but were not detectable in feces from conventional animals, but the role of cecal bacteria in mucin digestion in the rabbit is unknown.

1.4 Function of intestinal mucins

Mucus is a weak viscoelastic gel forming a continuous protective cover over mucosal epithelia. The gel components are held together by non-covalent interactions which resist solubilization but allow flow and annealing when disturbed. The composition of mucous gel is a complex mixture of secretory IgA and other plasma proteins, lipids, digested food material, desquamated epithelial cells, and digestive enzymes. But the most important constituent is the mucin molecule, responsible for the unique rheological properties exhibited by the gel. The many attributes accorded the mucous layer are concerned with protection of the underlying epithelial surface, either from physical damage by virtue of its slippery adhesive nature, or from chemical attack, as in the stomach, where its impermeability to ions prevents damage by gastric acidity. Dyscrasia of mucous production is also incriminated in several disease conditions in man and animals (Schrager & Oats, 1978). Comprehensive reviews by Allen (1981, 1984), Forstner (1978) and Forstner et al. (1984) list a number of other functions that have been proposed to reinforce the physiological importance of the mucous gel. It presents a barrier to potentially pathogenic microorganisms entering the GIT, by binding to bacteria or their toxins, thereby preventing access to receptor sites on the epithelium. At the same time it supports a dense flora of indigenous bacteria which also helps to exclude allochthonous microorganisms from the underlying tissue. The gel also actively binds iron and other cations, although the significance of these findings in absorption by the host is not clear.

1.5 Secretion of mucus

The mucin molecule is assembled in goblet cells originating in the intestinal crypts of cecal tubular glands. The cells secrete their mucin as they migrate along the basement membrane until they are desquamated from the tips of the villi. A peptide core is glycosylated in the golgi and is believed to be polymerized as it is transferred to vesicles for secretion. (Neutra, 1984). The stimuli for secretion of mucus from the goblet cells are complex and poorly understood. There seems to be a continuous discharge to maintain physiologically defined mucus levels, but apocrine-stimulated release also takes place when the epithelium is exposed to irritants or damage (MacDermot et al., 1974). Thus, treatment with mustard or neurotransmitter drugs induces enhanced secretion of mucous from intestinal goblet cells (Menguy & Thompson, 1967), although prolonged administration causes changes in the chemical structure of the mucin molecule.

As the thickness of the mucous blanket in any part of the GIT remains relatively constant between 50 and 500 μm (Forstner 1978), there appears to be a balance between secretion and loss into the lumen, controlled on the one hand, by the mechanisms influencing goblet-cell discharge, and on the other, by degradative processes at the outer surface of the gel.

1.6 Structure of intestinal mucins

Native mucins are glycoproteins of approximately 10^6 daltons undergoing a sol-gel transformation when their concentration approaches 20 mg ml^{-1} At least three models have been proposed to explain their functional and chemical properties and indicate the diversity of these molecules. Snary et al. (1970) found that reduction or proteolysis of native pig gastric mucin yielded four subunits of $5x10^5$ daltons, and proposed the windmill model illustrated in Figure 1 in which the subunits are held together by 78 disulphide bonds at the centre of each molecule (Starkey et al., 1974; Allen, 1978) It was subsequently found that although reduction with

mercaptoethanol did indeed yield four subunits, cleavage by proteolysis gave four slightly smaller subunits together with a peptide of 70 000 molecular mass (Roussel et al., 1984; Allen, 1983). A globular bead structure proposed by Robinson & Monsey (1975) has recieved little recognition other than in its original application to ovomucin, but Forstner et al. (1973) constructed a flexible-thread model, in which the molecule is held in its tertiary structure by many more disulphide bonds than in Snary's model. Forstner's model was proposed to represent the intestinal goblet cell mucins that were found to be resistant to disulphide bond reduction.

The structure of subunit mucins has been likened to that of a bottle brush (Allen, 1981), with a peptide core comprising up to 30% of the molecule and rich in serine and threonine in GIT mucins. It is to these amino acid residues that carbohydrate side chains are attached by O-glysosidic linkage via N-acetyl-galactosamine (Clamp et al., 1978). The peptide sequencing seems to be different for each species of mucin and determines the shape and overall structure of the molecule (Jabbal et al., 1976). Susceptibility of non-glycosylated peptides to proteolytic digestion and the difficulty in obtaining native mucins entirely intact is discussed by Horowitz, (1977).

It has been stated that the chemistry of mucin is the chemistry of its carbohydrates, as being the main reason for the marked polydispersity among mucin molecules (Clamp et al., 1978). Except for terminal structures which appear to be under gene control, supervision of glycosyltransferase activity associated with precursor synthesis in the goblet cell seems to be less critical, resulting in variability in the length and composition of mucins within the same species (Gallagher & Corfield, 1978). The side-chain structure of pig gastric mucin shown in Figure 2 is only one example of up to 800 such chains of varying length attached to the peptide core. The GIT mucins are composed of straight or branched

chains of galactose (Gal) and N-acetyl-glucosamine (GlcNAc), in mainly β1→3 linkages, anchored to serine or threonine by N-acetylgalactosamine (GalNAc). Fucose residues may be joined to the main chains by α1→2 linkage and are also found as part of terminal structures at the non-reducing end. Although some GIT mucins are sulphated on internal residues, the exact position of the esters is unknown for most of the species studied. Salivary mucins are also heavily sialated with a group of sugars based on N-acetyl- or N-glycol-neuraminic acids, but as with sulphation, the position of the small amounts present on lower GIT mucins is unknown. Both sulphate esters and sialic acids are supposed to confer resistance to digestion on the molecule by virtue of the negative charge they carry (Gottschalk, 1972; Roberton & Stanley, 1982).

Terminal structures of mucins have been of great value in mucin research, as they are the same as the carbohydrate structures that express the ABO blood group system on human red cells (Hoskins, 1967; Slomiany & Meyer, 1972). Under the influence of the Le secretor gene various sugars may be added to the non-reducing end of the carbohydrate side chain as shown in Figure 3 and are referred to as A, B or H substance. Mucins carrying these structures absorb homologous antibody out of an ABO hemagglutinating system, but antibodies, prepared in rabbits to purified mucins, do not recognize antigens of the ABO system (Jabbal et al.,1976). Hoskins (1967) successfully used this feature to monitor mucin secretion in various disease states in man, and Aminoff & Furukawa (1970) used the ABO structures to assess *in vitro* degradation of GIT mucins by loss of blood group substances.

Among the GIT mucins, the full structure of only pig gastric mucin has been determined, although the chemical composition of many others have been reported in major review articles, texts and research publications. Table 1 presents the available data on mucins from different anatomical

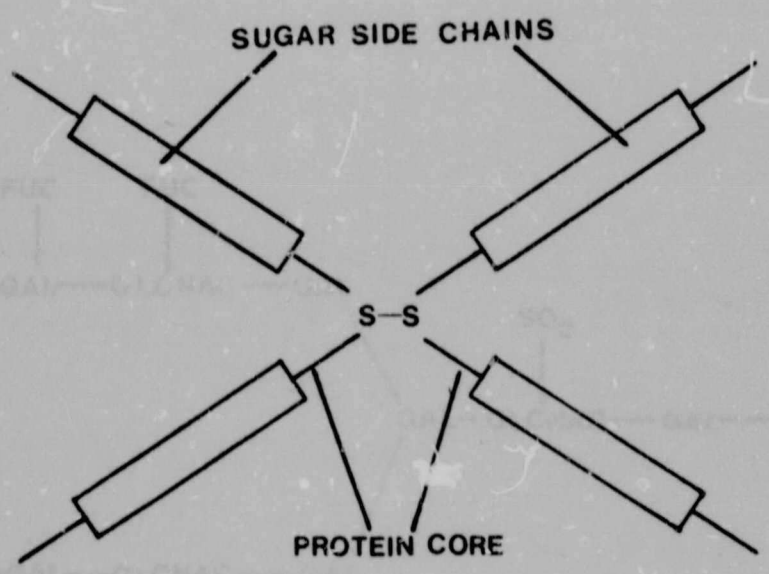

Figure 1. The windmill model of pig gastric mucin. (after Starkey et.al., 1974.) The sub-units consist of sugar side chains attached to the protein cores which are joined together by disulphide bonds

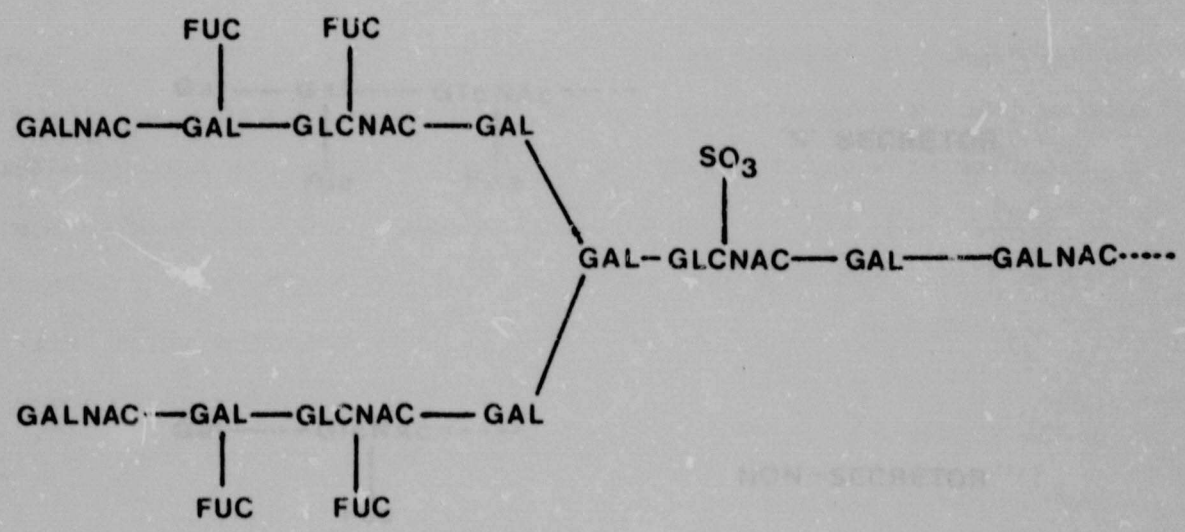

Figure 2. The structure of a carbohydrate side chain of pig gastric mucin (after Allen, 1978). The model is a representation of one of over 800 such chains on the mucin molecule.

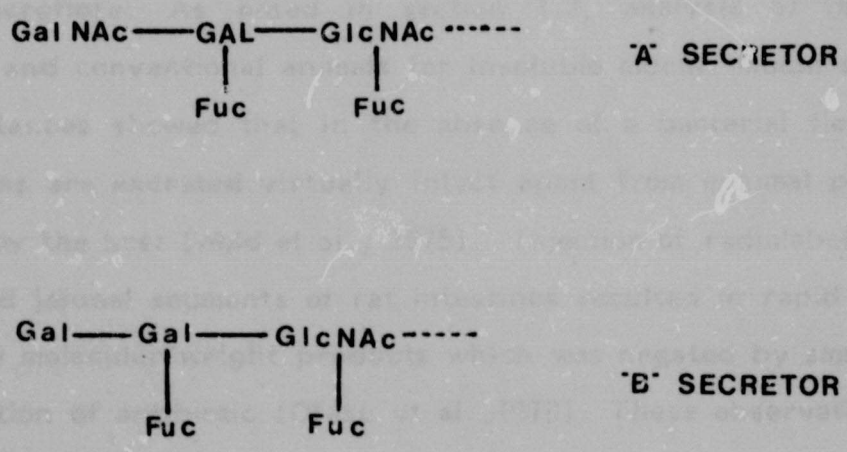

Figure 3. Terminal structures expressing blood group specificity on mucin molecules (after Slomiany and Meyer, 1972). Pig gastric mucins terminate in the A type structure.

sites, from a number of sources, to illustrate the diversity of this group of molecules.

1.7 Mucin degradation by gastrointestinal bacteria

There is ample circumstantial evidence to indicate that mucous glycoproteins are degraded in the gut by indigenous members of the enteric microflora. As noted in section 1.3, analysis of feces from germ-free and conventional animals for insolubie mucin, mucin sugars or ABH substances showed that in the absence of a bacterial flora in the GIT, mucins are excreted virtually intact apart from minimal proteolytic digestion by the host (Wold et al., 1975). Injection of radiolabelled mucin into ligated jejunal segments of rat intestines resulted in rapid degradation to low molecular weight products which was negated by simultaneous administration of antibiotic (Ofasu et al., 1978). These observations indicate quite clearly that degradation of mucin in the animal GIT is a function of the indigenous bacterial flora and that the host's digestive system plays a minimal role.

1.8 In vitro studies of mucin degradation

With the evidence presented above, it is surprising that so few studies have been made on the degradation of intestinal mucins by pure cultures of gut bacteria. In a series of publications over a number of years, Hoskins and his co-workers confirm the conclusions discussed in section 1.7 by demonstrating that consortia of human fecal bacteria in mixed culture digested all the carbohydrate and up to 50% of the protein in commercial pig gastric mucin. These findings explained the loss of ABO substances from the mucin substrate by the action of degradative bacteria found in earlier work. Subsequent studies showed that the enzymes responsible for degradation of the mucin molecule were extracellular glycosidases associated with bacterial populations present in human feces

Table 1. Published data on the composition of gastrointestinal mucins.

	Chemical composition						
	GlcNAc	GalNac	Gal	Fuc	NANA	Sulph	Prot
	---Molar ratios---				---%		
Saliva							
Human[d]	1.2	1	1.5	0.9	0.6	+	34
Pig[f]	nil	1	0.5	0.4	0.4	nil	36
Sheep[e]	nil	1	nil	nil	1.0	nil	46
Stomach							
Human[e]	2.4	1	3.1	2.1	0.2	7.0	17
pig[f]	2.8	1	2.9	1.9	0.2	3.0	13
pig[e]	1.5	1	1.5	0.4	0.04	nd	nd
Small Intestine							
Human[c]	1.0	1	2.0	0.6	0.2	nil	12
Pig[a]	0.6	1	0.7	0.3	0.5	2.6	18
Rat[c]	1.2	1	2.2	1.1	0.9	0.2	14
Rabbit[b]	1.0	1	1.7	0.3	0.2	0.1	21
Colon							
Human[e]	1.0	1	1.1	0.5	0.7	1.6	33
Pig[g]	2.9	1	2.5	1.5	0.2	3.0	13
Rabbit[h]	1.1	1	1.2	0 3	0.3	0.2	18

Inoue & Yosizawa, 1966[a]

Nemoto & Yosizawa, 1969[b]

Jabbal et.al., 1976[c]

Horowitz, 1977[d]

Forstner, 1978[e]

Allen, 1978[f]

Roberton & Stanley, 1982[g]

Munabata & Yosizawa, 1978[h]

at densities of 10^6 to 10^{10} per gram dry weight. (Hoskins, 1969; Hoskins & Boulding, 1976, 1981; Variyam & Hoskins, 1981).

The few workers who have attempted to study mucin degradation by pure cultures have not met with any great success. Salyers et al. (1977a) used reference cultures of human gut bacteria and monitored mucin fermentation by acid production. None of the strains studied utilized pig gastric mucin, although mucin monosaccharides were readily fermented by most. Similarly, Roberton & Stanley (1982), using reference cultures of *Bacteroides sp.* grown in media containing pig colonic mucin, demonstrated only limited degradation by analysis of the substrate before and after exposure to the bacterial cultures. Bayliss & Houston (1984) recovered 14 distinct bacterial isolates from human feces on enrichment media containing pig gastric mucin, but did not enquire further into the extent of substrate degradation. The evidence from this limited *in vitro* work on mucin digestion by gut bacteria indicates that although degradative activity is present in mixed cultures, separate species seem to be incapable of exerting any great effect when exposed to mucins in pure culture. Hoskins & Boulding (1976) suggest that to completely remove all the sugars from a typical side chain of pig gastric mucin, several α-L-fucosidases, β-D-galactosidase and both α- and β-N-acetylhexosaminidases would be required to work in sequence. They suggest the improbability that any single bacterial strain would produce all these enzymes.

1.9 Glycosidases of enteric bacteria

The extensive literature on bacterial glycosidases (or glycoside hydrolases) has been generated mainly through interests in plant fibre digestion by rumen microorganisms, or from purification and characterization studies on the enzymes in cell-free culture supernatant fluids. As a group, they have been demonstrated in a wide variety of bacteria

and fungi grown on an equally wide range of substrates. However, many authors have cautioned that a generic glycosidase may occur with different specificities, isoenzymes and physical requirements (Patel, 1978; Cabezas et al.,1983). Hoskins (1984) presents the accumulated data from several reports on the range of bacterial glycosidases detected in feces and intestinal contents of man and rats, among which are all those relevant to degradation of mucin polysaccharides. The only purified enzymes that have been studied in this context were recovered from *Clostridium perfringens* and tested against pig mucin. Aminoff & Furukawa (1970) found that an α-L-fucosidase removed ABH substance with attendant loss of serological specificity, and Roberton & Stanley (1982) report that clostridial neuraminidase effectively de-sialated gastric mucin. Berg et al. (1978) studied representative strains of a range of *Bacteroides* species that are widely distributed in the large bowels of animals. They found that all of them produced β-galactosidase, β-N-acetylhexosaminidase, and other glycosidases in culture supernates during late logarithmic and stationary phases of growth in batch culture on glucose as the sole energy source. The enzymes were tested on p-nitrophenol glycosides and it may be erroneous to assume that they would be equally effective on the glycoside bonds of complex glycoproteins.

1.10 Aims of this study

There has been a considerable revival of interest in the microbiology of digestion in the domestic rabbit in recent years, associated with an increasing awareness of the animal's importance as an economical source of protein. The studies generated by this interest have focussed mainly on the need for nitrogen supplementation of the rabbit's diet to improve weight gain and have been concerned almost exclusively, with the populations of ureolytic bacteria in cecal contents, or soft and hard fecal pellets. The animal is a herbivore, with hind-gut digestion of plant material and is therefore dependent on the bacterial populations in its

cecum to degrade complex polysaccharides to fermentable energy sources to the mutual benefit of both the host and its microflora. The part played by mucin in stabilizing these essential microbial populations has been suggested, but never investigated. This study proposes to examine the bacterial microflora of the rabbit cecum in greater depth than has been reported in previous investigations, with particular reference to the bacterial populations that are involved in degradation of mucin from the rabbit digestive tract. It is proposed that these bacteria are versatile polysaccharide-degraders that are able to use mucin as an alternative substrate during fluctuations in dietary input, thereby contributing to the stability of the cecal microflora in general. This proposal will be investigated through a study of the enzymes produced by bacteria involved in mucin degradation and their role in digestion of complex polysaccharides.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Animals

Adult New Zealand White rabbits of either sex were obtained from the university animal unit where they were housed under conventional conditions in racked cages with free access to water. They were fed a commercial pelleted diet of the following composition per Kg: protein, 160 g; fat, 25 g; fibre, 170 g; moisture, 120 g and supplementary trace minerals. Intravenous pentabarbitone was used for euthanasia and the gastrointestinal tract was dissected out *post mortem* as the source of all experimental material.

2.2 Electron Microscopy

Standard techniques were used in which small (0.5mm) and large (5mm) blocks of wall were removed from ceca after gentle washing in three changes of cold physiological saline. In all subsequent steps the tissues were handled as carefully as possible to retain mucous blanket material adhering to the epithelial surface. Blocks were fixed in cold 2.5% glutaraldehyde in 0.2M phosphate buffer at pH 7.1 for at least 24 hours. They were post-fixed in 1% osmium tetroxide in the same buffer for an hour, and dehydrated in ascending alcohol concentrations. For scanning electron microscopy (SEM) larger pieces of tissue were prepared by critical point drying in carbon dioxide and coated with 20nm of gold for examination in a Cambridge Stereoscan microscope. This routine technique was shown by Bayliss & Turner (1982) to be satisfactory for the preservation of the mucus blanket and its associated bacteria. Small

blocks for transmission electron microscopy (TEM) were embedded in Spurr's low viscosity resin for sectioning, stained with uranyl acetate-lead citrate sequence and examined in a Jeol 100S microscope at an accelerating voltage of 80Kv.

For negative staining of bacterial suspensions, a drop of culture was placed on a carbon-coated grid and mixed with a drop of a solution containing 0.3g dl^{-1} phosphotungstic acid. After 15 s the grid was drained and dried before examination in the electron microscope.

2.3 Anaerobic culture

Initial development of anaerobic culture techniques took place in a home-made flexible vinyl cabinet (Aranki & Freter, 1972: Dickman et.al.,1979), but this was later replaced by a commercial version (Forma Scientific). Separate gasses were piped into the system via flow meters to proportion the gasses to 12% CO_2 in nitrogen. Six percent hydrogen was maintained in the gas mix to activate palladium catalyst for scavenging residual oxygen in the cabinet and gas dispensing outlets. Redox potential was monitored by bubbling gas phase via an aquarium pump, through redox indicators. These were prepared by dissolving 1% methylene blue and indigo carmine separately in 4% glucose solution and boiling until colourless. They were taken into the cabinet and slowly brought to pH 7.0 with sodium carbonate as they equilibrated with the gas phase. Thus, reducing conditions in the cabinet could be confirmed by absence of colour change in the indicators (Costilow,1981). All sterile plastic-ware used in the cabinet were "aged" for a few days to remove adsorbed oxygen, and manipulations were done with a 12v electric loop powered with a transformer from within the cabinet.

The culture medium used throughout this work was based on that designed by Caldwell & Bryant (1966) for rumen microbiology. The original

M10 formulation was modified as described below to make it more suitable for cultivation of bacteria from the rabbit cecum.

Analysis of fresh cecal contents *in situ* indicated that the pH varied from 5.6 at the proximal end to 6.3 in the appendix. The carbonate-bicarbonate buffer was adjusted accordingly to a pH of 6.5 by trial and error by varying the amount of bicarbonate in the medium to give the required pH with the 12% CO_2 gas phase. The theoretical approach indicated by Hungate (1970) did not give satisfactory results in practice.

The volatile fatty acid composition of rabbit cecal digesta was measured by gas chromatography as discussed later in section 2.6, and was found to consist mainly of 63% acetic acid, 16% propionic acid and 21% butyric acid. A stock mixture was made up to give these proportions when added to media before sterilization. (see appendix 5).

Redox potentials of cecal contents were roughly assessed, in the absence of a redox measuring electrode, by opening ligated ceca into isotonic solutions of reduced indicators in the glove cabinet. According to Henrges and Maier (1972), no colour change in reduced indigo carmine solution after 30 min indicated that the cecum contents were below -160 mV and this reagent was used in all media at a concentration of 0.5 mg in 100 ml (Latham and Sharpe, 1971). Carbohydrate substrates for isolation of rumen bacteria were replaced by those most likely to be found in the rabbit cecum including glucose, galactose, maltose and lactose with the total carbohydrates not exceeding 2 g dl^{-1}. Mucins were also added to media, either in addition to the sugars mentioned above, or as the sole carbon source. Roberton & Stanley (1982) established that mucins are not altered by the autoclaving necessary to sterilize bacteriological media. Modified M10 medium for growth curve measurements and recovery of cell-free supernatants was made up as discussed above, in bulk

volumes of 200 ml, with single carbohydrate substrates at a concentration of 1.0 g dl^{-1}, added before autoclaving.

The other components of M10 medium such as salts, yeast extract, peptones, vitamins and reducing agent were not altered and are given in appendices 1 & 2, together with the method of preparation of media. In experiments to establish the suitablity of various media for primary isolation of cecal bacteria, formulations based on rumen fluid agar (Bryant and Robinson, 1961) and brain heart infusion agar (Holdeman et.al.,1977), were modified as described in appendices 6 & 8 for comparison with the modified M10 medium in terms of total numbers of organisms recovered and colony diversity. For detection of mucin degradation *in vitro*, basic modified M10 medium was supplemented with 500 mg dl^{-1} pig or rabbit mucin as the only carbohydrate. After 8 d incubation at 37° C mucin concentrations of tests and uninoculated control media were compared using the Periodic Acid-Schiffs method (PAS, appendix 12) and analysis by gas-liquid chromatography (GLC), (section 2.9).

Other media used in this study included Peptone-Yeast extract base medium (PY) to which was added 1 g dl^{-1} of various sugars for fermentation studies. (appendix 7). Solutions of 10 g dl^{-1} of D-galactose, L-fucose, N-acetylgalactosamine and N-acetylglucosamine were prepared in PY medium, filter-sterilized into bulk medium and dispensed aseptically into screw-capped vials under a stream of carbon dioxide. A drop in pH of 1 unit or more after inoculation and 48 h incubation, indicated positive fermentation (Holdeman et al.,1977). PY medium was also supplemented with 1 g dl^{-1} glucose (PYG) for culture of all isolates for detection of fermentation end-products by gas liquid chromatography (section 2.6).

2.4 Primary isolation and enumeration of cecal bacteria

2.4.1 Microscopic counts.

Mucosal scrapings and samples of lumen contents were diluted 1 : 100 by adding 0.1 ml to 10 ml of dilute crystal violet solution and agitating on a vibrator for 1 min. A Neubauer counting chamber was used to enumerate bacteria after they were allowed to settle for 30 min in a moist chamber.

2.4.2 Viable plate counts.

Ligated sections of ceca were placed in anaerobic diluting fluid (ADF, see appendix 4), gassed with CO_2 to exclude air (Patel et al., 1984), and passed into the anaerobic cabinet. Dilutions of digesta were made by adding 0.5 ml to a calibrated vial containing 4.5 ml of anaerobic diluting fluid. Cecal wall was washed three times in 20 ml ADF until clear of particulate matter and the mucous membrane scraped with a sterile microslide to give 0.5 ml of mucous material which was also added to 4.5 ml of ADF. Both samples were diluted 10-fold from 10^{-1} to 10^{-8} in ADF and 0.1 ml volumes of each dilution plated out in triplicate on pre-reduced agar media. After at least six days at 37°C plates were evaluated and discrete colonies picked off into M10 broth. These isolates were sub-cultured onto solid media, re-evaluated, and discrete colonies again picked off into liquid M10 medium. After a final check for purity by Gram's stain, sterile glycerol was added to each culture to give a final concentration of 10%, as a cryoprotectant, and the screw-capped vials stored at -80° C in a carbon dioxide refrigeration cabinet (Teather, 1982).

2.5 Tests for identification of isolates

As a matter of convenience, the MB24A system manufactured by Disposable Products, Australia. was used for basic tests to identify the