

anaerobic bacterial isolates. The system consists of a pre-prepared microtitre plate containing lyophilized substrates and the pH indicator, brom-thymol blue. A suspending medium supplied with the system was very similar to M10 medium and supported growth of all isolates tested. Bacteria were suspended in this medium to turbidity of a number 1 McFarland tube, (c. 3×10^8 per ml), and dispensed into the microtitre wells by pipette. After 48 h at 37° C in the anaerobic cabinet, pH changes were noted, after the addition of more indicator when necessary. Gram's iodine was added to the test well for starch hydrolysis and ferric citrate for esculin hydrolysis. Indole production was detected by the addition of Kovack's reagent, catalase with dilute hydrogen peroxide and nitrate reduction by sulphanilic acid reagent. The other fermentation substrates included in the system were arabinose, cellobiose, fructose, glucose, lactose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, ribose, salicin, sucrose, trehalose, xylose, and gelatin. Additional further tests included the fermentation of galactose, growth in M10 medium supplemented with 20 g dl⁻¹ ox bile (Difco) and detection of hydrogen sulphide production by suspending a lead acetate paper test strip above M10 broth cultures. The MB24A system was evaluated with *Clostridium perfringens* from our stock culture collection and *Actinomyces israelii* X372^T, and was shown to give concordance with published descriptions of these species.

Aerotolerance was checked by subculture on un-reduced M10 agar incubated for two days in air.

Isolates found to be motile by hanging drop method were examined by electron microscopy of negatively stained preparations. This method was less capricious than the flagellar stains viewed by light microscopy and is strongly recommended if facilities are available.

All bacterial names used in presumptive identifications were in accordance with Skerman et al. (1980).

2.6 Determination of fermentation end products

The method of Carlsson (1973) was used for gas chromatographic detection of volatile and non-volatile fatty acids. Cultures in PYG medium were passed through a small column of AG50W-X4 200 mesh cation exchange resin (Bio-rad Laboratories) and the clear eluate collected for chromatography. A standard mixture of volatile fatty acids (VFAs), each at a concentration of 1.0 meq dl^{-1} was also passed through a column with every batch of analyses. A Pye GC gas chromatograph was fitted with dual flame ionization detectors and dual $1.8 \text{ m} \times 3 \text{ mm}$ glass columns packed with Chromosorb 101 (Johns-Manville) leaving a 4.0 cm inlet space. Samples of $2 \text{ } \mu\text{l}$ were run isothermally at 210°C , with a nitrogen carrier gas flow rate of 60 ml min^{-1} . The acids chromatographed in the sequence, acetic (A), propionic (P), isobutyric (iB), butyric (B), isovaleric (iV), valeric (V), lactic (L), caproic (C) and succinic (S), in 16 minutes. Formic acid and alcohol could be detected, but not quantitated, as tail peaks emerging with the water peak. Major metabolic products were reported if peak areas were equal to or greater than those of the 1 meq dl^{-1} standard peaks and minor if they were less.

All isolates were placed into groups sharing common Gram's stain morphology and reaction, the presence of spores, motility, volatile fatty acid profiles and colonial characteristics. Approximately a third of the isolates of each group were fully identified and taken to be representative of the group as a whole. When anomalies arose, more isolates from the group were taken for identification.

2.7 Collection and purification of rabbit intestinal mucin

Ligated sections of small intestine, colon and cecum were stored frozen at -20°C and thawed in batches. Lumen contents were washed out with a gentle stream of tap water and the gut opened lengthways. The epithelium was scraped with a glass microscope slide into 0.2M NaCl in a flat dish and harvested in 50 ml centrifuge tubes. Sodium azide was added to the salt solution at a concentration of 0.02 g dl^{-1} to prevent any further bacterial degradation of samples to be used for chemical analysis. Increased yield of intestinal mucous in rabbits was obtained by intravenous inoculation of 0.2 ml of 1% pilocarpine (Alcon Laboratories Inc.) This caused profuse salivation and lacrymation within 10 minutes, and the animals were killed for mucin collection as described above. Secretion of cecal mucous was not affected by this treatment and yields remained too small for analysis, but mucous in the small intestines was increased two-fold and was used throughout this study.

Initial centrifugation at 2 100 g as suggested by Creeth (1978), separated the mucus into a soluble fraction in the supernatant and insoluble gel in the deposit. The former was stored and the latter subjected to homogenization with a Sorvall Omni-mixer at 16 000 rpm in 0.2 M NaCl with azide. Centrifugation again at 2 100 g for 30 min removed debris and the supernatant was pooled with the first soluble fraction. Sugar and protein analyses were carried out at each step to monitor mucous components in separated fractions (see appendices 9 & 10). To destroy the rheological properties inherent in native mucin, reduction of the disulphide bonds was attempted with 0.4 M β mercaptoethanol in phosphate buffered saline at pH 7.5 for 12 h at 4°C under nitrogen gas phase. Retention of gel forming properties encountered in subsequent steps confirmed Forstner's observations (1978), that some small intestine mucins are resistant to this type of reduction. However, successful digestion of the gel was achieved by pronase (Boehringer Mannheim GmbH, FRG.). A suspension of 100 mg in 5.0 ml of 0.2 M ammonium acetate buffer at pH 6.5 was added to 50 ml of mucin solution. A drop of toluene was

added as preserve and the mixture incubated for 24 h at 37°C. Another 5.0 ml of enzyme suspension was added 24 h later and incubation continued for a further 24 h. The preparation was dialysed against distilled water overnight at 4°C. and frozen at -20°C.

Crude sub-unit mucin was purified by precipitation with 60 % (v/v) ethyl alcohol saturated with sodium acetate (Roden et al. 1972). The precipitate containing over 90 % of the original mucin was collected by centrifugation at 2 100 g, dissolved in 50 ml of 0.2 M NaCl and lyophilised in 1 ml volumes (Variyam & Hoskins, 1981).

Hog gastric mucin was purchased from Sigma Chemical Corp., as batches 33f076 and 54f0154. It was dissolved in 0.2 M NaCl and dialysed against distilled water for 24 h before chemical analysis.

2.8 Analytical methods

Protein was estimated by the standard copper method of Lowry et al. (1951), using human albumen to prepare a calibration curve (appendix 9).

Hexose sugars were estimated with the anthrone method (Siefter et.al.1950) using galactose as the standard (appendix 10).

Sulphate concentration was measured by the technique of Lloyd (1966) after acid hydrolysis (appendix 11). As there is no really satisfactory method for sulphate determination (Horowitz,1967), the barium chloride test was chosen for its convenience.

Sialic acids were estimated by the thiobarbituric acid method of Warren, (1959), modified by Codington et.al.,(1976), using N-acetylneuraminic acid (Sigma Chem. Corp.) as standard (appendix 13).

Glycoproteins were qualitatively estimated by the modification of the periodic acid-schiff reaction (PAS) designed by Mantle & Allen (1978). No

standard reference was available, but the technique was only used to compare relative amounts of mucins remaining in culture media after digestion by bacterial strains (see appendix 12).

2.9 Gas Liquid Chromatography (GLC) of carbohydrates

2.9.1. Developmental background.

There are many techniques for gas chromatography of sugars. The alditol acetate method discussed by Laine et.al., (1972) was initially attempted as it is reputed to give chromatograms in which the sugar anomers do not separate, as is the case with other derivatizations. Results showed inexplicable loss of some standards, notably, galactose, and the technique was not pursued. Silyl derivatization was adopted (Clamp, 1977 and Chambers & Clamp 1971) with greater success when the following procedure was used.

2.9.2 Hydrolysis.

Methanolic-HCl reagent was prepared by bubbling HCl gas, generated from a mixture of calcium chloride and concentrated sulphuric acid, into AR grade methanol. Molarity was measured by titration with M NaOH and bromo-thymol-blue and the preparation stored under nitrogen at -20°C at a concentration of not less than 1.5 M. Dried mucins or standards were hydrolysed in 0.5 ml of methanolic HCl for at least 3 hours at 85°C (Chambers & Clamp, 1977).

2.9.3. Re-acetylation.

A "knifepoint" of silver carbonate was added to the hydrolysate to neutralize the acid. Any insoluble carbonate would have been effective in this step although the silver salt possibly adsorbs less organic material

from the hydrolysate. Acetic anhydride (BDH), 0.2 ml, was added and the mixture left to react for 2 hours at room temperature. The sugars were titrated at least twice with methyl alcohol and evaporated to dryness under vacuum.

2.9.4. Derivatization.

Of all the silyating reagents available, a mixture of trimethylchlorosilane and hexamethyldisilazine have been most widely used for sugars (Laine et.al., 1972). However trimethylsilylimidazole (TMSI) is recommended by the manufacturers (Supelco Inc.) for chromatography of sugars and was used in this study. To the dry deposit from the previous step, 0.1 ml of a mixture of TMSI and pyridine (1:2) was added half an hour before required, as the preparation was found to be stable for less than 8 h.

2.9.5. Chromatography.

A Pye GC gas chromatograph with flame ionization detectors and chart recorder was used throughout this work. Glass columns, 1.8 m by 3 mm internal diameter were packed in the laboratory. A recommended packing of 10% SP2330 on supelcoport was too polar in nature, giving excessive retention of derivatives in the column, and greatly extended chromatography times. SP2100 at 5% concentration on 80/100 supelcoport gave unacceptable peak tailing which was corrected by increasing the liquid phase to 10%. Physical conditions included a nitrogen flow rate of 60 ml min^{-1} with temperature programming, using balanced columns, for 3 minutes at 190°C followed by an increase of $18^{\circ} \text{ min}^{-1}$ until 320°C was reached. The major disadvantage of this method was that anomeric and ring forms of sugars gave multiple peaks, but these could be resolved by adequate standard controls (Spiro, 1972). Chromatograms were run in duplicate and molar ratios were derived by summation of peak areas for each sugar.

2.10 Identification of blood group substances

An application of a simple hemagglutination reaction was adapted from hematological practice to detect blood group substances in mucin preparations. Commercial antisera to blood groups A and B (Ortho Diagnostics), were separately absorbed with mucin preparations of approximately 100 mg ml^{-1} in 0.5 ml volumes. After incubation for 1 h at 37°C , both absorbed and unabsorbed antisera were diluted twofold to 1:32 and added to homologous washed red cells (donated by fellow staff). Agglutination was recorded microscopically with clumps of more than 20 red cells considered positive.

2.11 Growth curves

Flasks containing 200 ml of M10 medium were prepared with the following substrates: glucose, galactose, and lactose at 1 g dl^{-1} ; pig gastric mucin at 1.5 g dl^{-1} and medium without fermentable substrate. The media were inoculated with suspensions of test strains, standardized to the density of a number 5 McFarland tube, and incubated on a rotary shaker in the anaerobic cabinet. Every hour, duplicate 5 ml samples were removed for optical density measurements at 420 nm. The samples were pooled and centrifuged at $2\,100 \text{ g}$ for 15 min and supernatants decanted. Cells were washed once in physiological saline with 0.05 M dithiothreitol and centrifuged to a pellet. Both the cell pellets and supernatants were stored at -80°C until required for enzyme studies.

When the A_{420} of bulk cultures reached 0.9, 50 ml volumes were removed, centrifuged at $15\,000 \text{ g}$ for 30 min and processed as described above.

2.12 Dry mass determination

Thin 10 mm x 100 mm glass tubes were calibrated in a Bausch and Lomb spectrophotometer, dried at 110° C for 2 d and weighed on an analytical balance. Cultures remaining at the end of growth curve experiments were diluted 10-fold in saline and 10 ml volumes of each dilution added to calibrated tubes. The optical density at 420 nm of each tube was measured, then the tubes were centrifuged and supernatants decanted. After drying at 110° C for 2 d the tubes and contents were re-weighed and dry mass determined by subtraction. The graph of dry mass plotted against A_{420} is given in appendix 15 together with the theoretical graph drawn from the formula given by Koch (1981).

2.13 APIZYM system for bacterial enzymes

A plastic tray with 19 cupules each containing a dehydrated chromogenic substrate, forms the basis of this test system. The substrates used to detect glycosidases are coupled with either 1- or 2- naphthyl groups, which react with Fast blue salt BB to produce coloured formazans when released by enzyme activity. Colour intensity is quantitatively proportional to the amount of enzyme activity in the samples. Substrates included in the APIZYM system for detection of glycosidases are the naphthyl derivatives of the following: α -D-galactose, β -D-galactose, β -D-glucuronide, α -D-glucose β -D-glucose, N-acetyl- β -D-glucosamine, α -D-mannose and α -L-fucose. Bacterial strains tested by the APIZYM method (API systeme, SA France) were grown in pre-reduced trypticase soy broth without added carbohydrates (appendix 16). Cells were harvested by centrifugation, washed in anaerobic diluting fluid and resuspended to an optical density of 0.4. Suspensions were tested by addition of 0.2 ml to each cupule and incubating the API trays in the anaerobic cabinet for 4 h. Colour changes were developed by exposure of the trays to bright sunlight for 2 min and compared with a chart provided by the manufacturer.

2.14 Bacterial glycosidases

2.14.1. P-nitrophenol substrates for assay of glycosidases.

Specific glycosidases release chromogen from p-nitrophenol glycosides. The colour is enhanced by addition of alkali and optical density at 420 nm is proportional to the amount of enzyme present in the sample when all conditions of the test are kept constant.

2.14.2. Preparation of samples.

Frozen cell pellets from growth curve measurements were thawed by the addition of 2 ml of phosphate buffer at pH 6.5 containing 0.05 M dithioethretol. The cells were disrupted by sonication at one third power output in a 50% pulse cycle for 10 min with an Ultrasonics instrument. Cell debris was removed by centrifugation and disruption confirmed by phase-contrast microscopy.

Enzyme proteins were precipitated from supernatants with ammonium sulphate. Berg et al. (1980) used a two stage technique for partial purification of bacterial glycosidases. They precipitated all proteins out of solution by the addition of salt to a final concentration of 70% and followed this with a second step to recover proteins that precipitate out between 25% and 70% salt concentrations. With this process they gained a 20-fold increase in the amount of enzyme. In this study, the addition of solid ammonium sulphate to 50 ml of culture supernatant to give a final concentration of 70 g dl^{-1} was used for crude first stage separation of proteins. The mixture was stirred at 4°C for 1 h, then centrifuged at 2100 g for 10 min. The protein deposit was dissolved in 10 ml of buffer at pH 6.5 and dialysed against buffer at 4°C for 24 h.

The protein content of all samples was measured using the Lowry method (appendix 9), and corrected to 1.0 mg ml^{-1} with buffer.

2.14.4. Substrates.

The p-nitrophenyl glycosides of α -L-fucopyranose, β -D-galactopyranose, β -D-N-acetylglucosamide and α -D-N-acetylgalactosamide (Sigma) were used to detect the enzymes most likely to be involved in degradation of rabbit mucin. Stock solutions of 5 mM were prepared in 0.2 M phosphate buffer at pH 6.5. This pH was used for all substrates, as Berg et al. (1980) report maximal enzyme activities of glycosidases from *Bacteroides fragilis* to be at pH optima of 6.3 for α -L-fucosidase (EC.3.2.1.51), at 6.9 for β -D-galactosidase (EC.3.2.1.23) and 6.4 for N-acetylglucosaminidase (EC.3.2.1.52). As the pH of the contents of rabbit cecum at the mid point was between 6.3 and 6.5 (see section 2.3) a compromise pH of 6.5 this was chosen for enzyme tests in this study.

2.14.5 p-nitrophenol calibration.

Stock p-nitrophenol solution (Sigma Chem. Corp), at a concentration of $10 \text{ } \mu\text{M ml}^{-1}$, was diluted two-fold in 0.5 ml volumes in buffer. One ml of buffer was added and 2 ml of M sodium carbonate. The A_{410} of each dilution was measured in a spectrophotometer and the graph of optical density plotted against concentration. (appendix 14).

2.14.6. Optimum incubation time.

Cells from a culture of strain W13, showing strong reaction in the APIZYM test for β -galactosidase, were used to determine the optimum time of incubation for detection of glycosidases. Volumes of 0.5 ml of cells in buffer, were added to 0.5 ml of 5 mM p-nitrophenol- β -galactosidase and incubated at 37°C for 5, 10, 15, 20 and 30 min. After the addition of

2 ml of sodium carbonate soln., the A_{410} was measured. The optical density of the 20 and 30 min samples were 0.83 and 0.85 respectively and at 15 min the OD was 0.72 on the linear part of the curve of optical density plotted against time (Appendix 16). A time of 15 min was therefore chosen for use in the enzyme assay.

2.14.7 The standard test for measurement of enzymes.

Volumes of 0.5 ml of crude enzyme samples containing 0.5 mg protein, together with 0.5 ml of phosphate buffer at pH 6.5 were warmed to 37° C. Substrates as 0.5 mM solutions were also held at 37° C and 0.5 ml volumes added to reaction mixtures which were incubated at 37° C for exactly 15 min. Two ml of M sodium carbonate were added and the optical density measured at 410 nm. The amount of enzyme in the sample was calculated from the amount of p-nitrophenol released in 15 min and specific activity derived from enzyme activity per unit mass of protein, expressed as nkat mg^{-1} . Controls run with all tests included a volume of buffer replacing the substrate, buffer replacing enzyme preparation and enzyme preparation heated to 85° C for 15 min to destroy activity. The volume of 0.5 ml of buffer was replaced by solutions of sugars in buffer to retain constant reaction volumes in inhibition studies.

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CHAPTER THREE

RESULTS

3.1 Ultrastructure of the rabbit cecal mucosa

An overview of the mucus blanket covering intestinal epithelia is shown in Figure 4a, although it must be admitted that preservation of intact mucus was not as complete as this in all preparations. A higher magnification of the edge of the mucous layer in Figure 4b shows a dense mass of microorganisms in the mucus. The exposed epithelial surface is relatively free of adherent bacteria. This image, although permitting visualization of bacteria *in situ*, is an artefact of the preparatory method. When mucus is stabilized by treatment with specific antisera (Roze et al., 1982), or by low-temperature processing (Bayliss & Turner, 1982; Richards & Turner, 1984), no bacteria can be seen within the layer, but glutaraldehyde fixation reduces the gel to a fibrillar network and microorganisms in the mucus can be observed as in Figure 4b.

Low-power TEM photographs of the mucosal layer of the rabbit cecum taken from approximately half way along its length, showed a simple columnar epithelium with a fringe of microvilli. There was a layer of microvesicles of varying depth separating the microvilli border from the mucous gel, which was well preserved in most sections, approximately 50 μm thick and heavily colonized by different types of bacteria. In some sections, amorphous lumen material was still adhering to the outer surface of the gel (Figure 5). Tubular glands indenting the epithelial surface (Figure 6) showed a higher density of mucus secreting goblet cells than was observed in the surface epithelium and the gland lumens were also colonized by a mixed bacterial flora. Higher magnification showed the



Figure 4a. Low-power view of the mucous blanket covering gastrointestinal epithelium in the rabbit. Digesta has been removed by careful washing to leave portions of mucus intact. Bar = 25 μ m

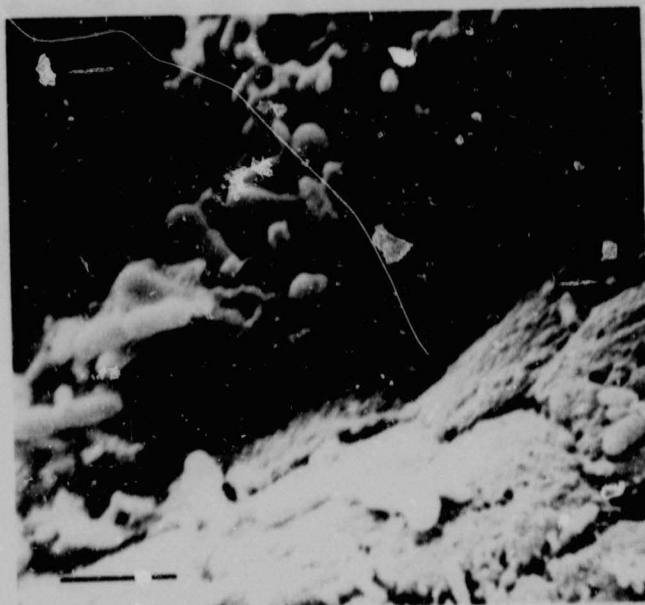


Figure 4b. Higher SEM magnification showing the edge of a fragment of mucous blanket as would be seen in the square marked on Figure 4a. Dense colonization of the mucus (above) contrasts sharply with the microvillus surface (below) with few attached bacteria. Bar = 1 μ m

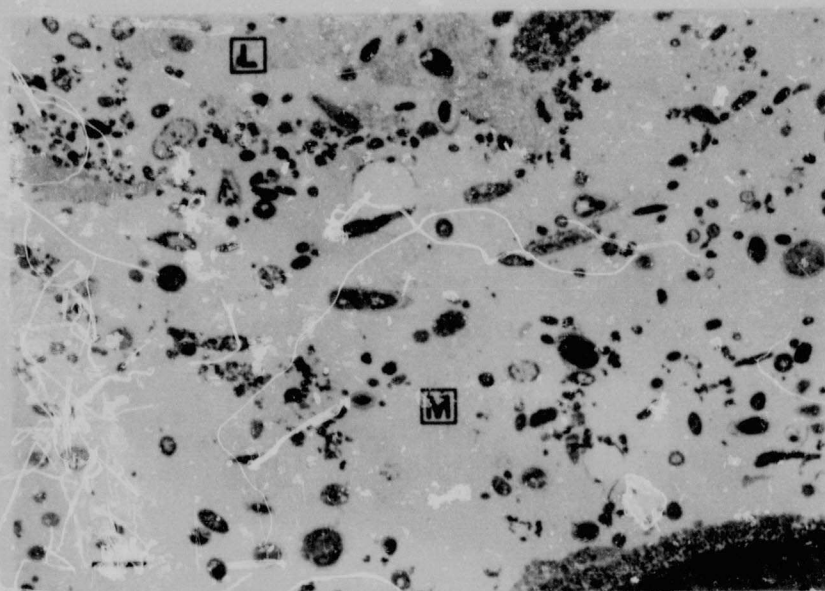


Figure 3. TEM section of rabbit cecum showing brush border (B), mucous blanket (M) and lumen contents (L). Bar = 2 μ m

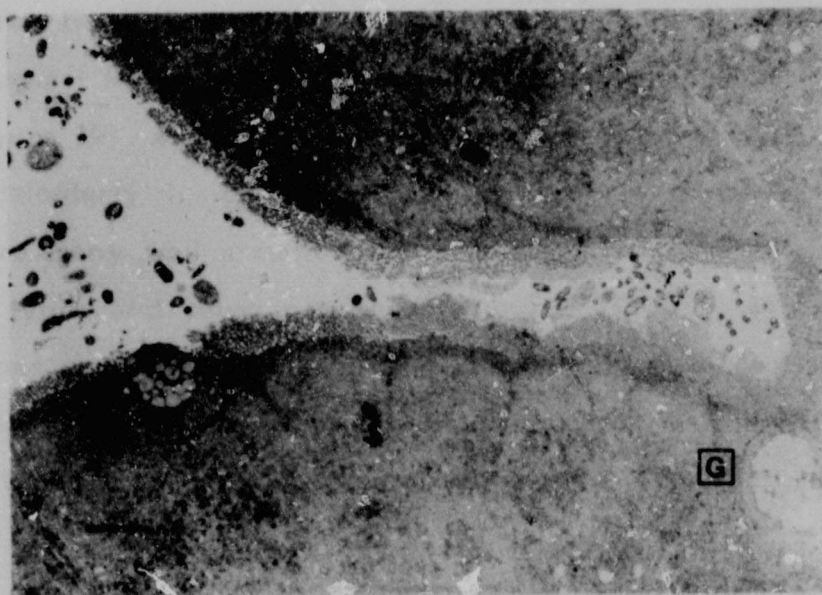


Figure 6. TEM section of tubular gland in the rabbit cecum. Two mucus secreting goblet cells (G) are to be seen in the gland epithelium. Bar = 2 μ m

microvesicular layer to be made up of small membrane-bound vesicles, 50 nm in diameter, infiltrating between the microvilli and sometimes free in the mucous gel (Figure 7). Although bacteria were rarely noted to be in contact with one or more microvesicles (Figure 8), none were seen to be adhering to the epithelial brush border in TEM sections.

Microorganisms embedded in the mucous gel were extremely diverse, judging from the different cell wall structures. There were many examples of the classical forms of Gram-positive (Figure 9) and Gram-negative cell walls (Figure 10), although there was considerable variation within each type, particularly in the nature and extent of glycocalyx formation. There were many wall structures that did not conform to either of the classical types and could not be categorized (Figure 11), and unusual morphotypes such as spores and spirillae were seen in sections. It was not possible, therefore, to make an assessment of the distribution of bacterial types by ultrastructural morphology in TEM sections.

Figure 12 shows a section in which bacteria are embedded in a gel matrix partially precipitated by gluteraldehyde fixation. The lucid zones around some bacteria may be artifacts, but they also suggest halos of cleared mucus, possibly formed by enzyme activity.

3.2 Bacterial populations associated with the rabbit cecum

Comparison of three culture media used for isolation of bacteria from cecal samples is given in Table 2. In general, the results of plate counts of samples on different media were very similar, although those on modified M10 medium were significantly higher and showed greater diversity than either the brain heart infusion agar or the rumen fluid-based medium. ($p=0.05$ when the data was analysed by the Student's *t* test) Total numbers of viable bacteria recovered from five rabbits by the plate count dilution method on modified M10 medium, varied between 10^8 and 10^{10} per ml of wet lumen contents with a mean of 10^9 . The mean of

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