material was associated with strongly mucinolytic rabbit isolates (Table 8). Salyers et al. (1978) using traditional fermentation studies did not detect acid production by strains of gastrointestinal tract bacteroides from type-culture collections when they were grown on mucin substrates. Roberton and Stanley (1982) also found minimal utilization of mucin by two reference cultures of *Bacteroides fragilis* using gas-liquid chromatography to monitor changes in mucin composition. However, it was subsequently found that isolates cultured directly from the pig cecum were more actively mucinolytic than the reference strains used previously. (Roberton, pers. comm.).

The other techniques used to detect changes in the mucin molecule after digestion have been used elsewhere. Loss of terminal structures was clearly shown by restoration of anti-A antibody titre after absorption with digested mucins (Table 9). Hoskins and Boulding (1976) argued that microbial degradation of mucin would probably start at the non-reducing terminals of the carbohydrate side chains, and used this to assess degradation by mixed fecal populations. Roberton and Stanley (1982) estimated that a 15% loss of sugars from pig gastric mucin would indicate the removal of merely one or two sugars from the end of each chain on the molecule. Gas-liquid chromatography of carbohydrates was equally successful in demonstrating alterations in mucin composition after microbial digestion (Figure 13). Changes in molar ratios of hexose components showed that the two mucins tested were degraded slightly differently. The rabbit small intestine mucin lost more fucose than pig gastric mucin and somewhat less galactose and N-acetylgalactosamine (Table 10). Roberton and Stanley (1982), from similar studies with pig colonic mucin, found that N-acetylgalactosamine was affected most by digestion with ATCC strains of *Bacteroides fragilis*.

This investigation, using different analytical techniques, has shown that bacteria isolated from rabbit cecal mucosa and lumen contents actively

digest mucins by removal of hexoses from carbohydrate side chains of the mucin molecule *in vitro*. The two mucins used as experimental substrates differed in their chemical composition and relative digestibility. Although rabbit small intestine mucin was more resistant to bacterial degradation than pig gastric mucin, the reason is not obvious from this study, but probably lies in the structure of the mucin molecule itself, as proposed by Forstner (1973). An important ecological implication of these findings is based on the increase in potential mucinolytic activity as the digesta approaches the ileo-cecal valve, together with the greater viscosity of upper digestive tract mucins. The rheological properties that protect the GIT epithelia against acidity in the stomach and trauma in the small intestines also seems to be associated with resistance to bacterial degradation in the lower part of the tract.

Most of the isolates from the rabbit cecum were presumptively identified by tests that included fermentation reactions with different carbohydrates and many were metabolically active on a wide range of substrates (Table11). Galactose was utilized by nearly all strains and most other monosaccharides were fermented by saccharolytic strains. The exceptions were fucose, used mainly by those strains degrading mucin, and the hexosamines, which were fermented by very few of the isolates studied. These sugars are not normally included in taxonomic profiles, but Salyers et al. (1977) reported that utilization of L-fucose was a variable feature of human colonic isolates from different genera, and was fermented by almost all strains of *Peptostreptococcus* and *Bacteroides* that were examined. These findings imply that utilization of monosaccharides likely to be released from mucus as the result of bacterial degradation is not confined to the degradative microorganisms, but can be used by most other bacteria in the environment. The ultimate disposal of the hexosamines, however remains unclear, as few organisms isolated in the present study could use these sugars as fermentable energy sources. The enzyme N-acetylglucosaminidase may have an important function in

synthesis or depolymerization of the peptidoglycan component of bacterial cell walls in cell division. Its presence outside cells and thereby freed of intracellular constraints maybe a significant factor in cell lysis of bacteria in the GIT micro-environment.

None of the bacterial isolates studied in this investigation could completely degrade mucin. Lindstedt et al. (1965) and Hoskins and Boulding (1981) showed that this could only be achieved by a mixed conventional fecal flora acting in consortia. The APIZYM test system showed that of all the isolates tested, those of the genus *Bacteroides* had the broadest spectrum of cell-bound glycosidases, amongst which were the enzymes most probably involed in mucin degradation (Table12). Several workers have shown that the genus contains closely related species that readily degrade a wide range of polysaccharides from different sources. The work of Bayliss & Houston (1984) and Salyers et al. (1978) demonstrates that many plant polymers are attacked and partially degraded by different species of *Bacteroides*. In the present study it was found that all strains of the *Bacteroides vulgatus - Bacteroides ovatus - Bacteroides distasonis* group contained a broad spectrum of glycosidases detected by the APIZYM system, and could be distinguished from the results of unrelated *Bacteroides* species with reasonable confidence. The pattern was clearly different from reactions obtained with other genera such as *Fusobacterium*. It has been suggested that the cell-bound enzymes detected by the APIZYM test may be used as a taxomonic system for general identification of bacteria, and it seems to be particularly suitable for the genus *Bacteroides* (Laughon et al., 1982; Knivett et al., 1983; Thragonnet et al., 1977).

The APIZYM system relies on the release of α-naphthol from synthetic glycosides to form coloured formazans with Fast blue salts. The p-nitrophenol assay used to estimate levels of enzymes in the current study, also detects enzyme activity by release of chromogen from syn-

thetic glycosides but although the technique has been in use for over half a century, there is some doubt whether the artificial substrates ideally model the reactions with natural glycosides. An α-L-fucosidase from *Clostridium perfringens* for example, was reported by Aminoff & Furukawa (1970) to have no effect on p-nitrophenol fucosides. In contrast, Hoskins & Boulding (1981) verified that β-galactosidase and N-acetylglucosaminidase from mixed fecal cultures showed good correlation between p-nitrophenol glycosidase activities and the release of monosaccharides from a defined glycoprotein. Results of inhibition studies confirm that the p-nitrophenol glycosides were suitable for use as substrates for the enzymes investigated in this study. Table 13 shows the specific activities of glycosidases when thay were incubated with substrates in the presence of different carbohydrates. The monosaccharides galactose and fucose were included to study the effect of probable end products on enzyme activity. Inhibition of 28% by 10 mM galactose on β-galactosidase and 75% inhibition of 20 mM fucose on α-L-fucosidase are in keeping with well documented product inhibition of bacterial glycosidases (Priest, 1977; Yem & wu, 1976). Cross-reactivity between these two enzymes, shown by 17% inhibition of galactose by fucose has also been reported previously for the glycosidases of *Bacteroides vulgatus* by Berg et al. (1980). The present study on the enzymes of a similar species of *Bacteroides* also revealed that the disaccharide lactose inhibited both galactosidase and fucosidase by over 20% when added at a concentration of 10 mM. The report by Cowan et.al.,(1984) of similar inhibition of a bacterial β-galactosidase by 10 mM lactose supports not only these findings, but also the indication that disaccharides may be inhibitory for glycosidase activity. Moderate inhibition of fucosidase by the polysaccharide component of sub-unit mucin observed in this study also suggests that intact glycosides can affect relevant enzyme activity, although it is equally possible that mild hydrolysis during the incubation period could release inhibitory monosaccharides. An explanation for the finding that some strains of

Bacteroides vulgatus were more active in degrading mucin, derives from a comparison of the amounts of different glycosidases produced by mucinolytic and non-mucinolytic strains. The mucin-degrading strains produced higher levels of cell-bound glycosidases and excreted them into the culture medium in late logarithmic growth by an active process rather than by cell lysis (Figure 16). The effect was particularly notable with N-acetylglucosaminidase and fucosidase and was enhanced when the organisms were grown on lactose as a substrate. Sub-unit mucin itself induced only slight increases in enzyme levels of both mucinolytic and non-mucinolytic isolates as did galactose and glucose. Constitutive baseline levels of glycosidases seem to be present in all strains of *Bacteroides vulgatus* isolated from the rabbit cecum , but in some cases, production of the enzymes, or slightly different isoenzymes, is increased in the presence of inducing polysaccharides and are ultimately excreted into the extracellular milieu (Figure 18).

Induction and export of elevated levels of enzymes have been well documented in the genus *Bacillus* and in some Gram negative aerobes typified by *Erwinia* (Priest, 1977; 1984). Several reports on the glycosidases of anaerobes have dealt with the enzymes found in *Bacteroides* species. Williams & Whithers (1982), studied a range of enzymes in *Bacteroides ruminicola*. The report by Salyers et al. (1977) was confined to β-glucanase of *Bacteroides fragilis* and Reddy et al. (1984) examined the hemicellulases of *Bacteroides ovatus*. Most of these reports were concerned with the enzymes associated with washed cells although some indications are given that activity was detected in culture supernatants. The reports all agree that the glycosidases of *Bacteroides* are substrate induceable, often in a non-specific manner and reach maximum levels in late logarithmic growth. Hoskins & Boulding (1981), in studies of the glycosidases in mixed human fecal cultures conclude that the enzymes involved in polysaccharide degradation are inducable and extracellular.

Berg et al. (1978) studied the glycosidases of several strains of *Bacteroides fragilis* and reported that one of them (B70) produced exceptionally high levels of a range of extracellular enzymes during growth on glucose. In a later report, details are given of purification of the glycosidases from stationary phase cultures of strain B70, which is now known as *B. vulgatus* The spectrum of glycoside hydrolases produced by mucinolytic strains of *Bacteroides vulgatus*, W12 and W13, investigated in the current study, was similar to the wide range of enzymes produced by strain B70. Furthermore, their levels were enhanced when the strains were cultured in media containing lactose rather than glucose (Figure 18). The list includes α- and β-galactosidase, α-glucosidase, N-acetylglucosaminidase, N-acetylgalactosaminidase and α-L-fucosidase (Table 12, Figure 18). Many of these enzymes may be relatively non-specific, some exhibiting cross reactivity with several substrates in different linkages (Berg et al., 1980; Cabezas et al, 1983). By virtue of these enzymes therefore, strains of *Bacteroides vulgatus* are capable of utilizing a range of substrates of different carbohydrate structures. Berg et al. (1980) even go as far to suggest that some strains of the species might be involved in cellulolytic activity. Because of the unique properties of the Gram-negative cell wall, Priest (1984) proposed that in this group of bacteria, the enzymes destined for export should be synthesised in the periplasmic space. Berg (1981) found this to be true for *Bacteroides vulgatus* in that most of the glycosidases produced by this species are associated with either the cell envelope or with the membrane. Forsberg et al. (1981) provide interesting ultrastructural evidence that the extra-cellular glycosidases of *Bacteroides succinogenes* are associated with membrane-bound vesicles released from the cells during growth. Although a search for similar vesicles was not made in studies of *B. vulgatus*, the vesicles seen in micrographs of the rabbit cecal epithelium, bear a striking resemblance to those illustrated by Forsberg et al. and interpretation of Figure 7 is approached with caution until more is known about the export of enzymes by *Bacteroides*.

The results of this investigation suggest that events leading to degradation of polysaccharides in the rabbit cecum may be initiated with the release of broad-spectrum glycosidases by export or lysis of bacteria similar to *Bacteroides vulgatus* As bacterial glycosidases are almost exclusively exoenzymes, sugars would be cleaved from the ends of polymer chains to release fermentable substrates for bacteria in the vicinity which would move into logarithmic growth. Intracellular levels of glycosidases and other enzymes might accumulate and the growth period would last as long as substrates were available. When these become exhausted locally, the cells would re-enter stationary phase and export more enzymes for the degradative process. Thus, the presence of a steady flow of mucins into the cecal environment ensures stability for the polysaccharide degrading bacteria upon which the rest of the cecal microflora depends. The bacteria involved in this process in the rabbit cecum have been investigated in the current study but the principle probably applies to the large bowels of other animals except that different species of bacteria are involved in the process of polysaccharide degradation.

CONCLUSIONS

Species diversity of the bacterial microflora in the rabbit cecum is similar to that found in the ceca of other animals. It is predominated by over 19 different cultivable species inhabiting either the lumen contents or mucosal blanket. Although species identification in this study was based only on phenotypic criteria, it was shown that population distributions in the two habitats are slightly different, for although some species were found throughout the cecum, others were confined to one habitat or the other. Bacteria colonizing the mucosa are likely to be limited to the availability of energy sources within the mucous gel, mainly endogenous cecal mucin, and several species were isolated that could degrade both pig gastric mucin and rabbit intestinal mucin. The microorganisms inhabiting the cecal digesta, however, have a far wider range of substrates available, mainly dietary polysaccharides and other polymers, dead biomass and upper digestive tract mucins. Rabbit small intestine mucin was more resistant to disulphide-bond reduction and to digestion by bacteria, than pig gastric mucin, and reaches the cecum almost intact. Bacteria that digest mucins have a broad range of glycoside hydrolases, some of which are found as extracellular enzymes. As approximately 17 % of strains from the rabbit cecum were able to digest mucin to a limited extent, it is concluded that mucin constitutes an important energy source for cecal bacteria, an alternative substrate in lieu of dietary polysaccharides and a stabilizing factor for the bacterial populations inhabiting this complex ecosystem.

CHAPTER FIVE

APPENDICES

Appendix 1. Preparation of pre-reduced culture media

Nutrients, Eh indicator, salts solution and growth factors were made up in the required volume of distilled water and boiled until indicator turned green. The solution was then cooled under a stream of 12% oxygen-free carbon dioxide in nitrogen, fed through flowmeters and de-oxycatalyst (Afrox Ltd.).

Autoclavable sugars, neutral volatile fatty acid solution, 0.05% cysteine, and 0.1% sodium bicarbonate were added and gas bubbled through until the pH stabilized. This was then corrected to pH 6.5 with M sodium hydroxide.

The prereduced, colourless medium was dispensed into gassed screw-capped containers each with a new rubber liner. Different media were identified by colour codes applied to caps with model enamels. Preweighed agar was added to bottles for media intended for slants or plates. All media were autoclaved for 15 min at 121°C and stored in the dark for a maximum of two weeks.

Solutions of additives that could not be autoclaved were added to media through a 0.45µ filter unit within the anaerobic cabinet before dispensing into petri dishes or sterile bottles.

Appendix 2. Modified M10 medium (after Caldwell and Bryant, 1966)

Yeast extract (Merck)	0.05g
Peptone (Merck)	0.1g
Tryptone (Merck)	0.1g
Hemin (Sigma)	0.1ml
Vitamin K (Sigma)	0.02ml
Indigo carmine (BDH) 0.05%	1.0ml
Salts solution A	7.5ml
Salts solution B	7.5ml
Cysteine hydrochloride (BDH)	0.05g
Sodium bicarbonate	0.05g
VFA mixture	2.4ml
Distilled water to make	100ml

This was prepared as described under pre-reduced media, correcting pH to 6.5. Carbohydrates were added as described in the text.

Appendix 3. Salts solutions

Solution A

Sodium chloride	6.0g
Potassium dihydrogen phosphate	3.0g
Ammonium sulphate	6.0g
Magnesium sulphate	1.2g
Calcium chloride	0.6g
Water to	500ml

Solution B

Dipotassium hydrogen phosphate	3.0g
Water to	500ml

Ingredients were dissolved in the order given. 7.5ml of each solution were added in 100ml of all media. Turbidity after autoclaving re-dissolved when cooled to room temperature.

Appendix 4. Anaerobic diluting fluid (ADF)

(after Holdeman et.al., 1977)

Salts solution A	7.5ml
Salts solution B	7.5ml
Indigo carmine; 0.05%	1.0ml
Sodium carbonate	0.63g
Water to make	100ml

The pH was brought to 6.5 and the solution autoclaved in sealed bottles.

Appendix 5. Volatile fatty acid solution

(Dr. A. Kistner, pers.comm.)

Acetic acid	10ml
Propionic acid	6ml
Butyric acid	4ml
Valeric acid	1ml
α-methylbutyric acid	1ml

10M NaOH was added very slowly to acids in a cooled flask until the pH was 7.0 and stored at 4°C. The solution was odourless and did not affect the pH when added to culture media.

Appendix 6. Pre-reduced Brain Heart Infusion medium (after Holdeman et al., 1977).

Brain Heart Infusion (Oxoid)	3,7g
Yeast extract (Oxoid)	0.5g
Indigo carmine, 0.5%	1.0ml
Cysteine	0.05g
Vitimin K soln.	0.02ml
Haem soln.	1.0ml
Salts soln. A	7.5ml
Salts soln. B	7.5ml
Water to	100ml
Agar	1.5g

This was made up as described in appendix 1, bringing the pH to 6.5 before sterilization.

Appendix 7. PY basal medium

(after Holdeman et al., 1977).

Peptone (Difco)	0.5g
Tryptone (Difco)	0.5g
Yeast extract (Oxoid)	1.0g
Salts solution A	7.5ml
Salts solution B	7.5ml
Hemin (VPI)	1.0ml
Vitamin K (VPI)	0.02ml
Sodium bicarbonate	0.05g
Cysteine (BDH)	0.05g
Distilled water to make	100ml

Medium was prepared as described under pre-reduced media and sugars added as required, either before autoclaving or through 0.45 µm filters into sterile media.