Table 13. Inhibition of bacterial glycosidase activity.

		Inhibitor				
		blank	Galactose	Fucose	Lactose	Mucin
			10	20	10	8
			mM	mM	mM	mg/ml
Enzyme		Enzyme activity, (nKat/mg) & inhibition (%)				
Fucosidase	(W11)	.98	.85(15)	.25(75)	.75(24)	.72(27)
Galactosidase	(W22)	.72	.52(28)	.60(17)	.57(21)	.73
Hexosaminidase	(W11)	.82	.79	.80	.83	.79

Inhibition was tested on the enzyme activity of washed cells of enzyme-producing *Bacteroides vulgatus*-like strains. Inhibitors were included in the standard enzyme assay in place of the buffer volume to give different final concentrations. The A_{410} of the standard tests (blanks) are compared to the A_{410} of reactions in the presence of inhibitory concentrations of carbohydrates.

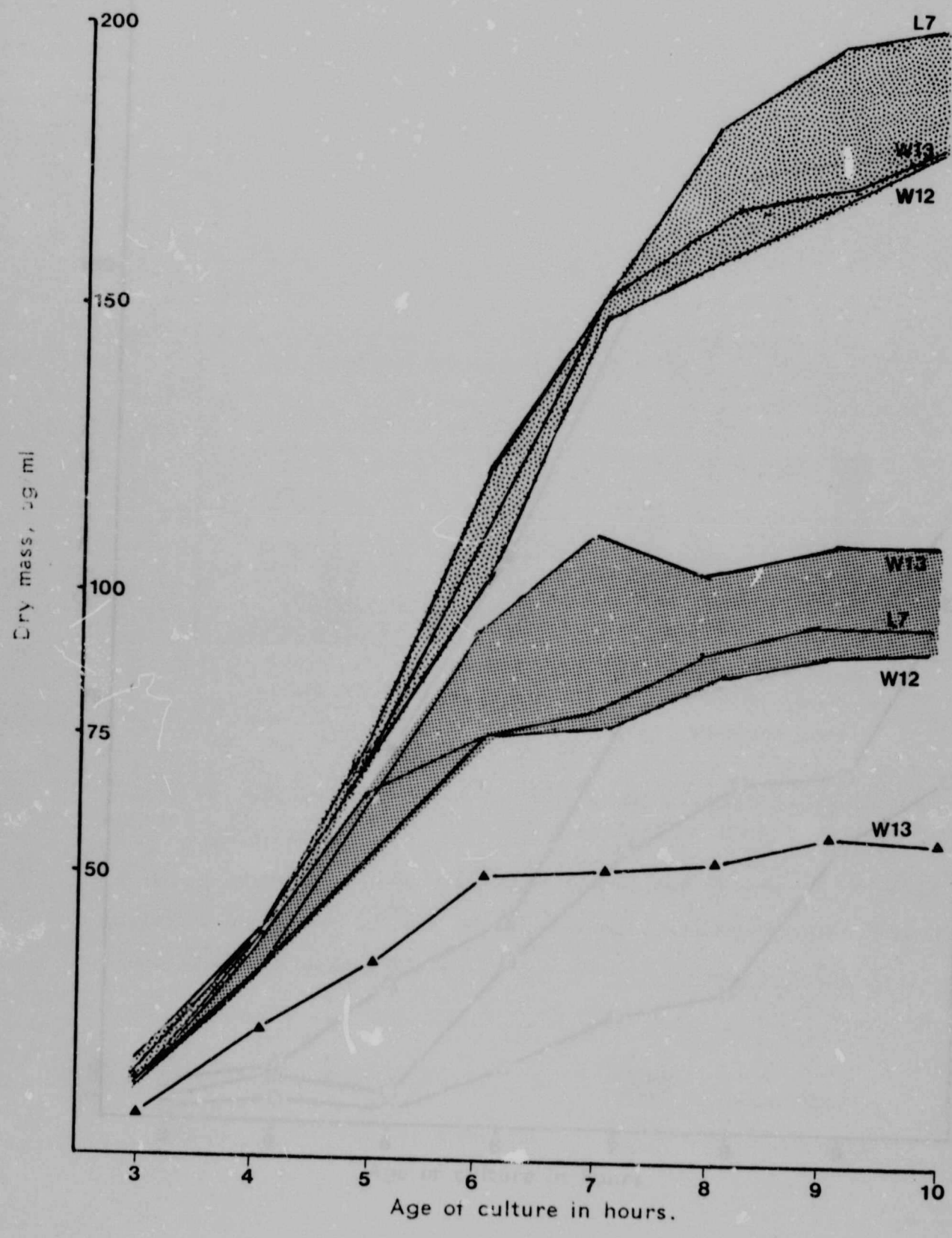

Figure 15. Growth curves of *Bacteroides vulgatus* strains W12, W13 and L7 grown on 1% galactose and 1% fucose Growth of W13 on 1% N-acetylglucosamine ▲——▲

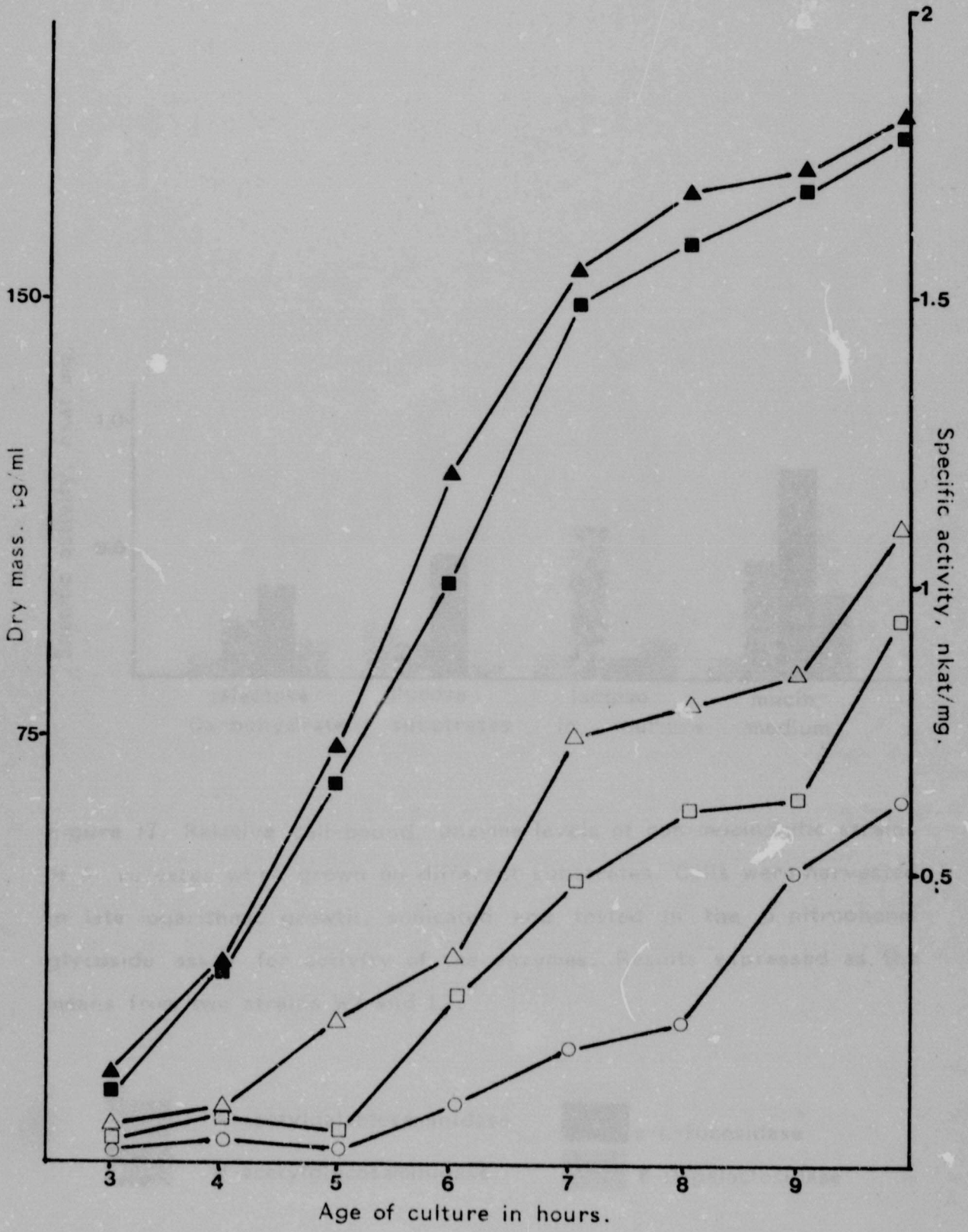

Figure 16. Enzyme activity in cell-free supernatants of two strains of *Bacteriodes vulgatus*-like strains W12 and W13, grown on 1% glucose as substrate. Dry mass of culture W12 ■---■ and W13 ▲——▲ Specific activities of galactosidase □——□, N-acetyl glusosaminidase △——△ and fucosidase ○——○

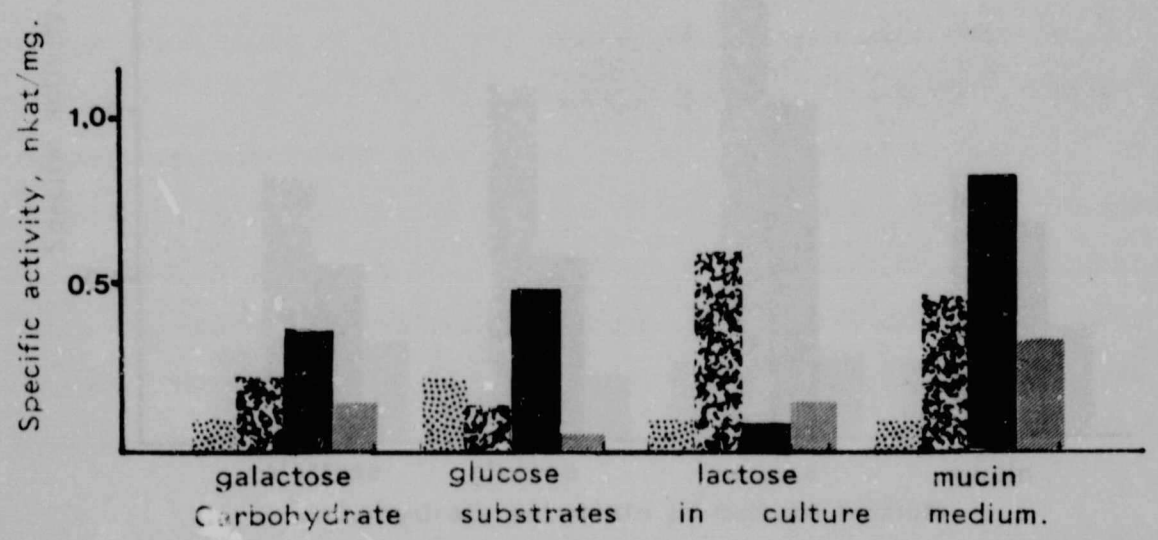

Figure 17. Relative cell-bound enzyme levels of non-mucinolytic strains of *B. vulgatus* when grown on different substrates. Cells were harvested in late logarithmic growth, sonicated and tested in the p-nitrophenol glycoside assay for activity of the enzymes. Results expressed as the means from two strains W2 and L7.

N-acetylgalactosaminidase
N-acetylglucosaminidase
α-L-fucosidase
β-D-galactosidase

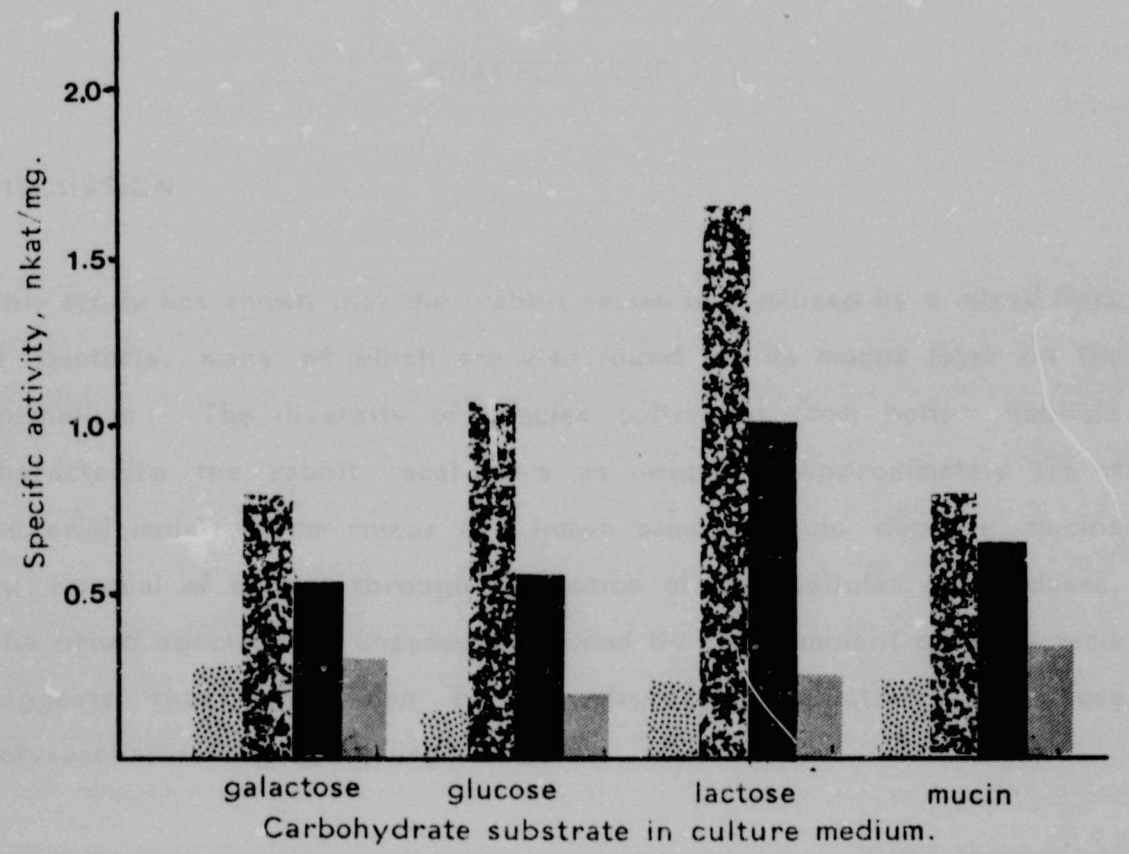

Figure 18. Relative cell-bound enzyme levels of mucinolytic strains of *B. vulgatus* when grown on different substrates. Cells were harvested in late logarithmic growth, sonicated and tested in the p-nitrophenol glycoside assay for activity of the enzymes. Results are expressed as the means from two strains W12 and W13.

CHAPTER FOUR

DISCUSSION

This study has shown that the rabbit cecum is colonized by a mixed flora of bacteria, many of which are also found in the mucus layer on the epithelium. The diversity of species cultivated from both habitats characterize the rabbit cecal flora as unique. Approximately 17% of bacterial isolates from mucus and lumen samples could degrade mucins by removal of sugars through the action of extracellular glycosidases. The broad spectrum of enzymes produced by the dominant cecal bacteria suggests that mucin can be an alternative substrate for these polysaccharide-degrading organisms.

Mucin is a complex glycoprotein with an inner protein core protected by extensive glycosylation with polysaccharides chains (Allen 1984). In the gastrointestinal tract, similar structures are found in dietary material, particularly plant polysaccharides, animal cells desquamated from the gut epithelium and dead microbial biomass. These, together with mucins from the proximal gastrointestinal tract survive to the large bowel where stasis allows sufficient time for at least partial degradation into soluble oligosaccharides (Vercellotti et al. 1978). The rabbit cecum, therefore proves to be is an ideal model to investigate interactions of the autochthonous flora of the gastrointestinal tract with mucins that may be washed into the area from the upper reaches of the tract or secreted locally from cecal epithelia and to determine the role of mucin as an energy source for cecal bacteria. Compared with the thick layer of mucus encountered for most of the length of the small intestine, the cecum gave a very poor yield when the mucus was harvested by scraping the opened mucosal surface. Furthermore, initial centrifugation of cecal mucus gave

a small, insoluble pellet with free sugars detected in the supernatant fluid. In contrast, small intestine mucus consisted of a substantial insoluble gel, with a smaller soluble sugar component in the supernatant. Yosizawa and his colleagues in their studies of rabbit mucin (Nemoto & Yosizawa, 1969; Munabata & Yosizawa, 1978), do not state how many animals were sacrificed to recover sufficient colonic or cecal mucin for chemical analysis, but in this study the cecal epithelium of five rabbits yielded insufficient insoluble, native mucin for use as a culture medium substrate, or even for chemical analysis. Sellars et al. (1983) showed that there is a progressive loss of structural strength and increased solubility in samples taken in descending order from gastric to colonic sites in the pig and this may well explain why the composition of cecal mucins has been studied mainly in large animals in which the yield is likely to be greater. Davis et al. (1977), in an SEM study of ileal and colonic mucous membrane in rodents, reported a thick mucous blanket in germ-free animals, but very thin or absent in conventional rats. Partial solubilization of cecal mucus *in vivo* is offered as substantial evidence for degradation by bacteria inhabiting the mucosal layer.

Recovery of cecal mucous after parenteral treatment of the animals with the neurotransmitter drug Pilocarpine was not enhanced, although there was an almost immediate increase in duodenal secretion, lacrymation, and salivation within 10 min of injection of the drug. Menguy & Thompson (1967) found no effect of the drug on dog gastric secretions, but Neutra (1984), reported that response to pilocarpine in rabbit mucosal explants was restricted to crypt goblet cells from small and large intestines. It would appear therefore, that apocrine stimulated release of goblet cell mucin in the gastrointestinal tract is limited to specific target epithelia and seems to be confined to the proximal GIT with little or no response in the large bowel.

TEM sections of the rabbit cecum wall showed very few active goblet cells, as in Figure 6, and the mucous layer covering epithelial surfaces was only 25 - 50 µm thick in places. The paucity of mucin the cecum would appear to be physiologically normal and the functions attributed to mucins in other GIT systems do not seem to be so important in the cecal context. Compared with other regions of the gastrointestinal tract, the cecum is minimally affected by invasion by pathogenic bacteria, although many components of mucus are reputed to have a strong influence on the infectious process. Furthermore, a pH of 5.6 measured in the distal end of the cecum is sufficiently low to indicate that even a thin layer of mucin is adequate for protection of the epithelium against adverse acidity. It is concluded from these observations that little mucin is produced in the cecum and that most of the lumenal mucin in the cecum originates from the upper gastrointestinal tract, where transit time is too short for significant degradation of polysaccharides to have taken place. Apart from the inability to harvest sufficient mucus from the cecum for analysis, there is adequate justification for the use of small intestine mucin in studies on utilization of mucin by cecal bacteria.

There is substantial evidence to indicate that the structure of the mucin molecule strongly influences degradative mechanisms operating in the gastrointestinal tract. Before consideration of microbial-mucin interactions therefore, it was necessary to define the chemical composition of rabbit small intestine mucin to be used as a substrate in digestion studies. It was found to be similar to previously published data on rabbit intestinal mucins as shown in Table 6 (Nemoto & Yosizawa, 1969; Munabata & Yosizawa, 1978). There was less sulphate esterification found in the mucin derived from rabbits in this study and exceptionally high protein component. Allen (1981) discusses the contamination of mucous by tissue proteins when it is collected from epithelial scrapings. Starkey et.al. (1974) also report that pig gastric mucus scrapings may contain over 50% protein wet weight and small intestine mucus as little as 15% carbohydrate

component. The high proportion of protein found in this study is most likely to be unidentified material remaining bound to mucin during the separation techniques. Table 1 shows that the protein concentrations of gastrointestinal mucins lie between 12 and 33%, but three separate analyses of rabbit small intestine mucin consistently gave between 50 and 55% protein. Ceca were frozen at -20° C and thawed in batches for analysis. It is possible that degradation and loss of carbohydrate components could have taken place during the thawing process, but retention of A - type terminal structures, as shown by absorption of anti-A antibody from blood group specific sera means that *post mortem* degradation was minimal. This technique was used by Hoskins & Boulding (1976) to monitor fecal digestion of exogenous mucin. The demonstration of B absorbing structures on rabbit small intestine mucin preparations is not definative, as A and B substances are similar to the chemical structures that express ABO blood groups on red cells and as the red cells of three rabbits used in this study were typed as group A, it is concluded that the absorption of anti-B by rabbit mucin was due to a non-immunological cause, possibly associated with the anonymous protein discussed above. Some confirmation of this is found in digestion studies in which bacterial degradation of rabbit small intestine mucin preparations failed to restore anti-B titre as it did with anti-A.

Resistance to disulphide-bond reduction in the initial steps of purification indicates that rabbit small intestine mucin is structurally different from the more easliy reduced pig gastric mucin upon which Starkey's model was based (Starkey et al., 1974). Forstner et al. (1973) remodelled a flexible thread structure discussed by Horowitz (1967) after encountering difficulty in disulphide bond reduction of rat small intestinal mucin. In this model, tertiary structure is maintained by many more disulphide-bonds, yet susceptibility to proteolytic digestion at non-glycosylated portions of the peptide core is retained. Hoskins (1978), referring to a report made twenty five years earlier, mentions an enzyme,

cysteine reductase, produced by unknown GIT bacteria, and involved in cleavage of disulphide bonds. The enzyme appears in modern literature as EC.1.6.4.1, effecting NADH - NAD conversion in the presence of its substrate cysteine. The enzyme may well occur in the intestines in sufficient amount to effect premature solubilization of the mucous gel unless the mucin molecule were resistant to its activity.

The carbohydrate components of the rabbit mucin preparations were similar to those of other mucins within the limitations of polydispersity and analytical techniques (Table 6). The range of sugars in rabbit small intestine mucin preparations revealed by gas-liquid chromatography(Figure 13) was the same as that reported for other gastrointestinal tract mucins and conforms to the model of the side chain structure proposed for pig gastric mucin. However, the molar ratios of the component sugars were not the same as those found in pig gastric mucin and it is concluded that rabbit mucin contained less fucose and more galactose and N-acetylgalactosamine in proportion to N-acetylglucosamine. Thus the rabbit small intestine mucin has either shorter or fewer side chains on the molecule. No sulphate was detected in rabbit small intestine mucin preparations and there was moderate sialation compared with other mucins. These moieties are thought to confer a negative charge on the mucin molecule and greater resistance to enzyme attack (Allen, 1978; Flowers & Sharon, 1979). Roberton (personal communication) found some isolates of GIT bacteria that could cleave ester linkages thereby releasing sulphate from the mucin molecule. Bacterial neuraminidases have also been extensively studied in the context of desialation or binding to acid polysaccharides, in pathogenesis of infectious disease. The protective role accorded sulphation and sialation of GIT mucins in general is supported by substantial evidence but rabbit small intestine mucin has little of either, and some other mechanisms must operate to resist premature degradation and solubilization in excess of baseline mucus secretions. As there was minimal sialation and no sulphate

detected in rabbit mucin preparations, these residues were not monitored during degradation studies by gut bacteria.

Increased solubility of mucin after proteolytic cleavage of the peptide core results in loss of the intrinsic rheological nature of the gel (Scawen & Allen, 1977). Bacteria inhabiting the lower gastrointestinal tract and utilizing mucins as energy sources may therefore be involved in two important processes. Those that colonize the mucous layer may remove carbohydrate side chains by the action of glycoside hydrolases, thereby rendering the mucin more readily susceptible to proteolytic activity and contributing significantly to mucin turnover in the cecum. The mucinolytic bacteria inhabiting the lumen also use similar enzymes to remove all evidence of mucin from the digesta before it is excreted in the feces (Lindstedt et al. 1965). Polysaccharide-degrading bacteria of the lower gut are considered to be beneficial to the host, as the epithelia absorb many products of microbial metabolism for assimilation from the cecum where vitamins, deconjugated bile products and growth factors are made available through the action of cecal and colonic microorganisms. Bacteria responsible for this activity derive much of their nutrition from the complex polysaccharides, including mucins, reaching the large bowel (Draser & Hill, 1974). The polysaccharide-degrading bacteria of the large bowel therefore, may be considered to be primary producers in the complex GIT ecosystem.

The bacterial flora of the ceca of rabbits fed exclusively on a maintenance pelleted diet was shown in this study to be diverse. Over 19 dominant species were recovered in culture, in counts between 10^6 to 10^8 per ml (Table 4). The numbers isolated were to some extent dependent on the culture media used, as was found by Kelly (1983), in a study of isolation media for chicken intestinal bacteria. The modified M10 medium simulated the cecal environment as closely as possible and gave higher counts and greater diversity than the other media with which it was compared. Total

viable numbers recovered on M10 medium represented less than 10% of the microscopic count but reached 10^{10} per ml of wet lumen contents in one rabbit. These numbers are similar to the viable counts found by Bonnafous & Reynaud (1970) in the cecum and by Emaldi et al. (1979) in rabbit soft feces, which are comparable to cecal contents. There were fewer bacteria cultured from mucous scrapings, with mean total counts of 10^6 per ml consisting of dominant species recovered at levels of 10^4 to 10^5 per ml. Anomalous results from viable-cell counts and direct microscopic counts of gastrointestinal systems has always been a problem in GIT microbiology. Undoubtedly, many dead cells are counted in the microscope, but also, there must be many types of bacteria that can only be isolated under specific conditions and many more that do not respond in artificial culture. The bacteria isolated in this study therefore, represent only the least fastidious types constituting only part of the total flora. TEM and SEM micrographs of the mucous layer (Figures 5 & 7) showed that the gel was densely populated by many different morphotypes although none were seen adhering to the epithelial surface. Almost all other parts of the gastrointestinal tract of animals supports an adherent microflora that bind to specific receptor sites on epithelial cells (Savage, 1983). This study has demonstrated that bacteria do not adhere to the cecal epithelium because of the presence of a layer of small membrane bound vesicles covering the brush border, shown in Figure 8. They have been reported before in both man and rabbits (Toner et al., 1971) but no special significance was given to them except that they were thought to arise from blebbing of the microvillus tips. They were not seen in SEM preparations (Figure 4b), although their size should have been just within the resolution of a good SEM. The few bacteria seen to be in contact with microvesicles suggested receptor-ligand adherence but continual shedding of microvesicles into the mucous gel, as postulated by Toner et al. (1971) would ensure that any adherence by bacteria to the epithelial surface would be temporary.

An attempt was made to categorize the bacteria in mucous gel by transverse section of cell wall structures. Although classical Gram-positive and Gram-negative cell walls were seen, there were many bacteria with cell envelopes that did not conform to either of the two groups, and clear sub-division of the bacterial flora of the mucous gel on morphological criteria could not be made. There is an urgent need for techniques to identify microorganisms *in situ* in complex systems with high adherent components such as the GIT, aquatic biofilms and soil, to be able to relate to functional characteristics established *in vitro* Immunological techniques using fluorescent, radioactive or electron dense labels have not fulfilled all their promise and it was hoped that ultrastructural microanatomy might be of some help in this study. As shown in Figures 9, 10 and 11, bacteria *in situ* have few clearly differentiating features and comparison with preparations from artificial cultures would be far from satisfactory. It is now generally accepted that "Gram-positive" cell wall ultrastructure is not necessarily synonymous with Gram-positivity in the classical staining technique, as was clearly shown by Mead & Jones (1981) in their studies of the anaerobic bacteria associated with the sheep rumen wall.

The difficulties in cultivation and identification of gut anaerobes was not underestimated in this study, and the limitations discussed by Moore & Holdeman (1974) were noted before results were interpreted. Problems lie with the suitability of culture media for maximum recovery of the broad range of bacterial species present in gut samples; in the assurance that sampling techniques are adequately representative of the natural system and in the validity of techniques used to enumerate bacteria. The methods used in this study were based on standard practice currently in use for gastrointestinal microbiology and identification of strains should be considered to be merely presumptive. Recent evidence using genetic criteria for relatedness among GIT bacteria has shown that strains grouped together on purely phenotypic characteristics may well be quite heterogeneous genetically (Shah and Collins, 1983). Thirty one taxonomic

criteria were used to presumptively identify isolates, with weighted characteristics of Gram's stain reaction, metabolic end-products and motility defining initial generic grouping. When more than 29 out of the 31 characters were in accordance with published species descriptions from various sources, isolates were given a taxonomic name and strain number.

The cultivable bacterial flora of the rabbit cecum was found to be original, with a combination of dominant species that is not found in other animals (Table 4). The most frequently isolated species had already been described, although there were minor populations that could not be identified. There was considerable variation in the distribution of species between individual rabbits and between the lumenal and mucosal floras (Table 5). The rabbit cecum is not unusual in this as studies on other animals have shown similar variations. The colonic flora of four human cadavers were quite different (Croucher et al., 1983), and the fecal flora of man, considered to represent that of the colon, also varies between individuals and even within an individual from day to day (Holdeman et al., 1976). Similar findings have been reported in dogs by Davis et al. (1977), and in pigs by Robinson et al. (1981).

Strains similar to *Bacteroides vulgatus* were the most frequent isolates from all ceca and present in lumen contents and mucosae, as was *Bacteroides ruminicola* and the facultative streptococci represented by *Streptococcus intermedius*. These species, together with *Fusobacterium* and absence of *Lactobacillus* are characteristic of the rabbit cecal flora. The diversity of genera recorded from the rabbit cecum was comparable to that found in the ceca of other animals such as dogs, pigs, rodents and man. In the dog, over 18 different species were found in one group of animals with the dominant genera *Streptococcus*, *Lactobacillus*, *Fusobacterium* and *Bacteroides*, although it was shown that housing conditions stongly influenced the composition of the dog cecal flora. In contrast, the fecal flora of man, also an omnivore, generally contains

Bacteroides, *Bifidobacterium*, *Streptococcus*, *Peptostreptococcus*, and *Lactobacillus* as dominant genera (Holdeman et al.,1976). The rabbit cecal flora is more similar to the distribution of bacterial species in the ceca of other hind-gut fermenting herbivores such as rodents and pigs. *Bacteroides*, *Lactobacillus* and *Fusobacterium* are most frequently isolated from rodents (Macy et al.,1982) and *Bacteroides*, *Lactobacillus*, *Peptostreptococcus*, and *Streptococcus* are reported as being dominant genera in the pig (Robinson et al., 1981; Russell, 1979).

The distribution of bacterial populations in cecal mucosae investigated in this study was not the same as in lumen contents. Although *Bacteroides vulgatus* and streptococci were dominant in both, there were some species present that were not found in the lumen, particularly *Coprococcus eutactus*, and various *Clostridium sp.* Robinson et al. (1984) reported a similar finding in the pig, with *Streptococcus* and *Bacteroides* species the most frequent isolates from the mucous layer, but *Leptotrichia*, *Peptostreptococcus* and *Selonomonas* found exclusively in the mucosal environment. In other animals however, there is considerable evidence for colonization of the cecal wall by unique bacterial populations. The filamentous organisms of the rodent gastrointestinal tract for example, and spirochaetes of the primate colon, are intimately attached to the epithelial surface rather than loose in the mucus blanket (Savage, 1983), but no evidence of populations of this nature was found in the rabbit cecum, nor were lactobacilli nor coliforms found in any of the rabbits in this study.

The animals were adult conventional stock from a university facility maintaining high standards of care and nutrition. Christ-Vietor (1973) found a comparable type of microflora in laboratory rabbits maintained under similar conditions, but it was later shown that fresh vegetables fed as a regular component of the diet, induced marked changes in the cecal microflora characterized by the persistence of lactobacilli and

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coliforms in the adult animal (Weber et al., 1974). The cecal flora of rabbits, as in other animals, is therefore susceptible to environmental factors, and the descriptions given in this study apply only to animals maintained under the general conditions described above.

Bacteria inhabiting the mucus layer of the rabbit cecum are presumably using components of this secretion as a source of nutrients, but whereas some were indistinguishable from isolates found in the digesta, others exclusively colonized the mucosal habitat. *Bacteroides vulgatus*-like strains and the streptococci were not only among the species occurring in the highest numbers in the cecum but are also found in both digesta and mucus, implying versatility in their nutritional requirements. The influence of mucus on the distribution of bacteria in the cecal ecosystem is obviously significant. Mucinolytic bacteria were isolated from lumen (L isolates) and mucus (M isolates) in more or less equal numbers but whereas the M isolates were living in a milieu almost entirely composed of locally secreted cecal mucin, the L isolates would have been exposed to a range of exogenous polysaccharides, including mucins from small intestines and even stomach.

The choice of methods used for detection of mucin degradation by bacteria is important for success. The periodic acid-Schiff's method was originally a histological technique for demonstration of certain polysaccharides such as glycogen, goblet cell mucin and other PAS-positive material of unknown composition. Mantle and Allen (1978) modified the technique to a biochemical test for monitoring and measuring mucins in their important studies on the composition and structure of the mucin molecule. The periodic acid opens glycosidic bonds to expose aldehyde groups which restore leuco-basic fuchsin to its coloured dye conformation. The test is therefore a measure of the number of glycosidic bonds present in the molecule being studied. When used to monitor the amount of mucin in culture media after bacterial digestion, up to 40% loss of PAS - reactive