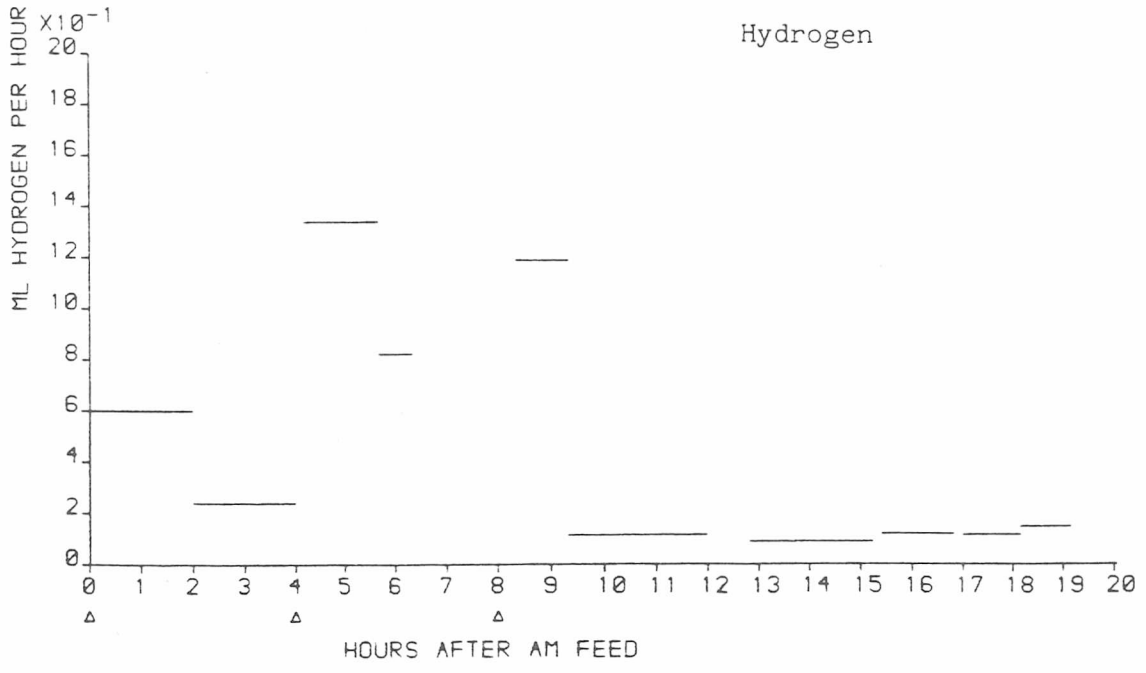
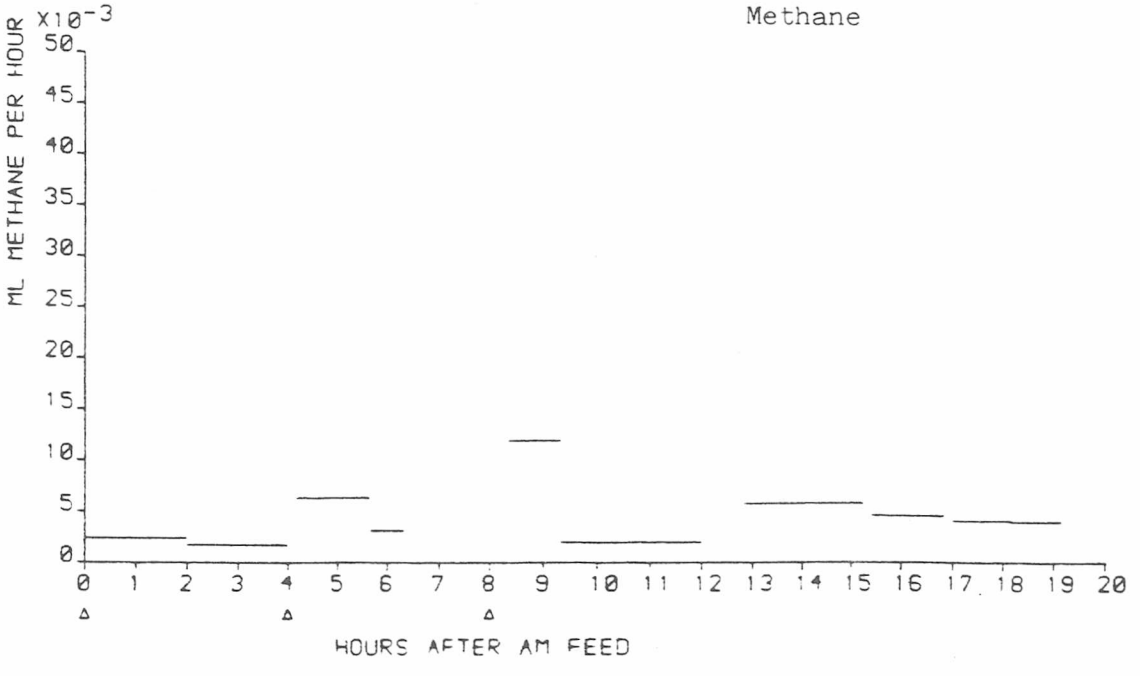


Figure 3-5-6 Hydrogen and methane production ($ml.hr^{-1}$) during experiment 3j (Cabbage deletion)





were produced during experiments 3i and 3j respectively. The proportions of the total VFA represented by acetate, propionate and butyrate are shown in figures 3-5-3 and 3-5-4. The average proportions of each acid are shown in table 3-5-2. The total VFA and proportions of VFA were stable throughout experiment 3i. The slight variation in the proportions of acetate and propionate in experiment 3j were not reflected in the total VFA which remained constant throughout the experiment. More than 75% of the total VFA was produced within the first 4 hr in experiment 3i and within the first 2.5 hr in experiment 3j after which acetate constituted most of the increase.

3.5(2) Hydrogen and Methane Gases

The amounts of hydrogen ($ml.hr^{-1}$) and methane ($ml.hr^{-1}$) collected over a period of 18 hr in experiment 3i and 20 hr in experiment 3j are shown in figures 3-5-5 and 3-5-6. Hydrogen production during experiment 3i was within the range 0.011 - 0.054 $ml.hr^{-1}$ and during experiment 3j, 0.088 - 1.34 $ml.hr^{-1}$. Methane production was within the range 0.00318 - 0.03 $ml.hr^{-1}$ and 0.00173 - 0.012 $ml.hr^{-1}$ for experiment 3i and experiment 3j respectively. Methane production patterns appear to mirror that of hydrogen production except in experiment 3i where only methane rose steadily between 14-18 hr after the morning feed. Estimates of the amounts of hydrogen and methane produced per 24 hr were 0.73 ml H_2 and 0.196 ml CH_4 in experiment 3i and 11.46 ml H_2 and 0.112 ml CH_4 in experiment 3j. In total the amount of hydrogen

Figure 3-5-7 Ammonia-N, Nitrate-N and Nitrite-N concentrations (ppm) during experiment 3i (Cabbage deletion)

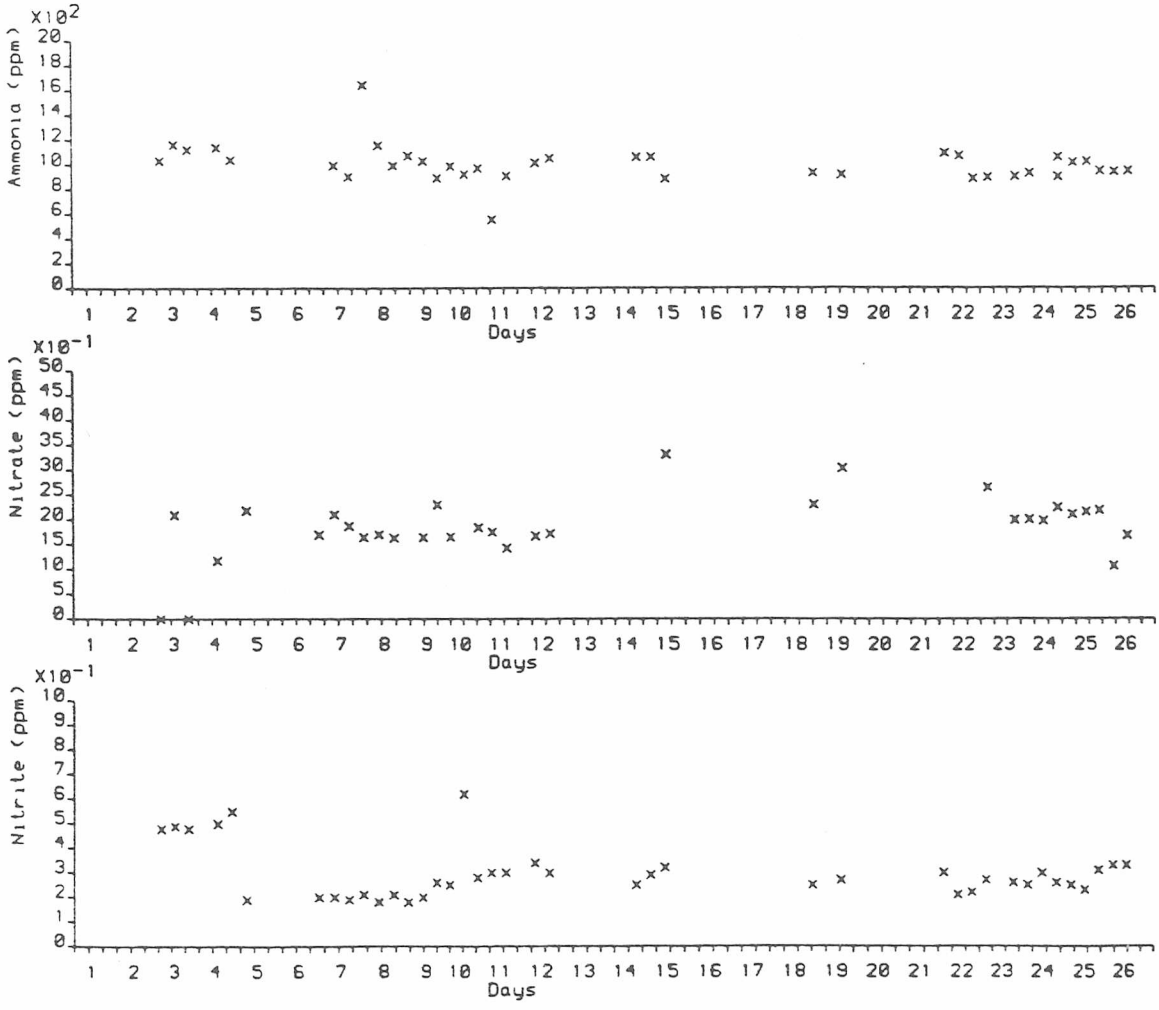


Figure 3-5-8 Ammonia-N, Nitrate-N and Nitrite-N concentrations (ppm) during experiment 3j (Cabbage deletion)

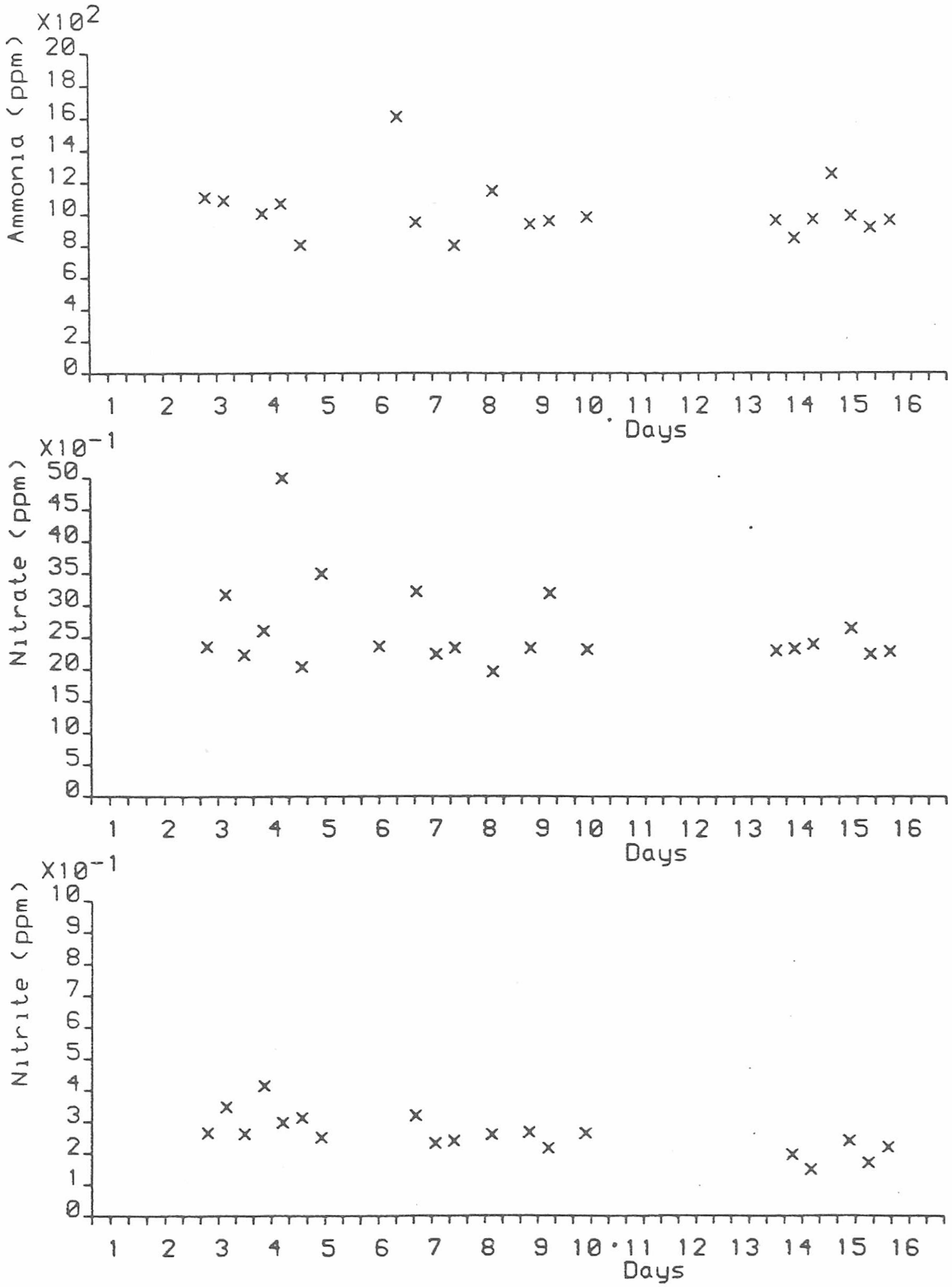
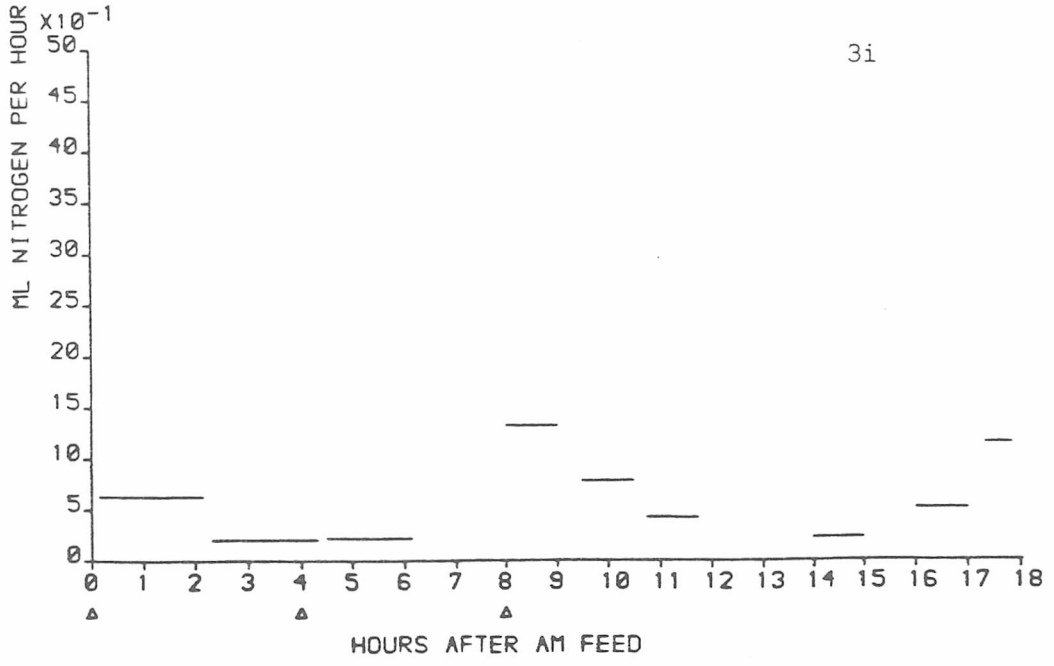
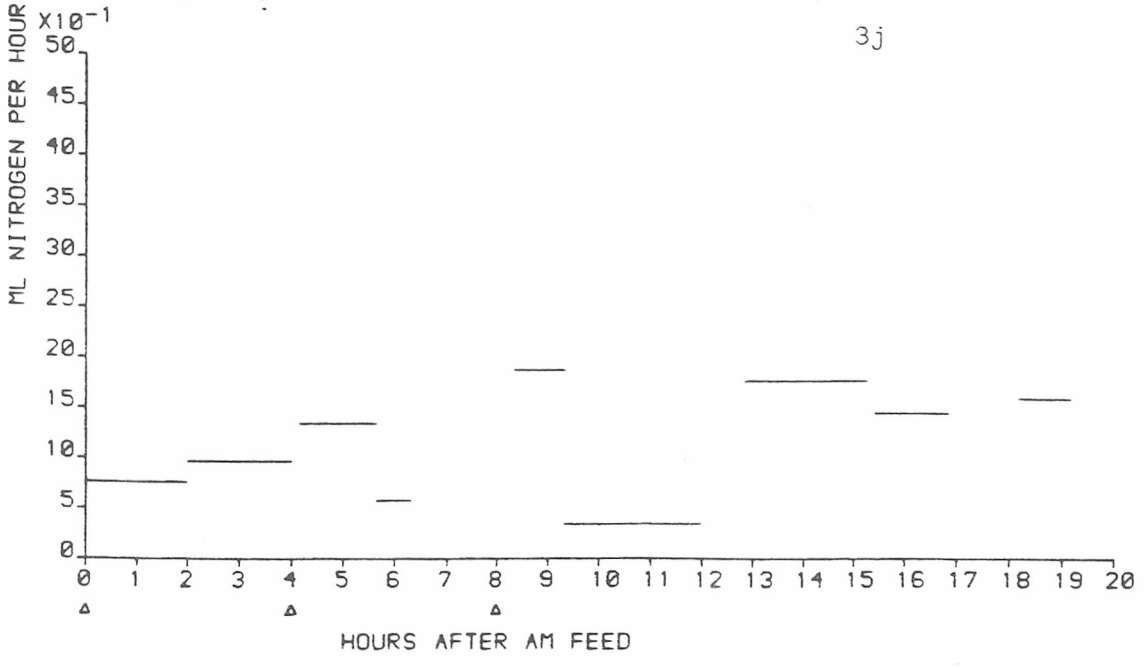


Table 3-5-3: Ammonia-N, Nitrate-N and Nitrite-N concentrations (ppm) in feedstock and fermentor samples.

	Ammonia-N (ppm)	Nitrate-N (ppm)	Nitrite-N (ppm)
Feedstock experiment 3i	324	139	0.05
Fermentor samples experiment 3i	994 ± 81.32	1.83 ± 0.65	0.25 ± 0.05
Feedstock experiment 3j	311	135	0.07
Fermentor samples experiment 3j	1017 ± 176	2.64 ± 0.73	0.26 ± 0.06

Figure 3-5-9 Nitrogen gas production ($ml.hr^{-1}$) during experiments 3i and 3j (Cabbage deletion)





collected plus that required for the formation of CH_4 accounted for 1.54 ml H_2/24 hr in experiment 3i and 11.91 ml H_2/24 hr in experiment 3j. The theoretical production of H_2 calculated from volatile fatty acids produced was 709.58 ml in experiment 3i and 880.82 ml in experiment 3j. Taking into account hydrogen consumed during nitrate and sulphate reduction, methane formation and the hydrogen collected, 6.55 mmoles of acetate could have been produced by homoacetic acid fermentation according to equation (1) in section 3.1(3).

3.5(3) Nitrogen

Ammonia-, nitrate- and nitrite-N concentrations during experiment 3i and 3j are shown in figures 3-5-7 and 3-5-8 respectively. Feedstock and average fermentor sample levels of ammonia- nitrate- and nitrite-N concentrations are shown in table 3-5-3.

The net increase in ammonia-N concentrations in fermentor samples represents an average of 87.0 ppm in experiment 3i and 91.79 ppm in experiment 3j. Nitrate-N concentrations were decreased by an average of 17.81 ppm each feed in experiment 3i and by 17.19 ppm each feed during experiment 3j. If this nitrate was completely reduced to nitrogen gas then an average of 9.54 ml N_2/feed and 9.22 ml N_2/feed would be produced from the nitrate available in experiment 3i and experiment 3j respectively. The results of N_2 gas measurements during experiments 3i and 3j are shown in figures 3-5-9(i) and 3-5-9(j) respectively. The amount of N_2

Figure 3-5-10 Hydrogen sulphide production ($ml.hr^{-1}$) during experiment 3j (Cabbage deletion)

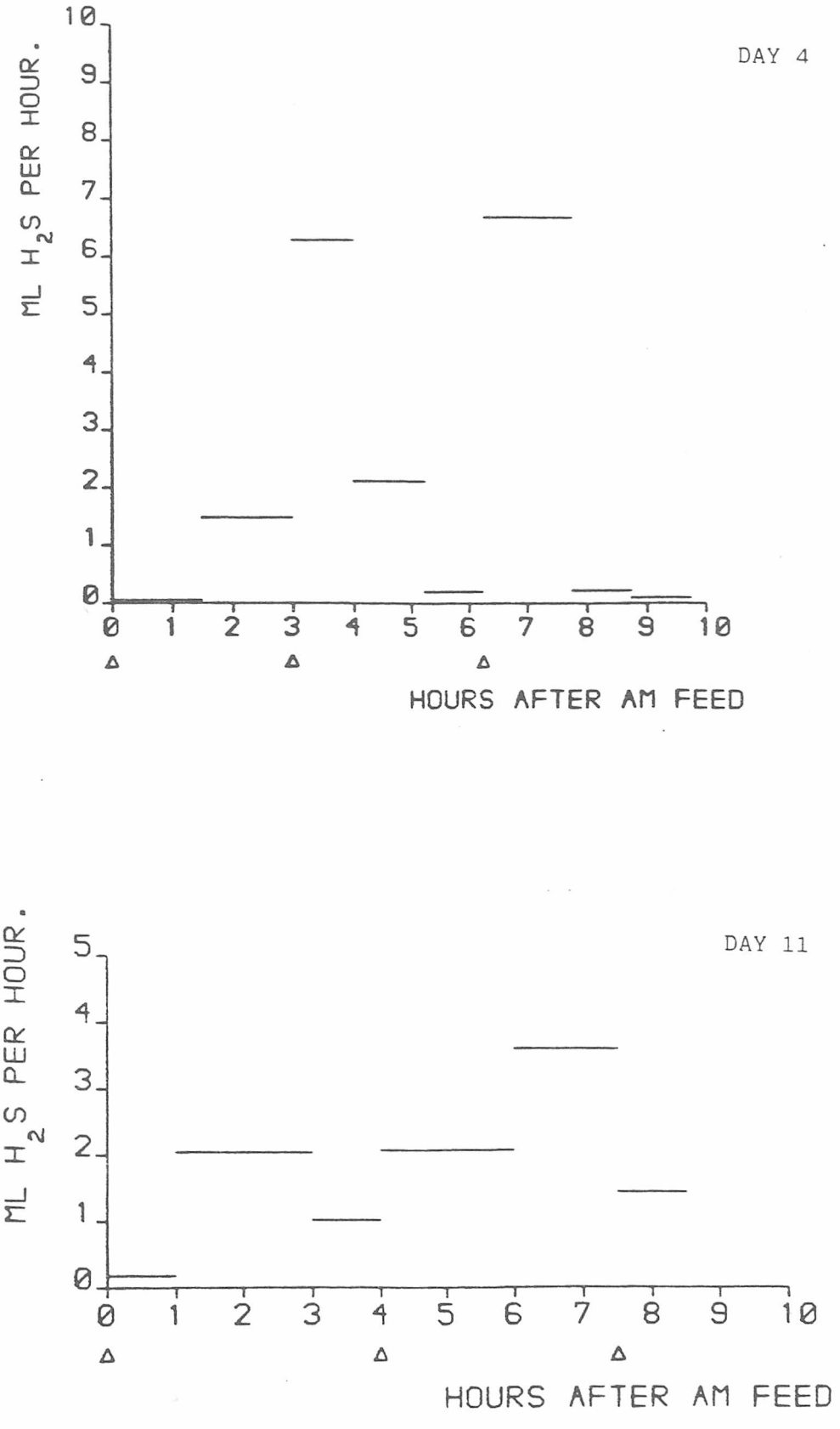


Table 3-5-4: Composition of dietary fibre ($mg.ml^{-1}$) in feedstock and fermentor samples.

	Starch	Uronic Acids	Arabinose	Xylose	Mannose	Galactose	Glucose
Feedstock							
experiment 3j	1.723	1.248	0.856	0.813	0.196	0.715	2.054
Fermentor samples							
experiment 3j	0.204 ±0.041	0.053 ±0.013	0.529 ±0.325	0.454 ±0.277	0.013 ±0.007	0.035 ±0.002	0.134 ±0.127

Table 3-5-5: Enumeration of bacteria in experiment 3i and 3j.

	Bacteria/ml of fermentor contents	
	Experiment 3i	Experiment 3j
Total Anaerobic Counts	$2.56 \pm 1.22 \times 10^9$	$3.52 \pm 1.52 \times 10^9$
Total Aerobic Counts	NT	$2.5 \pm 1.0 \times 10^7$
Enterobacteriacea	$2.18 \pm 1.02 \times 10^7$	$1.9 \pm 1.31 \times 10^7$
Xylanolytic bacteria	4.0×10^7	$7.96 \pm 3.5 \times 10^6$
Pectinolytic bacteria	1.2×10^9	$1.25 \pm 0.8 \times 10^9$
Starch digesting bacteria	$5.75 \pm 2.5 \times 10^8$	$5.3 \pm 2.7 \times 10^8$

Anaerobic : Aerobic Ratio	NT	140 : 1
Anaerobic : Xylanolytic Ratio	64 : 1	442 : 1
Anaerobic : Pectinolytic Ratio	2.13 : 1	2.81 : 1
Anaerobic : Starch digesting ratio	4.45 : 1	6.64 : 1

NT - Not tested

gas produced over a 24 hr period was estimated to be 19 ml (experiment 3i) and 28.6 ml during experiment 3j.

3.5(4) Sulphur

During experiment 3j sulphate concentrations decreased by 0.293 ± 0.107 mM each feed. If this amount of sulphate was reduced to sulphide approximately 4.4 ml of H_2S/feed would be produced. H_2S was collected on days 4 and 11 of experiment 3j and the results are shown in figure 3-5-10. A total of 18.0 ml H_2S and 15.65 ml H_2S were produced from 3 feeds on days 4 and 11 respectively. Based upon the amount of sulphate available from each feed a total of 13.0 ml H_2S could have been expected on each day. it is possible that the discrepancy in H_2S produced on day 4 resulted from the chemical release of H_2S from cysteine hydrochloride. H_2S was detected in the feedstock headspace in the first few days of all experiments.

3.5(5) Fibre Digestion

The starch, uronic acid and sugar composition of the dietary fibre residues in fermentor samples and feedstocks during experiment 3j are shown in table 3-5-4. An average of 0.840 mg/ml carbohydrate were consumed from each feed. This was equivalent to 1.69 g/fermentor/24 hr or 9.39 mmoles/hexose equivalents/fermentor/day.

3.5(6) Microbiology

Table 3-5-5 shows microbiological data recorded during

Table 3-5-6: Identification of bacteria in experiment 3j

		Bacteria/ml of fermentor contents
1.	Fusobacterium	
	Selenomonas	0
	Leptotrichia	
	Vibrio succinogenes	
2.	Clostridium	
	Eubacterium	9.54×10^7
3.	Bifidobacterium	
	Actinomyces	2.86×10^8
4.	Bacteroides	1.52×10^9
5.	Lactobacillus	4.77×10^8
6.	Propionibacterium	9.55×10^8

experiment 3i and 3j. The numbers of xylanolytic bacteria were somewhat lower in experiment 3j compared with experiment 3i and experiment 3a when all fibres were included. Table 3-5-6 shows the numbers of bacteria in experiment 3j assigned to the various Genera according to volatile fatty acid production and antibiotic sensitivity. The numbers of bacteria belonging to group 1 were drastically reduced compared with experiment 3a. The numbers of Bacteroides spp. Propionibacterium spp were significantly higher in experiment 3j compared with experiment 3a. The total numbers of bacteria in experiment 3j represent a cell turnover of 8.0×10^{11} cells/fermentor/day. This represents approx 1.06 g dry weight cells per day. If 3-4 g of carbohydrate are required to produce 1.0 g dry weight of cells then between 3.18 - 4.24 g carbohydrate would have been required to sustain the observed turnover. Alternatively if the dry weight of cells represents 20-30% of the carbohydrate fermented 3.53 g - 5.3 g of carbohydrate would have been digested. In fact the amount of carbohydrate digested each day was 1.69 g (section 3.5(5)).

SECTION 4

DISCUSSION

4.1 Volatile Fatty Acids

The volatile fatty acids acetate, propionate and butyrate were the major acids produced during the *in vitro* fermentation of various mixtures of dietary fibres. Branched chain VFA, which were also produced represented approximately 5% of the totals.

In most cases the total concentrations and proportions of individual VFA stabilised very quickly after inoculation and were maintained for the duration of each experiment. On the two occasions when cultures were not reinoculated following the previous experiment *ie* experiment 3c (turnip deletion) and 3h (apple deletion) the total VFA and/or the proportions of individual VFA were much more variable. During experiment 3c the total concentration of VFA and the proportion of butyrate increased and in the case of experiment 3h total VFA concentrations did not appear to be affected although the proportions of acetate increased while butyrate decreased. These results suggest a degree of 'adaptation' to the preceding feedstock requiring an extended period of 'readaptation' before a steady state was reached on a new feedstock.

The fermentor was reinoculated in order to minimise the effects of previous diet (feedstock) thereby decreasing the length of time taken to reach a new steady state and to re-establish any species which may have been washed out

during the preceding experiment. The marked effects of a previous diet on the production of VFA in faecal incubates from subjects given a range of different fibres was highlighted by Ehle et al. (1982).

Total VFA concentrations during experiments 3a - 3j ranged from 77.63 ± 2.1 mM to 102.16 ± 5.0 mM and daily production rates which are proportional to VFA levels were between 28.55 and 38.87 μmoles/ml fermentor contents/24 hr. These production rates are substantially less than those which may be inferred from human studies from which it can be estimated that between 500 - 600 mmoles of VFA are produced per day in the colon (McNeil, 1984). The addition of lactulose to a final concentration of 0.5% in the fermentor did demonstrate that the bacterial population was capable of greatly increased rates of VFA production when a readily fermentable carbohydrate was supplied. (138.24 μmoles/ml/24 hr).

Approximately 20 g of dietary fibre is consumed per day on a normal British diet although recently Cummings et al. (1986) suggested that it may be as low as 13-14 g/day. In addition the colon receives unspecified amounts of unabsorbed dietary carbohydrate and proteins and endogenous substrates. Thus on a weight per volume basis the amount of dietary fibre fed to the fermentor (3 g per 670 ml/day) was low compared with fibre intakes in vivo and, apart from amino acids added in the feedstocks, dietary fibre was the only source of carbon and energy available to bacteria.

Table 4-1-1: Results of the Mann-Whitney Tests for significance of diferences between the 9.00 am samples in each experiment.

	All fibres b	Carrot e	Carrot f	Cabbage i	Cabbage j	Turnip c	Turnip d	Apple g	Apple h
All fibres a	NS	< 0.001	NS	< 0.005	NS	< 0.05	< 0.005	NS	NS
All fibres b		< 0.01	NS	< 0.01	NS	< 0.05	< 0.05	NS	NS
Carrot e			< 0.05	< 0.01	< 0.005	NS	NS	< 0.001	<0.005
Carrot f				NS	< 0.05	NS	< 0.05	NS	NS
Cabbage i					< 0.005	NS	< 0.005	< 0.001	NS
Cabbage j						< 0.05	< 0.01	NS	NS
Turnip c							NS	< 0.01	NS
Turnip d								< 0.001	<0.01
Apple g									NS

Table 4-1-2: Results of the Mann-Whitney Tests for significance of diferences between the 1.00 pm samples in each experiment.

	All Fibres b	Carrot e	Carrot f	Cabbage i	Cabbage j	Turnip c	Turnip d	Apple g	Apple h
All fibres a	NS	< 0.001	NS	< 0.005	NS	< 0.05	< 0.005	NS	NS
All fibres b		< 0.01	NS	< 0.01	NS	< 0.05	< 0.05	NS	NS
Carrot e			< 0.05	< 0.005	< 0.005	NS	NS	< 0.001	<0.005
Carrot f				< 0.05	NS	NS	< 0.05	NS	NS
Cabbage i					< 0.005	NS	< 0.005	< 0.05	NS
Cabbage j						< 0.01	< 0.01	NS	NS
Turnip c							NS	< 0.05	NS
Turnip d								< 0.001	<0.01
Apple g									NS

Table 4-1-3: Results of the Mann-Whitney Tests for significance of diferences between the 5.00 pm samples in each experiment.

	All fibres b	Carrot e	Carrot f	Cabbage i	Cabbage j	Turnip c	Turnip d	Apple g	Apple h
All fibres a	NS	< 0.001	<0.05	< 0.05	NS	< 0.005	< 0.005	NS	NS
All fibres b		< 0.01	NS	NS	NS	< 0.05	< 0.05	NS	NS
Carrot e			< 0.05	< 0.05	< 0.005	NS	NS	< 0.001	< 0.01
Carrot f				NS	NS	< 0.05	< 0.05	NS	NS
Cabbage i				< 0.05	< 0.05	< 0.005	< 0.05	NS	
Cabbage j						< 0.01	< 0.01	NS	NS
Turnip c							NS	< 0.005	< 0.05
Turnip d								< 0.001	<0.01
Apple g									NS

Tables 4-1-1 - 4-1-3 show the results of a statistical analysis of the comparison (Mann-Whitney) between VFA concentrations at 9.00 am, 1.00 pm and 5.00 pm in all experiments. In brief, VFA levels in both turnip deletion experiments (3c and 3d), carrot deletion experiment 3e and cabbage deletion experiment 3i were significantly different from experiments 3a and 3b (all fibres). There were no significant differences between VFA levels during the all fibres experiments and the two apple deletion experiments 3 g and 3h or between all fibres and experiments 3f or 3j. The significant differences between 3e and 3f (carrot deletion) were possibly due to differences between faecal inocula.

The starch content of the dietary fibre used in experiment 3j was substantially higher than that of the previous batch probably as a result of seasonal and variety changes. This may have been responsible for the much higher VFA production rate, 39.87 μmoles/ml fermentor contents/day, in experiment 3j compared with 33.64 μmoles/ml fermentor contents/day in experiment 3i.

In some cases statistical analysis of the comparison between VFA levels at 9.00 am, 1.00 pm and 5.00 pm within the same experiment showed that there were significant differences in total VFA levels throughout the day (Wilcoxon test). When the mean production rates were calculated for periods 9.00 am - 1.00 pm, 1.00 pm - 5.00 pm and 5.00 pm - 9.00 am a steadily

increasing rate was observed. This was not always confirmed by statistical analysis probably due to the extent of variation in the total VFA levels at a given time. The mean VFA production was always highest during the overnight period when the bacteria had 16 hr to digest the dietary fibre. These results suggest that the extent of fibre digestion was determined by the retention time, complete turnover of fermentor contents was effected every 2.23 days.

When the time course of VFA production was studied no lag period was evident at any time and the rate of production was most rapid within the first 2-4 hr.

The range of proportions of the major VFA recorded throughout the experiments were acetate, 59.88% - 75.5%, propionate 11.89% - 19.2% and butyrate 8.12% - 18.99%. The proportions in the fermentor were similar to those recorded in the *in vitro* model system used by Miller and Wolin (1981). In their experiments acetate represented between 68 - 77% propionate 4-15% and butyrate 10-17%. The proportions of acetate, propionate and butyrate in the present work were similar to those recorded for faecal samples (see section 3.1(1)). Proportions of VFA in faecal samples recorded in the literature are generally approx. Acetate : propionate : butyrate 60 : 24 : 16 (Cummings, 1981; Ruppin *et al.* 1980). At present it is assumed that the proportions of VFA in faeces represent those of the colonic milieu since

VFA are thought to be absorbed by passive diffusion. As yet no difference has been found between the rates of absorption of acetate, propionate, or butyrate in man (McNeil, 1984) but there is a suggestion from studies using rats (McKay and Eastwood, 1983) and the rabbit (Marty and Vernay, 1984) that differential absorption of VFA may be a feature of mammalian systems.

There appeared to be two phases of acetate production between feeds. The first was associated with the rapid burst in fermentation immediately after a feed, the second less rapid phase of VFA production was often entirely due to increased levels of acetate. Miller and Wolin (1981) found two distinct rates of acetate production in their in vitro model. The first most rapid rate occurred for approximately 2 hr after feeding and the second slower rate continued for the remaining 10 hr of the feeding period.

In the present study the proportions of propionate were comparatively constant for any set of deletion experiments whereas an inverse relationship between the proportions of acetate and butyrate was observed in some instances eg in experiments 3c and 3d (turnip deletions). In the former, acetate and butyrate were 75.5% and 10.28% respectively and in the latter acetate represented 61.18% and butyrate 18.99% of the total VFA. Dietary manipulations of acetate and butyrate levels in vivo may be of particular consequence to the welfare of the colonic mucosa. Roediger (1980) showed

that butyrate was a preferred energy source for suspensions of isolated colonocytes and Marty *et al.* (1985) demonstrated that acetate was extensively metabolised by the mucosa of the rabbit hindgut.

4.2 Lactate, Formate, Ethanol and Methanol

Lactate did not accumulate in the fermentor during any of the experiments. Although lactate is an intermediate product of the colonic fermentation *in vivo* it does not normally accumulate except in infants. Ethanol and methanol were almost completely exhausted by the fermentation under all experimental conditions. It is thought that these alcohols are not subject to bacterial metabolism *in vivo* but are rapidly absorbed across the colonic mucosa (Cummings, 1981). However, *in vitro* studies show that the colonic population can metabolise ethanol and methanol. Members of the genus *Eubacterium* have been shown to be capable of growth using methanol (Sharak Genthner *et al.* 1981) and *Eubacteria* were amongst the predominant species in the fermentor. Ethanol is converted to a variety of higher alcohols by human faecal homogenates. The same toxic alcohols, were then shown to be rapidly absorbed across the rat colonic mucosa. (Levitt *et al.*, 1981) The authors suggest that differences in the metabolism of ethanol may play a role in determining the susceptibility to organ damage in alcoholics. Formate did not appear to be metabolised by the bacterial population. This was surprising since formate is considered to be a

rapidly utilised transient intermediate in fermentations in the intestinal tract of other mammals, eg the ruminant. Concentrations of formate in faeces are generally low between 1-2 mmol/l.

4.3 Hydrogen and Methane Gases

Hydrogen and methane gases were produced by the fermentations in all experiments. From the volumes of methane gas produced and the calculated theoretical H_2 production (see section 3-1-3) it was obvious that methanogenesis did not create a substantial demand upon available H_2. For this reason the relationship between hydrogen and methane gas production was difficult to interpret. Methane did not appear to be stimulated by hydrogen at the concentrations recorded in the present study. The lowest range of hydrogen production was recorded during experiment 3f (carrot deletion) 0.0058 - 0.03 $ml.hr^{-1}$ and the highest during experiment 3j (cabbage deletion) 0.088 - 1.34 $ml.hr^{-1}$ and these hydrogen production values coincide with the lowest and highest VFA production rates. The corresponding ranges of methane production were 0.0017 - 0.014 $ml.hr^{-1}$ and 0.00173 - 0.012 $ml.hr^{-1}$ respectively. The hydrogen production rate of 12.34 $ml.hr^{-1}$ measured during experiment 3j was 38 times higher than the highest rate recorded for experiment 3f and yet methane production in the two experiments was similar. Within a particular collection period hydrogen and methane increased or decreased in a synchronous manner. There was no evidence

of the product/precursor relationship normally seen between the two gases if H_2 is a substrate for methane formation.

In many cases the time course of peak production rates was erratic and not obviously related to feeding patterns. This suggests that the major hydrogenotrophic reaction used hydrogen as quickly as it was produced thereby dampening the response to feeding.

In contrast the successful use of the hydrogen breath test in human subjects depends upon a pronounced increase in breath hydrogen concentrations with the onset of colonic fermentation of unabsorbed carbohydrate, malabsorbed sugar or commercial preparations such as Palatinit and lactulose. Indeed several groups of workers have found a linear relationship between the amount of substrate ingested or infused into the colon and the magnitude of the breath hydrogen response (Fritz et al., 1985 and Bond and Levitt, 1972). The breath test has also been used as an indication of the extent of the digestion of certain dietary fibres preparations (Tadesse and Eastwood, 1978) and Hanson et al., 1985) and with limited success to measure intestinal transit time (La Brooy et al., 1983 and Read et al., 1985). In many breath hydrogen studies methane production is ignored as it is generally believed to be independent of the supply of exogenous substrates (Wolin and Miller, 1983a). Methane production in healthy subjects was not affected by inclusion of a variety of dietary fibres or lactulose in the

experiments described by Tadesse and Eastwood (1978) and Bond et al. (1971). In contrast McKay et al. (1981) found that methane production correlated with the dietary intake of non-cellulosic polysaccharide pentose, lignin and with the administration of D(+) xylose and L(+) arabinose. Methane production was higher on xylan and pectin diets than on cellulose, corn bran or a fibre free diet (Marthinsen and Fleming, 1981).

In the present studies the highest rate of methane production was found in experiments 3d and 3h during which the percentage fermentation of xylose and arabinose was substantially higher than in any other experiment see (table 4-1-6). Hydrogen and methane production was markedly increased when lactulose was added to the fermentor (see section 3.1(9)) H_2 production rose to 8.05 $ml.hr^{-1}$ and methane production to 1.062 $ml.hr^{-1}$ in the first two hours after the addition. The rapid and dramatic response of both hydrogen and methane to the inclusion of a readily fermentable substrate may suggest that when the partial pressure of hydrogen rises above a certain threshold value methane production becomes significant.

Alternatively, methanogenesis may have been stimulated by increased concentrations of bacterial metabolite(s) other than H_2.

As mentioned earlier methane formation from hydrogen and

carbon dioxide could not account for the disappearance of the large amounts of hydrogen which it was estimated, on the basis of volatile fatty acid production, could have been formed. The utilisation of H_2 and CO_2 during homoacetic acid fermentation is postulated to account for the theoretical deficit in hydrogen apparent with feeding each type of feedstock (see table 4-1-6). In methanogenic individuals methane production from H_2 and CO_2 is generally assumed to have a central role in the balance between hydrogen produced and hydrogen consumed (Wolin and Miller, 1983a). Epidemiological studies reveal that approximately one third of individuals produce detectable amounts of breath methane. Recently McKay et al. (1985) have suggested that 'all healthy subjects may produce methane but only when the production reaches a threshold does it appear in the breath.' Homoacetic acid fermentation in the fermentor may have maintained H_2 concentrations below the 'threshold' levels which are necessary for significant methane production.

4.4 Nitrogen

A net production of ammonia was measured in the fermentor throughout the experiments which could not be accounted for by hydrolysis of urea supplied in the feedstocks. The production ranged from 43.6 ppm per feed to 107 ppm per feed. Evidence was available to suggest that ammonia was derived from the fermentation of amino acids and/or proteins supplied in the feedstocks. Branched chain VFA which are common

products of amino acid fermentation were produced at constant levels throughout the experiments and growth in anaerobic dilution solution indicated that amino acid utilising species were amongst the predominant organisms present. Further, theoretical calculation of the amount of VFA produced from the fermentation of dietary fibre was always less than the actual amount of VFA measured suggesting that a variable proportion of VFA was derived from amino acids.

Ammonia may also be produced during nitrate reduction. It has been suggested that the proportion of nitrate reduced to ammonia increases as the concentration of nitrate decreases and as the need for an effective electron sink increases (King and Nedwell, 1985 and Tiedge et al., 1982). The hypothesis is based on the fact that reduction of nitrate to ammonia is more effective in consuming reducing equivalents than partial reduction to nitrite or gaseous products. There was no evidence of ammonia being derived from nitrate in the present work.

All the evidence points to the use of nitrate in dissimilatory nitrate reduction to N_2 gas. The findings of Samuelson (1985) suggest that in Pseudomonas putrefaciens the redox potential of the growth medium influences the end product of nitrate reduction. Nitrate is reduced to ammonia if the redox potential is high and to nitrogen gas in media of low redox potential.

Anaerobic bacteria may use amino acids either as a source of nitrogen (Maczulak et al., 1985) or as carbon and energy sources (McFarlane et al., 1986; Nagase and Matsuo, 1982; Nanninga and Gottschal, 1985). Under conditions of energy limitation and adequate ammonia supplies, such as existed in the fermentor during these experiments, it is reasonable to assume that amino acids were metabolised primarily to obtain sources of carbon and energy. Ammonia concentrations at any given time reflected consumption by bacteria (nitrogen source) and production as a result of fermentation and the balance may depend upon such factors as the availability of fermentable carbohydrate. Ammonia concentrations decrease in faecal incubation systems when an energy source such as lactulose is added (Vince and Burridge, 1980). The fermentor population in the present studies was undoubtedly energy limited and therefore, under these circumstances it is reasonable to expect amino acid and carbohydrate fermentation to be taking place simultaneously. The toxicological effects of high ammonia concentrations are reviewed by Rowland et al., (1986).

The evidence collected in the present study was consistent with the reduction of nitrate to nitrogen gas. Nitrite never accumulated above 3 ppm except when nitrate concentrations were increased during the nitrate experiment (see section 3-1-8). The appearance of N_2 accounted for 95% or more of the nitrate reduced. There are no figures available in the literature which give average daily intakes of nitrate. Nitrate is present in many fruits and vegetables and in some

areas nitrate in water supplies is above normal levels. Only a fraction of the ingested nitrate reaches the colon as it is efficiently absorbed in the upper intestinal tract. The small amount which does reach the ileocaecal valve is subsequently available to the colonic bacteria and implicated in the formation of nitrosamines and other potentially toxic metabolites. (See review by Rowland et al., 1986).

During the nitrate experiments nitrite accumulated to maximum levels of between 8 and 32 ppm (see figures 3-1-10 - 3-1-13). Nitrite and nitrate patterns show a typical product-precursor relationship. It appears that the faecal microflora in vitro metabolise high concentrations of nitrate and that nitrite accumulates proportionately. Relatively high nitrite concentrations appear after a delay and are transient, supporting the view that it was an intermediate of dissimilatory nitrate reduction. The production of nitrous and nitric oxides was not investigated. The magnitude of the increase and decrease in ammonia levels during the nitrate experiments suggests that it was a reflection of the changing balance between production and consumption as dictated by the population growth kinetics.

Nitrogen represents the largest proportion of colonic gas. The origin of colonic N_2 is generally believed to be either swallowed air and/or N_2 which has diffused into the colon from the blood supply (Levitt et al., 1981). The results of the present in vitro study suggests that the colonic

microflora are capable of producing N_2 in quantities proportional to the concentration of nitrate.

4.5 Sulphur

Sulphate was supplied in the feedstocks at constant levels and was metabolised by the bacterial population during all experiments.

Sulphate is available to the colonic bacteria in the form of dietary and endogenous sulphated mucins eg chondroitin sulphate, sulphated bile acids and inorganic dietary sources. Bacteria capable of degrading these compounds have been studied by Salyers *et al.* (1977b) and Salyers (1983) and faecal sulphatase activity was measured by Rhodes *et al.* (1985). The colon is thought to be impermeable to sulphate and this is the rationale of including sulphate in ionic fluid absorption studies (Ruppin *et al.*, 1980). Magnesium sulphate is prescribed for its cathartic effects which result from massive secretion of fluid in response to the presence of an unabsorbed ionic compound.

The product of dissimilatory sulphate reduction at physiological pH is hydrogen sulphide gas. Quantitative analysis of the amounts of sulphate reduced in the fermentor were used to predict the volume of H_2S produced and sulphate reduced was generally consistent with dissimilatory sulphate reduction. The production of H_2S was occasionally higher

than expected probably due to the evolution of H_2S from sulphur-containing amino acids in the feedstocks. Hydrogen consumption due to sulphate reduction was included in the calculations of the overall fermentation balance. Hydrogen sulphide gas is known to be extremely toxic. The maximum safe atmospheric level for repeated exposure is 10 ppm. If the colonic microflora were exposed to increased levels of sulphate, the evidence from the in vitro studies in the present work suggests that toxic levels of H_2S could be produced.

Obligate and facultative sulphate-reducing bacteria were isolated from human faeces by Bereens and Romond (1977). The number of sulphate-reducing bacteria was approximately 10^7/g wet weight faeces. Desulfovibrio desulfuricans was a predominant species. Rumen strains of this organism can use nitrate as an alternative terminal electron acceptor. Dissimilatory nitrate reduction is not a feature of most sulphate-reducing bacteria and may reflect a particular adaptation to an environment in which the supply of sulphate is erratic. In the present study sulphate reduction was completely inhibited by nitrate reduction at the levels recorded during the nitrate experiments. The nature of the inhibition could not be concluded from the information available. Inhibition due to nitrite would appear unlikely given that sulphate was completely inhibited when nitrite concentrations were only 4 ppm above normal levels and none of the other metabolic functions investigated, eg VFA production and fibre digestion, were affected.

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Table 4-1-4: Composition of dietary fibre ($g.l^{-1}$) in the feedstocks.

CARBOHYDRATE g/l

	All Fibres (Expt. 3b)	Turnip Deletion (Expt. 3d)	Cabbage Deletion (Expt. 3h)	Carrot Deletion (Expt. 3f)	Apple Deletion (Expt. 3g)
ARABINOSE	0.82	0.92	0.86	0.84	0.75
XYLOSE	0.89	1.54	0.87	0.90	0.88
MANNOSE	0.19	0.20	0.20	0.20	0.17
GALACTOSE	0.66	0.54	0.72	0.47	0.64
GLUCOSE	2.42	1.89	2.05	1.92	2.10
URONIC ACIDS	1.15	1.15	1.25	1.26	1.15
STARCH	1.71	1.52	1.72	1.43	1.54

Table 4-1-5: Starch, Uronic acid and sugars $mg.ml^{-1}$ metabolised between each feed period

	ARABINOSE	XYLOSE	MANNOSE	GALACTOSE	GLUCOSE	URONIC ACIDS	STARCH
All fibres Experiment 3a	0.070	0.076	0.024	0.087	0.301	0.153	0.203
Turnip deletion Experiment 3d	0.069	0.168	0.026	0.066	0.244	0.146	0.160
Carrot deletion Experiment 3f	0.034	0.072	0.025	0.056	0.244	0.164	0.182
Cabbage deletion Experiment 3j	0.045	0.058	0.025	0.094	0.264	0.145	0.209
Apple deletion Experiment 3h	0.073	0.091	0.021	0.085	0.272	0.150	0.201

Table 4-1-6: Percentage starch, uronic acids and sugars metabolised between each feed period

	ARABINOSE	XYLOSE	MANNOSE	GALACTOSE	GLUCOSE	URONIC ACIDS	STARCH
All fibres Experiment 3a	18.05	18.35	67.01	76.24	55.84	78.41	46.90
Turnip deletion Experiment 3d	14.39	34.53	61.55	50.87	67.18	61.21	30.72
Carrot deletion Experiment 3f	5.41	16.35	61.16	46.18	61.76	69.44	63.69
Cabbage deletion Experiment 3j	7.84	11.28	65.97	72.79	66.36	41.96	50.50
Apple deletion Experiment 3h	25.34	28.98	48.42	80.90	76.78	72.53	71.53

4.6 Dietary Fibre

On a weight per volume basis the fermentor received a concentration of fibre (3 g/day) below the average UK daily intake of 20 g/day.

From table 4-1-4 it can be seen that the composition of the dietary fibres in each feedstock was very similar. The composition of dietary fibre preparations compared well with those of Southgate *et al.* (1978) and Nyman *et al.* (1986). The actual amounts of individual sugars, uronic acids and starch fermented between each feed period is shown in table 4-1-5. The proportion of the available sugars, starch and uronic acids which were fermented during the experiments is shown in table 4-1-6. From the data in table 4-1-6 it is obvious that while the composition of each feedstock is similar the percentages of the different components of dietary fibre which were fermented depend upon the mixture in which they were supplied. The physical characteristics of the mixture may have a direct effect on digestibility or the changes in the extent of the fermentation of individual components may be mediated by changes in the colonic microflora or their metabolic response to the different mixtures. The lowest percentage fermentation results were recorded for arabinose and xylose. Approximately 50% of the pentoses present in the feedstocks were contained in bran which is generally poorly digested. Bran was the most abundant fibre observed in fermentor effluent. Southgate *et*

Table 4-1-7: Theoretical fermentation balance for experiments 3a, 3d, 3f, 3h and 3j.

	(1) Hexose equivalents mmoles/fermentor/day	(2) Theoretical VFA production mmoles/fermentor/day	(3) Theoretical production of acetate from H_2/CO_2 mmoles/fermentor/day	(4) Sum of (2) + (3) mmoles/fermentor/day	(5) Measured VFA production mmoles/fermentor/day
All fibres Experiment 3a	10.22	18.023	4.82	22.85	24.8
Turnip deletion Experiment 3d	9.81	16.388	3.46	19.85	20.17
Carrot deletion Experiment 3f	8.67	15.013	3.83	18.48	24.44
Cabbage deletion Experiment 3j	10.03	18.38	4.01	22.39	24.34
Apple deletion Experiment 3h	9.39	17.29	6.55	23.84	26.71

al. (1978) found a correlation between the pentose content of fibre and faecal bulking in human subjects. The results for glucose, which was mainly derived from cellulose, indicate that cellulose was extensively metabolised in the fermentor. The percentage degradation was between 35.84 and 76.78%. Since bran is only poorly digested most of the glucose probably came from the fruit and vegetable cellulose. The amounts of carbohydrate fermented were sufficient to sustain the observed turnover in bacteria in all experiments except 3f (carrot deletion) and 3j (cabbage deletion).

Table 4-1-7 shows the overall balance between carbohydrate fermented and VFA produced including the theoretical production of acetate from H_2 and CO_2. The theoretical yield of VFA calculated from the carbohydrate fermented was very close to VFA actually measured in all experiments except 3f (carrot deletion) and 3j (cabbage deletion). These results suggest that carbohydrate furnished most of the carbon and energy requirements of the bacteria during the fermentations. Between 1 and 6 mmoles VFA/fermentor/day could not be accounted for by the theoretical amounts of VFA produced from the amount of hexose fermented. It is likely that amino acids to a greater or lesser extent furnished the carbon and energy required to account for the 'gap' (1 - 6 mmoles) between carbohydrate fermented and VFA produced.

If amino acids were being fermented it would be reasonable to

assume that the concentration of ammonia would increase. However, ammonia concentrations during experiments 3f and 3j were not unusually high. In fact the levels of ammonia during experiment 3f were 50% lower than normal.

4.7 Microbiology

The numbers of bacteria present in the fermentor during the experiments was remarkably constant and close to the bacterial counts recorded by Wolin and Miller (1981). There is no clear cut evidence which demonstrates diet-induced changes in the microflora except in the most severe circumstances (Hentges, 1980). From the literature it seems more likely that the colonic bacterial population as a whole is capable of considerable metabolic diversity which accounts for the lack of variation in total numbers and species. Cummings (1983) estimated the numbers of bacteria to be between 10^{11} - 10^{12}/g dry weight of colonic contents. Between 1.49 $\pm$ 0.57 - 4.41 $\pm$ 1.36 x 10^{9} bacteria were produced per ml of fermentor contents during the experiments. These values would represent bacterial numbers between 1.5 - 5 x 10^{10}/g dry weight of fermentor contents.

The numbers of xylanolytic bacteria were between 5.0 $\pm$ 1.0 x 10^{6} bacteria/ml and 4.4 $\pm$ 2.73 x 10^{7} bacteria/ml, pectinolytic bacteria were 1.52 $\pm$ 0.5 x 10^{8}/ml - 9.7 $\pm$ 4.01 x 10^{8}/ml. There was no obvious correlation between the numbers of pectinolytic, xylanolytic and starch degrading bacteria

and the amount of uronic acids, arabinose, xylose and starch fermented. This would tend to indicate that despite changes in the amounts of these components fermented (see table 4-1-6) the numbers of bacteria responsible remained unchanged. Many of the predominant species were able to use combinations of all three polymers (xylan, pectin and starch). Those which were exclusively pectinolytic; xylanolytic or starch digesting were in the minority. (See section 3.1(7)). The number of aerobic (facultatively anaerobic) organisms was similar in all experiments and members of the Enterobacteriacea were always predominant within the group. The numbers of bacteria tentatively identified as belonging to group 1 was similar in all experiments except 3j when numbers dropped dramatically. Bacteroides spp. and Propionibacteria spp. were always the most numerous except in experiment 3 h when the numbers of Propionibacteria were low. The numbers of faecal isolates belonging to the major genera were approximately equal. There appeared to be no major differences in the numbers of bacteria belonging to each of the 6 groups. Although the antibiotic method employed gave a good impression of the predominant organisms present it was not found to be a satisfactory means of identifying organisms from such a diverse range as was present in the fermentor. The method was improved by the inclusion of volatile fatty acid analysis in the identification. Amino acid fermenting bacteria were always detected in the highest dilution of fermentor contents indicating that the predominant species were capable of amino

acid fermentation. Macfarlane *et al.* (1986) found that members of the genera *Bacteroides* and *Propionibacterium* were the predominant proteolytic species in faecal samples.

Cabbage dietary fibre liquid medium was the only medium in which cellulolytic activity was observed. Ball-milled filter paper preparations were successfully used to isolate human faecal cellulolytic bacteria (Betian *et al.*, 1977 and Bryant, 1978) but the cellulolytic species present in faeces and in the fermentor were incapable of growth on any of the media containing filter paper as the source of cellulose.

Acetate was the major product in all minimal media incubated under an atmosphere of H_2:CO_2.80%:20%. These results support the postulate that homoacetogenic fermentation was a major hydrogenotrophic reaction in the fermentor and that the organisms are capable of using extracellular H_2. In this capacity homoacetogenic bacteria could compete with methanogenesis for transferred hydrogen.

In conclusion the experimental evidence collected from these studies suggests that the *in vitro* system provided a reasonable model of certain aspects of the colonic fermentation of mixtures of dietary fibres. In most cases a steady state fermentation was reached within the first two days after inoculation and the numbers and types of bacteria and their metabolic end-products were similar to those observed *in vivo*.

In future the model may be used to try to reproduce the effects on colonic microbial fermentations of certain malabsorption syndromes or of unabsorbed nutrients such as 'resistant starch'. It may also be useful to study the effects of different concentrations of terminal electron acceptors eg sulphate using faeces from methanogenic and non-methanogenic individuals.

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