Old Herborn University Seminar Monograph

1. MICROBIAL ECOLOGY OF THE HUMAN DIGESTIVE TRACT

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I. INTRODUCTION

This is a report of the first of a series of 'Old Herborn University Seminars'. These seminars will deal with the digestive tract and its complex microflora and will regard the total of mucosa and flora as a complex and vitally important organ. Like any other organ, the digestive tract has its specific functions of which digestion and preparation of food and control of colonisation by pathogens are well known. In addition, however, the digestive tract microflora may be involved in the pathogenesis of certain auto-immune phenomena due to malfunction of interactions with the immune system. Furthermore, it appears to have several endocrine functions, e.g. influences on several remote organs and organ systems. The complex organ - the digestive tract including its mature flora - differs from other organs as its composition is the resultant of chance and not merely of genetic factors. The composition of the flora, as we may hear in a future seminar in greater detail, is greatly determined in the months after birth. Finally, the composition of the diet, stress, disease and antimicrobial treatment may modulate or even alter the composition of the flora and therewith the function of this part of the organ. Differences in composition of the flora involve differences in influence; not only on processes inside the intestinal canal, but also on function and activity of remote organs.

To obtain good insight in this complex matter, it was realised that it had to be reviewed in a systematic way. Therefore, the first seminar was devoted to the most central issue; e.g. the 'micro-anatomical' structure and some local aspects of the functioning of this 'organ'. This regards the composition of the lumen flora as well as host factors, which influence its composition. Since the stability of the digestive tract ecosystems relies predominantly on the composition at the mucosal site, the first meeting was mainly focussed on the site where microflora and host organism meet; e.g. on activities in and around the mucous membrane.

In subsequent seminars, interactions of the flora with other organ systems than the gut itself will be discussed as well as consequences of various types of antimicrobial treatment for the microflora.

The second seminar will be devoted to the immune system. This organ system interacts intensely and continuously with the digestive tract and its microflora. Information about the mechanisms involved in the continuous controlling and guarding role of the immune apparatus on the microflora, is essential for the understanding of the fact that the flora of the digestive tract is: 1. stable for long periods of time and 2. inter-individually different. Only with this information, it may become better understood that the response to contamination with infectious agents differs between individuals. Also other less well-known influences and activities of the gastrointestinal tract flora may greatly differ between individuals of the same species. This regards for example the influence of the flora on bone marrow activity, clotting time, metabolic rate, etc. More specifically related to host-flora interactions, is the fact that antigens common to (autochthonous) bacteria and the host organism could play a role in the pathogenesis of certain autoimmune phenomena.

In the present report concerning the first seminar, we will start with some remarks made in the meeting concerning immune system-flora interactions at the mucosal site. The mucus as a nutritional source will subsequently be discussed, since the mucus layer and its composi-

tion are perhaps equally important for the understanding of stability and composition of the microflora of the digestive tract. After presentation of this basic information, we will discuss reported reviews regarding the composition of the flora of the alimentary canal and the occurrence of inter-individual differences. Finally, mechanisms will be discussed which cause, or predispose to, alterations of the flora.

The various aspects discussed in these last paragraphs may become subject of future seminars. They should therefore not be regarded as an attempt to comprehensively review the matter, but rather as a bridge to other aspects which may become of primary importance to the understanding of the pathogenesis of several diseases.

II. INTERACTION OF FLORA, IMMUNE SYSTEM AND MUCOSAL CELLS

1. Structure and Maturation Cycles of Digestive Tract Tissue

Lymphoid cells of different kinds are to be found in the mucosa along the whole length of the digestive tract. Some lymphocytes can be found in the loose connective tissue, the lamina propria, others between the epithelial cells as well as at various sites where organised lymphoid tissues are located; e.g. tonsils and Peyer's patches.

The intestinal lining formed by a sheet of enterocytes along the villi has the unique function of absorption of nutrients, water and electrolytes. In addition, it forms a tight boundary between microflora and underlying tissues. This boundary function may explain the presence of large numbers of cells of the immune system in the intestinal mucosa. These cells are dispersed singly or in small groups among the epithelial and connective tissue components of the intestines. The cells comprise T and B lymphocytes as well as NK cells together with monocytes, mast cells and eosinophils. Thus the gut has the (immune) cellular capacity for truly protective local immunity. Epithelial, stromal and lymphoid cells are continuously renewed, either because of their short life span or because they migrate away from the mucosa (*Parrott*, 1987). The crypts of Lieberkühn are the sites where enterocytes are produced. The degree and speed of cell extrusion at the tip of the villi on the other hand, regulates the length of the villi.

It is known that autochthonous flora influence the length of intestinal villi from experiments on germfree mice (*Abrams*, *Bauer* and *Sprinz*, 1963). It is also known that bacteria appear to adhere more readily to immature enterocytes (near the crypts) than mature enterocytes (*Stokes*, *Miller* and *Bourne*, 1987). It is tempting to postulate that the rate of crypt cell division is the consequence of dynamic interactions between autochthonous flora and the local immune system, but this postulate presupposes: a. that the local immune system can influence that rate of crypt cell division and b. that immune responses are mounted against autochthonous flora. This brief review summarises evidence that the rate of crypt cell division is influenced by local cell mediated immune responses.

Vital early experiments were carried out by Drs. Anne Ferguson and Tom MacDonald (*Ferguson* and *Parrott*, 1973; *MacDonald* and *Ferguson*, 1977, 1978): When grafts of foetal mouse intestine - which are empty and have no antigens within the lumen - were implanted heterotopically into adult mice of the same inbred strain, they grew and retained their morphology for several months. If donor and host animals were from different strains, grafts grew only for a few days. Then the grafts got infiltrated by lymphocytes and were rejected. Rejection of foetal mouse allografts is an apparent thymus dependent phenomenon, as it did not occur in thymectomised recipients. The most striking histological effect of rejection was seen before the mucosa was completely destroyed. Grafts became infiltrated by lymphocytes. Villi had become short or absent but the crypts of Lieberkühn appeared normal. These intestinal changes are very similar to those seen in intestinal biopsies in human coeliac disease and parasitic infection.

Because foetal intestinal allografts provided only small pieces of tissue for

examination, subsequently graft-versus-host disease (GvHD) was used to investigate more closely the time course changes in crypts and villi. GvHD was induced in five days old CBAxBalb/C F1 hybrid neonatal mice by intraperitoneally (i.p.) injection of parental spleen cells. A detailed analysis was then made of mucosal cytokinetics upon contact with bacteria. Comparisons between villi, crypts and crypt-mitosis in GvHD mice and untreated littermates used as controls, revealed striking crypt hyperplasia in mice aged 10 days. However, the villi were about the same height in both groups although there was some shortening in GvHD mice. Both studies could be regarded as providing evidence that crypt hyperplasia may be a compensatory response to loss of part of the mature enterocyte population. However, in the GvHD studies a 3 to 4 fold increase in crypt mitosis preceded shortening of the villi by several days. It can therefore be concluded that the crypt stem cell mitosis resulted from implanted (donor) lymphocytes. Later investigations with *Giardia* infected euthymic and athymic mice, indicated that these lymphocytes were presumably T cells. In *Giardia* infected animals, increased numbers of intra-epithelial lymphocytes were found and these cells appeared to be T cells. Like in GvHD mice, in these infected animals with increased (doubled) crypt mitosis rates

Table 1: Immunologically mediated enteropathy after injecting CBA spleen cells into CBAxBALB/c F1 mice*

	EL increase	Crypt depth increase	CCPR increase	Villus atrophy	Reference
1. Unirradiated	+++	++	++	-	*Mowat* and *Ferguson*, 1981
2. Lyt-2	++	+	+	-	*Mowat* et al.,
Lyt-1	-	-	-	-	1986
3. nu/+	++	++	++	-	*Mowat* et al.,
nu/nu recipients	++	+++	+++	++	1987
4. Lethally irradiated recipients		*Before Day 3*			*Mowat* et al.,
	++	++	++	-	1988
		After Day 3			
	-	-	-	++	
5. Unirradiat. recipients	++	++	++	-	*Mowat* and *Felstein*,1987
NK cell depleted recipients	+	-	-	-	

*All mice received untreated spleen cells from CBA donors except (2) when Lyt-2 depleted or Lyt-1 depleted spleen cells were used.

more rapid transit of enterocytes along the sides of the villi was seen, but no villus atrophy.

More recently, the GvHD model has been used to examine the relative contribution of helper/inducer, cytotoxic T cells or NK cells to the phenomenon of immunologically mediated enteropathy (including numbers of intra-epithelial lymphocytes (IEL), crypt depth, villous atrophy and crypt cell production rate) see Table 1 (*Mowat* and *Ferguson*, 1981; *Mowat* et al., 1986, 1987, 1988; *Mowat* and *Felstein*, 1987).

In summary, these experiments showed that the helper induced T cells (Lyt2-cell) is primarily responsible for the proliferative enteropathy consisting of crypt hyperplasa and increased numbers of intra-epithelial lymphocytes though natural killer (NK) cells also have a contributory role. Nude athymic and irradiated mice show the additional feature of villus atrophy, which is probably the consequence of (injected parent) cytotoxic T cell activity. The studies indicated that pre-T cells, T cells and NK cells are involved in controlling crypt cell proliferation rate (CCPR) and villus height in a complex way probably by the release of growth factors or interleukins. The GvHD model has therefore been a very useful model of pathological changes in the intestine. Further studies presently in progress using untreated athymic mice indicate that nude mice have a lower crypt renewal cell rate than normal mice. Mucosal T cells therefore may also regulate enterocyte growth under normal conditions, and normal conditions include autochthonous flora.

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2. Interrelationship of Digestive Tract Bacteria with Host Tissues and Immune Systems

The role of the immune system is commonly perceived as protector against infections and toxins and therewith maintains self-integrity. In this concept, autoimmune reactions are considered aberrant and harmful for the individual. It is generally accepted that the immune apparatus has a good recognition mechanism, which prevents it to respond with cellular or humoral immunity to many ‘self’ antigens to which it, comes frequently in contact. This specific unresponsiveness is related to the phenomenon of immunologic tolerance.

It has been known for many years that auto-immunisation with antibody formation is readily achieved with certain special tissues, in particular brain, lens, uvea, thyroid, testes, adrenals and pancreas. Also following a severe trauma with massive tissue (muscle) damage, (auto-) antibody production may be evoked transiently (*DeVay* and *Adler*, 1976; *Schattner*, 1986). These organs were said to contain 'organ-specific' antigens. No unusual manipulations of organ material were required to render the tissue constituents antigenic upon injection; immune responses are invariably transient in these cases. It is a tenet of immunology that reactions are determined in character by the category of the immunologic phenomenon and not by the identity of the antigen. This consideration applies to 'autoimmune' phenomena, all of which must be manifested as cytotoxic, humoral or combinations of these two. The degree to which immune responses become manifest depends on the antigen but also on the condition of the immune apparatus of the host. If an infectious agent lowers

the activity or turns off regulating cells an aberrant immune response may occur to 'self', to altered 'self', and to exogenous antigens. Within these categories, they give rise to lesions, which are indistinguishable from lesions produced by a response to exogenous antigens or to a complex of exogenous allergens with a tissue constituent.

Non-lnfectious Exogenous Antigens

Erythrocytes may be agglutinated or lysed by the action of antibody directed against antigens, which are normal constituents of the red cell blood groups. Such a reaction, of course, is the basis for cell damage in transfusion reaction, haemolytic disease of the new-born but also in some cases of 'acquired haemolytic anaemia'. The antigen involved can be either bacterial, e.g. pneumococcal polysaccharide which is capable of adhering to red cells and cause *in vivo* agglutination and/or lysis, or can be a certain drug (*Schattner*, 1986; *Springer*, 1971). This shows that bacterial antigens can cause an unfavourable immune response in the host by binding to host cells.

Bacteria with Antigens Common to Host (Connective) Tissues

There is accumulating evidence that bacteria and human organ specific antigens may have antigenic determinants in common. In other words bacteria may induce an immune response to auto-antigen upon infection. Unlike lesions associated with viruses, which involve a variety of parenchymal tissues, bacterial induced antibodies tend to affect predominantly connective tissue (*Wong*, *Skelton* and *Feeley*, 1985). Particularly in the joint synovialis, in which venous plexus exist in which haematogenous fragments of organisms or soluble immune complexes are readily trapped, immune cross reactivity may come to expression (*Schwab*, 1965). The clinical course of (experimental) post-infectious 'autoimmune' lesions is in general sub-acute, presumably being terminated by the gradual disappearance of bacterial antigen. However, the disease may recur following renewed infections with the organism.

Chronic Remittent Diseases

The chronic, remittent diseases that are presumably associated with bacterial infections are chronic reactive arthritis, ankylosing spondylitis and arthritis associated with psoriasis (*Mielants* et al., 1985). A fourth candidate is possibly chronic inflammatory bowel disease (*Cooper* et al., 1988).

Particularly the reactive arthritis including ankylosing spondylitis have been studied in relation with Gram-negative infections in several centres since the beginning of the present decade (*Ebringer*, 1983). It appears that this disease is associated with the HLA B27 tissue antigen. For example, in Europe this disease has been found to have a 90% relation with HLA B27. In Japan, an association of 66% among B27 positives has been reported versus 1% in a control population. Gram-negative bacilli such as *Klebsiella*, *Yersinia*, *Shigella* and *Salmonella* appear to have proteins in their outer membrane, which closely resemble HLA B27.

Van Bohemen and colleagues have investigated a family with diarrhoea as well as a larger outbreak of infectious diarrhoea in association with HLA B27 and arthritis (*Van Bohemen* et al., 1986a). Some of the family members were B27 positive, others not. All except one family member developed dysentery. Interestingly, only some family members developed arthritis. The two parents remained free but three of four children developed reactive arthritis. Notably, this was not confined to children who were B27 positive, while the

father - who did not develop arthritis - was found B27 positive.

In a larger outbreak of dysentery by *Shigella flexneri* in 62 patients, fortynine B27 negative patients did not develop the disease while seven B27 positive patients experienced also no or only rare transient pains of the joints. In five B27 positive patients with the HLA B27 antigen, reactive arthritis did develop. The *Shigella* appeared to be B27 positive. An interesting observation was that the HLA B27 positive patients who all developed the disease, displayed marked (mostly IgA) serum antibody titres against the *Shigella* strain (*Van Bohemen* et al., 1986b). Antibodies were found in these cases to belong to all major classes of antibody isotypes (IgA, IgM and IgG). Particularly the IgA antibody titre was much higher in the reactive arthritis patients than in those who remained free of the disease (*Van Bohemen* et al., 1986a).

Hypothesis

To explain the observed phenomena, three hypotheses have been postulated:

a. *Ebringer* and colleagues (1977) presume that antigens on the cell surface of these Gram-negative bacteria (*Enterobacteriaceae* species) carry HLA B27 antigen, which would induce an immune response to 'self'.
b. *Geczy* et al. (1983) presume that antigens on the cell surface of Gram-negative bacteria can be associated with receptors on tissues and these receptors are either B27 antigen or structures closely related to them.
c. *Geczy* and co-authors (1987) have recently put forward a complementary theory in which they propose that parts of a plasmid of Gram-negative bacteria, which code for certain antigens, can be transfected into human tissue cells. This would cause expression of the bacterial B27 antigen on the cell surface.
d. *Van Bohemen* and co-workers (1986 a,b) presume that their observations in diarrhoeal patients indicate that reactive arthritis is not only correlated with the presence of HLA B27 antigen on connective tissue cells. They state that if there are antigens on bacteria which resemble B27 antigen, this means that these antigens may not exclusively resemble cross-reactive antigens on synovial cells, as these antigens also exist on, for example, B and T cells. During the process of induction of immunity to the bacteria, both B cells and T helper as well as T suppressor cells are influenced. The latter are turned off by the infection due to enhanced contra-suppressor cell formation, while high antibody titres can be expected as were indeed found in their study.

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III. THE INTESTINAL MUCUS BLANKET

1. Effects of Specific Bacteria on Mucus Secretion

Mucus is secreted throughout the gastro-intestinal tract by a variety of specialised cells located in glands or in the surface epithelium. The prime function of the mucus layer is to protect the delicate underlying mucosa from damage by potentially harmful agents in the lumen (such as digestive enzymes and bacteria) and by the mechanical forces associated with digestion and the passage of solid material through the gut. Mucus exists in two distinct physical forms: 1. a thin layer of stable, water-insoluble gel firmly adhering to the mucosal surface and 2. a soluble layer mixed with luminal material that overlies the gel. The soluble mucus, although very viscous, can be removed from the mucosa by gentle washing. Both the adherent gel and the soluble mucus are composed of mucus glycoproteins (or mucins) which are responsible for the characteristic visco-elastic and gel-forming properties of the secretion. The relationship between the soluble and gel forms of mucus has not yet been clarified but, since proteolytic enzymes are capable of solubilising mucus gel and degrading mucus glycoproteins, it is presently believed that the sol layer is likely derived from the gel by a combination of enzymatic and mechanical erosion (*Allen* and *Carroll*, 1985). Therefore, to maintain an intact mucus barrier, erosion from the luminal surface must be balanced by secretion.

In mucosal sections fixed and stained for light or electron microscopy, the adherent mucus layer on the epithelial surface is difficult to visualise and often appears discontinuous. This is because many of the fixatives used, such as ethanol and glutaraldehyde, dehydrate the mucus causing denaturation and shrinkage of the gel. However, gastric mucus can readily be observed in situ on unfixed mucosal sections mounted transversely under the light microscope. The gel appears as a thin but continuous translucent layer of varying thickness (50 to 450 µm in man) between the surface epithelium and the luminal solution (*Allen* and *Carroll*, 1985). In the intestine and colon, a mucus 'blanket' (30 to 400 µm thick, dependent on species and region of the gut) lines the crypts and covers the villi and surface mucosa (*Sakata* and *Engelhardt*, 1981; *Rozee* et al., 1982). Observation of the continuity of the mucus layer in the intestine and co-

Table 2: Methods used for measurement of mucin secretion

1. *In vitro* or *in vivo* incorporation of radiolabelled precursors (e.g. $^{14}C/^{3}H$-monosaccharides, ^{35}S-sulphate) into tissue and release of high molecular weight labelled glycoproteins into secretions.
2. Colorimetric assays of glycoprotein or individual monosaccharide constituents (e.g. galactose, sialic acid) in secretions.
3. Determination of total carbohydrate content of secretions by gas liquid chromatography.
4. Ultrastructural morphologic assessment of mucus-producing tissue by light or electron microscopy, including autoradiography (presence/absence of full/empty goblet cells).
5. *In situ* determination of adherent mucus gel thickness over the surface mucosa.
6. Specific enzyme-linked or radio-immunoassays of mucin in secretions.

lon is particularly difficult but has been achieved at the electron microscopical level following stabilisation of the gel with anti-mucus antibody (*Rozee* et al., 1982). In these same studies, it was found that the surface mucus blanket contains a rich and varied population of indigenous bacteria and protozoa that are randomly distributed throughout the mucus layer but are physically separated from the underlying mucosa by the gel. Although most micro- organisms do not penetrate beyond the mucus barrier, some filamentous bacteria are anchored in the epithelium and are buried under or project into the mucus gel (*Rozee* et al., 1982).

Most of what is known about the effects of specific bacteria on intestinal mucus secretion is based on observations during particular infections. There are very few systems where goblet cells and mucin secretion have been studied in detail and any changes accurately quantitated. This is in part due to the multitude of difficulties associated with measuring mucin secretion. The gel layer adheres strongly to the mucosa and is therefore difficult to sample. Thorough washing of the tissue may not remove all the adherent gel, particularly that trapped in the crypts, and scraping of the mucosa inevitably damages the underlying epithelium leading to contamination by intracellular mucin released from ruptured goblet cells. Measurement of soluble mucin in the lumen is not a satisfactory index of secretion since an increase in the amount present may reflect greater erosion of the insoluble gel layer rather than actual mucin release. Notwithstanding these problems, a variety of methods have been used to assess gastro-intestinal mucin secretion (Table 2). Many, however, are associated with pitfalls and require careful interpretation. The major drawback to most of the techniques is their lack of sensitivity and/or specificity. For example, precursor radiolabelling and colorimetric assays measure all glycoproteins present in secretions and not just mucus glycoproteins (*Forstner*, *Maxwell* and *Romui*, 1981; *Specian* and *Neutra*, 1982). Ultrastructural, morphologic assessment of mucus-producing tissue may be used to determine whether goblet cells have emptied (*Specian* and *Neutra*, 1982; *Neutra*, *O'Malley* and *Specian*, 1982) but has severe

Table 3: Effects of cholera toxin on rat intestinal mucin secretion

	Amount of toxin	Potency ('blueing dose')	µg mucin released / mg tissue protein
Crude filtrate:	50 mg/ml	60	25
	25 mg/ml	30	22
Pure toxin:	50 µg/ml	950	15
	20 µg/ml	380	16
	10 µg/ml	190	17
Control:	no toxin	0	5

Rat intestinal tissue slices were incubated in vitro in modified Krebs-Ringer solution, pH 7.4, at 37°C, in a shaking metabolic incubator, in the presence of varying amounts of crude dialysed cholera filtrate or purified enterotoxin. The amount of mucin secreted into the incubation medium at the end of the 90 min incubation period was determined using a radio-immunoassay and mucin release was calculated relative to the protein content of the incubated tissue. Data taken from: Forstner et al., 1981.

quantitative limitations in terms of the amount of mucin released. Actual measurement of mucus gel thickness is possible in the stomach (*Allen* and *Carroll*, 1985) but cannot be reliably carried out in the intestine and colon due to the presence of crypts and the fragility of the tissue. Recently, however, the development of highly specific and sensitive immunoassays for intestinal mucins have allowed for accurate and reliable quantitation of secretion (*Forstner*, *Maxwell* and *Roomi*, 1981; *Forstner* et al., 1981; *Roomi* et al., 1984; *Mantle* et al., 1989).

There are two studies in which intestinal mucin secretion (as assessed by immunoassays) has been thoroughly investigated in response to specific bacteria, namely *Vibrio cholerae* (*Forstner* et al., 1981; *Roomi* et al., 1984) and *Yersinia enterocolitica* (*Mantle* et al., 1989). The first case represents an acute rapid response in mucin secretion to a bacterial toxin, while the second study examines the more long-term effects of an entero-invasive organism.

Following exposure of the rat small intestine to cholera toxin, mucin secretion is rapidly increased within 30 minutes (*Forstner* et al., 1981). Since crude cholera filtrate stimulates mucin secretion to a greater extent than purified enterotoxin (Table 3), it appears that the crude extract contains another potent (but as yet unidentified) mucin secretagogue. Cholera toxin binds to GM1 ganglioside in the cell membrane and ultimately activates adenylate cyclase, leading to increased intracellular cAMP levels and elevated fluid and electrolyte secretion from the epithelium into the intestinal lumen. However, a variety of agents that are known to raise intracellular cAMP levels and/or promote intestinal fluid secretion have no effect on mucin secretion (Table 4),

Table 4: Effects of agents that stimulate fluid secretion and/or increase intracellular cAMP on mucin secretion in the rat small intestine

Agent	% change in mucin secretion
Control	100%
Crude cholera filtrate (125 mg)	1000%
Pure cholera enterotoxin (20 µg)	950%
Dibutyryl cAMP (10^{-2}M and 10^{-3}M)	120%
+theophylline (10^{-3}M)	120%
Theophylline (10^{-3}M)	100%
Isoproterenol (10^{-3}M)	85%
+theophylline (10^{-3}M)	85%
VIP (2.10^{-7}M and 3.10^{-8}M)	100%
+theophylline (10^{-3}M)	100%
Mannitol (450 mosmol/1)	170%

In situ loops were constructed in the small intestine of anaesthetised rats. Control or test solution was injected into the loop lumen, the abdomen was closed and the animal allowed to regain consciousness. After 4 hours, the rat was sacrificed, the loop was removed, opened and carefully washed to collect all secretions. Control secretion (0.8 - 1.2 µB mucin protein/mg tissue protein) was normalised to 100% and test solutions were calculated relative to this baseline. All the above agents (with the exception of mannitol) increase intracellular cAMP and promote intestinal fluid secretion. Theophylline is a phospho-di-esterase inhibitor. Isoproterenol is a ß-adrenergic agonist. VIP, vaso-active intestinal peptide. Hyperosmotic mannitol stimulates fluid secretion but without increasing intracellular cAMP concentration. Data taken from: *Roorni* et al., 1984.

Table 5: Effect of *Yersinia enterocolitica* on rabbit intestinal and colonic mucin production

Mucin		Upper small intestine	Mid small intestine	Distal small intestine	Proximal colon
Tissue content (µglmg)	INF	86±12*	106±24*	121±13*	200±19*
	PFC	41±7	49±13	64±20	90±20
	CON	30±7	32±8	43±9	59±15
Gobletcells per 100 enterocytes	INF	7.2±0.2	8.7±0.1*	11.1±02*	ND
	PFC	7.5±0.1	7.0±0.2	5.5±0.1	ND
	CON	6.0±0.1	5.7±0.1	7.0±0.2	ND
Secretion (µg/mg)	INF	11±2*	12±2*	18±2*	22±3*
	PFC	6±1	6±1	7±1	7±3
	CON	5±1	5±1	6±1	7±2
Synthesis (^{14}C-dpm uptake/mg)	INF	37±45*	547±90*	900±200*	2375±375*
	PFC	128±20	144±19	158±23	783±178
	CON	165±32	164±39	159±42	470±106

Rabbits infected with *Yersinia enterocolitica* (INF) were compared on day 6 to pair-fed, malnourished (PFC) and unmanipulated, weight-matched control (CON) animals. Tissue samples from the upper, mid and distal small intestine and the proximal colon were homogenised and analysed for mucin content by an enzyme-linked immunoassay. Tissue mucin content (in µg) is shown expressed relative to tissue protein content (in mg). Further tissue samples were fixed and stained with periodic acid-Schiff reagent and haematoxylin and eosin. Goblet cells were counted and are expressed as the number per 100 enterocytes in a crypt/villus unit. The remaining tissue was incubated in vitro in modified Krebs-Ringer solution, pH 7.4, containing 25 µCi (^{14}C)-glucosamine, at 37°C in a shaking metabolic bath. After 90 minutes, mucin in the tissue and medium was measured by the immunoassay and secretion into the medium (in µg) was calculated relative to total (tissue + medium) protein content (in mg). Total glycoprotein synthesis was estimated from the amount of protein-bound radiolabel found in the tissue after 90 minutes of incubation and is calculated relative to total (tissue + medium) protein content (in mg).

ND: goblet cell and enterocyte numbers could not be determined in the proximal colon. No differences were detected between PFC and CON groups.

* $p<0.05$ for INF compared to PFC and CON groups.

suggesting that cholera toxin-induced mucin secretion, unlike fluid and electrolyte secretion, is not controlled via the adenylate cyclase-cAMP system (*Roomi* et al., 1984). In subsequent studies, it was shown that cholera toxin-induced fluid/electrolyte and mucin secretion in the small intestine could actually be dissociated by a number of drugs. Cycloheximide (a protein synthesis inhibitor), cytochalasin B (a microfilament disrupter), colchicine (a microtubule disrupter), and verapamil (an inhibitor of Ca^{2+} transport) all abolished cholera toxin-induced mucin secretion but had no effect on fluid/electrolyte secretion. Conversely, acetazolamide (a carbonic anhydrase inhibitor) decreased fluid/electrolyte secretion without inhibiting cholera toxin-induced mucin secretion (*George* and *Leitch*, 1983; *Njoku* and *Leitch*, 1983).

Thus, the secretory effects of cholera toxin are not all mediated by the same intracellular second messenger mechanism.

While similar rapid increases in fluid/electrolyte and mucin secretion are also observed following challenge with *Escherichia coli* enterotoxin (*Moon*,

Whipp and *Baetz*, 1971), not all enterotoxins behave in the same manner. For example, the toxins produced by various *Salmonella* and *Shigella* species promote small intestinal fluid secretion much more slowly (with a lag period of ~100 minutes) and do not apparently induce mucin release, as assessed by morphologic techniques showing no depletion of goblet cell mucin content (*Steinberg* et al., 1975; *Formal*, *Hale* and *Sansonetti*, 1983). In general, enterotoxin production causes fluid/electrolyte secretion in the small intestine without damaging the mucosa and it seems likely that this fluid secretion, either with or without concomitant mucin secretion, is a relatively early, non-specific mucosal response to bacterial toxins in an attempt to 'wash away' the organism and prevent colonisation of the gut.

Yersinia enterocolitica (YE) is an entero-invasive organism that causes severe gastro-enteritis. Rabbits infected with a human pathogenic strain of YE develop diarrhoea and weight loss by about day 3 (*O'Loughlin* et al., 1986). In the early stages of the disease, the organism penetrates the mucosa forming micro-abscesses throughout the small intestine and colon. By day 6 post-infection, mucosal abscesses become more localised to the ileo-caecal region, crypt hyperplasia occurs throughout the small intestine with villus atrophy in the ileum, fluid and electrolyte absorption are decreased, and the activities of brush border membrane di-saccharidases are markedly depressed. Thus, the diarrhoea developed by YE-infected animals likely results from maldigestion and malabsorption of nutrients, rather than active secretion (*O'Loughlin* et al., 1986; *O'Loughlin*, *Pai* and *Gall*, 1988).

Tissue mucin content is significantly elevated in the upper, mid and distal small intestine and in the proximal colon of infected animals compared to pair-fed, malnourished and unmanipulated, weight-matched control animals (Table 5). These results suggest either goblet cell hypertrophy or hyperplasia. In fact, a significant increase in goblet cell numbers could only be demonstrated in the mid and distal small intestine (but not in the upper small intestine) and, even then, the increase was not in proportion to the rise in tissue mucin content, suggesting that goblet cell hypertrophy also occurs during the disease. Mucin secretion along with total glycoprotein synthesis is markedly elevated in infected animals compared to pair-fed and unmanipulated control animals (Table 5). Interestingly, a graded response is observed down the intestinal tract of infected animals with the greatest impact on tissue mucin content, mucin synthesis and secretion occurring in the distal small intestine and the proximal colon where mucosal injury is most severe (*Mantle* et al., 1989).

Goblet cell proliferation and increased mucin secretion in the small intestine and colon are not unique to YE infections, since a variety of other entero-invasive organisms have been observed to produce similar effects, including *Escherichia coli* (*Khaviin* et al., 1980), *Salmonella typhimurium* (*Rout* et al., 1974), *Shigella flexneri* (*Rout* et al., 1975), and *Treponema hyodysenteriae* (*Hughes*, *Olander* and *Williams*, 1975).

Since stimulation of an inflammatory response by intestinal invasion accompanies enhanced mucin production and secretion, it appears that there is close integration between the immune component and mucus secretion in the host's mucosal defence system. The reason for the enhanced mucin production is not yet clear but it likely represents an attempt by the mucosa to push away a damaging organism and clear it from the body, to prevent the organism from gaining access to and further dam-

aging the tissue, and/or to allow epithelial recovery under a thickened mucus blanket, protected from potentially injurious agents (such as digestive enzymes and bacteria) in the lumen.

Little is known about the effects of indigenous bacteria on mucus secretion. Since the gut flora is capable of degrading the mucus barrier by production of extracellular and membrane-bound proteases and glycosidases, a natural balance must exist *in vivo* between bacterial erosion and mucin secretion. Indigenous microflora may also contribute to mucosal protection by a. modifying potential receptors in mucus for pathogenic organisms, and/or b. competitively inhibiting the attachment of pathogens to mucus and their subsequent multiplication in or penetration of the mucus layer. If the normal situation is compromised, for example, by malnutrition or a change in the gut flora by antibiotic therapy, tissue mucin content and mucin secretion decreases (*Sherman* et al., 1985). This can result in reduced mucosal protection in the host and therefore enhanced susceptibility to epithelial damage by indigenous bacteria or increased vulnerability to enteric pathogens. During bacterial overgrowth, mucin secretion is increased (*Sherman* et al., 1987), indicating that the normal gut flora can affect mucin production in particular circumstances. Whether interaction of these bacteria and the immune system is required for the stimulation of mucin secretion is not known at present.

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2. Mucus Structure and Physiology

The mucus gel creates a stable unstirred microenvironment at the mucosal surface which is - when measured under stirred condition - about 70 to 80 microns thick in the rat and about 180 microns in man. In the rat stomach topical administration of prostaglandin increases mucus secretion. Therefore, it is thought that increased mucus secretion is often mediated by prostaglandins.

Macroscopic and Microscopic Localisation of Mucus Production

Mucus is produced throughout the length of the gastrointestinal tract by a variety of cell types: oesophagus mucous glands, cardiac, fundic and pyloric glands of the stomach, Brunner's glands of the duodenum, goblet cells of the small and large intestines and superficial mucous cells found all through the tract (*Filipe*, 1979). The control of mucus production by these cells can occur at two levels:

a. secretion of preformed mucus, and
b. mucus biosynthesis.

In a normal mucus-secreting cell, one might expect these two processes to be interdependent, since an increase in secretion alone would soon exhaust the cell of mucus unless there was also an increase in its biosynthesis. However, stimulation of mucus secretion by excessive mechanical or chemical irritation might exceed the rate of biosynthesis and exhaust the mucus within the cells. This ultimately would cause a breakdown of the mucosal barrier. Scanning electron microscopy studies of anaesthetised dog gastric mucosa by *Zalewsky* and *Moody* (1979) have shown three mechanisms by which mucus is released:

a. a continuous exocytosis of a few granules at a time,
b. an explosive release of mucus by

apical expulsion of the older cells in the interfoveolar area and

c. the relatively rare event of cell exfoliation.

The explosive release of mucus from mucosal cells in contact with irritants is in keeping with apical expulsion (*Kelly* et al., 1979; *Zalewsky* and *Moody*, 1979), which is maximal on gastric mucosa away from the foveolae; this is the site of maximal drainage in gastric injury.

Stimulation of Mucus Secretion and Release

There is a large body of evidence that the output of mucus can be increased in the luminal juice by nerval, hormonal or chemical (irritants) stimulation. The production of a thick mucous secretion in the stomach in response to acid was first observed by *Ivy* and *Oyama* (1921) and has been observed with a number of other chemical irritants, e.g., mustard oil and ethanol (*Dinoso, Ming* and *Meniff*, 1976; *Forstner*, 1978).

Salivary mucus is stimulated by either sympathetic or parasympathetic stimulation. Two different glycoproteins or groups of related glycoproteins are formed, depending on which nerve is stimulated to evoke submaxillary secretion. Thus the ratio of sialic acid to fucose or hexosamines was higher and the size of the salivary glycoproteins smaller in secretion evoked by sympathetic than parasympathetic stimulation (*Dische* et al., 1970).

Stimulation of the splanchnic or vagus nerves or topical application of acetylcholine produces a copious gastric mucous gel in dogs (*Horowitz* and *Hollander*, 1961). Acetylcholine stimulated radioactive mucus release from rabbit and human colonic biopsies *in vitro* but did not affect the rate of glycoproteins biosynthesis (*MacDermott, Donaldson* and *Trier*, 1974).

The two best-documented hormonal stimulants of gastric mucus production are secretin and prostaglandins. Secretin increases the sugars of mucous glycoproteins in gastric juice from humans (*Andre, Lambert* and *Descons*, 1972), cats (*Vagne* and *Perret*, 1976) and dogs (*Kowalewski* et al., 1979). Prostaglandins, which, as judged visually, cause an increased production of viscous mucus (*Domschke* et al., 1978), produce a rise in the glycoprotein-bound sialic acid content of gastric washouts in man when given with pentagastrin (*Domschke* et al., 1978). Prostaglandins also increase the soluble mucus in rat gastric juice, although no change in the glycoprotein content of the surface mucous gel was observed (*Bolton, Palmer* and *Cohen*, 1978). Finally, various other gastrointestinal hormones, including gastrin, CCK-PZ, and histamine have been shown to increase the fucose and galactose content of gastric juice from cats prepared with fistulas or pouches (*Vagne* and *Perret*, 1976), although there was no information on viscosity or on whether the glycoprotein was degraded and therefore the result of peptic erosion. Both 5-TH and carbachol increased mucus production by the rat colon, as measured by increased hexose content of the perfused lumen *in vivo* (*Black, Bradbury* and *Wyllie*, 1979). Atropine abolished the action of carbachol and application of either histamine or isoprenaline alone had no effect on the hexose content.

Chemical and Physical Characteristics of Mucus

Mucus is a gel like agar. If one cuts agar it remains cut, but if one cuts mucus it reforms. Its protective function may rely on this ability to reform and to flow slowly along the epithelial lining. These properties enable mucus to make a continuous cover over the mucosal surface. The mucus gel is made up of complicated molecules, which are re-

sistant to pH 1 to 8 and are not dissolved by bile. Mucus is, however, affected by proteolysins - in particular pepsin and bacterial proteolysins - by reduction of disulphide bridges. By breaking these bridges, one dissolves the mucus. The quantification of mucus gel secretion poses special problems since it is necessary to distinguish qualitatively the adherent layer from both soluble mucus in the lumen and from the intracellular reserves of unsecreted, preformed mucus. To measure the mucus gel one of the most successful techniques is called mechanical spectroscopy (*Kerss, Allen* and *Garner*, 1982). In this technique, the mucus gel is put between two plates of a rheogoniometer. The top plate is oscillated under controlled computerised conditions. Very small oscillations do not break down the gel but disturb its structure. These oscillations are picked up by the plate below. If there is a solid substance between both plates, the two plates will move at the same frequency and phase. Frequency and phase of the bottom plate change when the substance between both plates becomes viscous to liquid. Purified glycoprotein of intestinal mucus has the same curve as the native gel. Treatment with pepsine changes the structure and therewith the curve registered by the bottom plate substantially. Studies with this apparatus have revealed that the gel glycoprotein consists of approximately 20% protein and some 80% carbohydrate chains. The latter chains are up to 20 chivers long surrounding the central protein core so protecting it from proteolysis.

The molecular weight of undegraded glycoprotein from pig gastric mucus, which is $2x10^6$ daltons, is markedly decreased in size to $5xl0^5$ daltons following proteolysis by pepsin and other proteases (*Allen* and *Snary*, 1972).

Furthermore, the undegraded glycoprotein is dissociated on reductive cleavage of the disulphide bridges with mercapto-ethanol into four equal sized subunits of MW $5xl0^5$ daltons (*Snary, Allen* and *Pain*, 1970), the same size as those obtained by proteolysis. However, there is also a bit of the protein core that has no carbohydrate around it. It has been shown that a separate protein of 70,000 MW joined to the glycoprotein subunits by disulphide bridges is the main component of the naked protein of each glycoprotein molecule (*Pearson* and *Allen*, 1980). The importance of naked protein and disulphide bridges to the gel-forming structures of glycoproteins from a wide variety of mucous secretions is clear from the ready solubilisation of these secretions by proteolytic enzymes and thiol reagents.

The structure of the carbohydrate chain consists of five negatively charged sugars in defined order. With this structure of carbohydrate chains of up to 12 sugars in size, it may become understood that mucus gels can in fact do differ between animal species and even between different sites in the digestive tract. These differences in carbohydrate chain cause a great antigenic variation. The terminal sugars of human mucus are alike glycoproteins of the blood group (ABH) system on erythrocytes and other cells.

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3. Gut Mucus as a Nutrient Milieu for Enteric Bacteria

Degradation of the Oligosaccharide Chains of Mucin Glycoproteins in the Colon

The gastrointestinal mucus layer's principal constituents are mucus glycoproteins (*Allen* and *Hoskins*, 1988). Each of these macromolecules is comprised of numerous oligosaccharide chains linked at their reducing end to a common polypeptide core, and each chain is comprised of from 2 to 18 glycosidically linked monosaccharides. Since the oligosaccharide side chains account for 70% or more of the dry weight of mucus glycoproteins the monosaccharides comprising the side chains would constitute an excellent endogenous carbon and energy source for enteric bacteria providing that their glycoside linkages could be cleaved. The longer oligosaccharide chains of mucin glycoproteins have a backbone of branched and unbranched sequences of alternating ß -galactose and ß -N-acetylglucosamine residues. Each chain is linked at the reducing end to the core polypeptide by a glycoside bond between a terminal α-N-acetylgalactosamine residue and the hydroxyl group of serine or threonine (Figure 1). In glycosphingolipids of gastrointestinal epithelium similar chains are linked to the ceramide lipid moiety via a "lactosyl" (galactosyl-ß-(1-4)-glucosyl-) sequence (Figure 2) (*Gustafsson* et al., 1986; *Bjork* et al., 1987).

The outer, non-reducing end of fully completed chains in mucin glycoproteins terminate in α-linked monosaccharides that include sialic acid and saccharides conferring ABH and Lewis blood group antigenic specificities. Similar α-linked glycosides are present on the non-reducing terminus of many of the oligosaccharide chains of gut mucosal

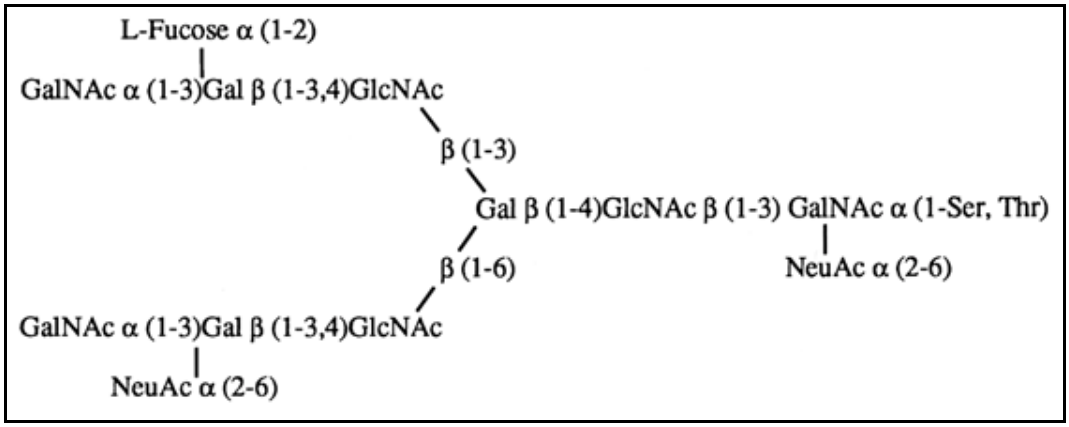

Figure 1: Structure of a typical branched oligosaccharide chain of mucin glycoproteins from rat (*Slomiany* et al., 1980) and human (*Podolsky*, 1985) colonic mucus showing the core N-acetylgalactosamine in an α-linkage with a serine or threonine residue in the polypeptide core on the right, a branched backbone of ß-linked saccharides, and α-linked saccharides at the outer, non-reducing end of the chain that include the blood group A and H antigenic determinant N-acetyl-galactosaminoyl-α(1-3)- and L-fucosyl-α(1-2)- moieties.
Abbreviations: GalNAc = N(acetyl)galactosamine, GLCNAc = N(Acetyl)glucosamine, Gal = galactose, NeuAc = N(acetyl)neuraminic acid, Ser,Thr = serine, threonine.

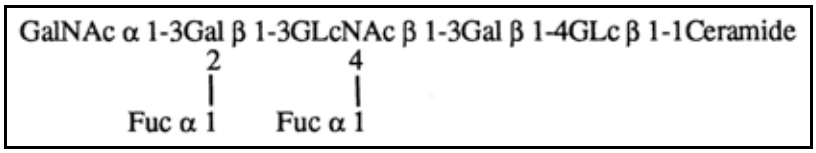

Figure 2: "A-7", a representative neutral glycosphingolipid isolated from human meconium and rat intestinal mucosal epithelium showing the galactosyl-ß(14)glucosyl-core sequence linked to a ceramide, and blood group A, H and Lewis-b a-linked saccharides at the non-reducing end. Abbreviations as in Figure 1.

glycolipids. Catalytic hydrolysis of each glycoside linkage requires a glycosidase, each of which exhibits a high degree of specificity for its particular substrate glycosidic linkage. This specificity is determined by the structure of the monosaccharide, the anomeric configuration of the linkage, the location of the carbon on the penultimate monosaccharide to which it is bonded, and, frequently, the structure of the penultimate monosaccharide itself. Thus, chain degradation requires the simultaneous presence of several glycosidases, each having the requisite specificity to degrade a specific glycoside linkage as it is exposed during sequential degradation of the chain. In the absence of a particular glycosidase further chain degradation will cease at the glycoside linkage it would have otherwise cleaved.

It is now clearly established that the enteric microbiota degrade the complex oligosaccharide side chains of secreted mucin glycoproteins and cell membrane glycolipids that are normally sloughed into the gut lumen. Studies have shown that large amounts of undegraded mucin glycoproteins are excreted in faeces of germfree animals but not in faeces of conventional animals nor in faeces of germfree animals monocontaminated with certain strains of enteric bacteria (*Lindstedt*, *Lindstedt* and *Gustafsson*, 1965; *Hoskins* and *Zamcheck*, 1968; *Gustafsson* and *Carlstedt-Duke*, 1984). Analysis of mucus constituents in gastrointestinal contents of healthy humans autopsied shortly after sudden death revealed high molecular weight glycoconjugates in ileal contents where indigenous bacteria are sparse and loss of these large molecules in the colon where bacterial populations are large (*Vercellotti* et al., 1977). Mucin glycoproteins added to sterile, anaerobic, bacterial culture media are extensively degraded during growth of bacteria following inoculation with faeces (*Hoskins* and *Zamcheck*, 1968; *Variyam* and *Hoskins*, 1981), and the cell-free supernates of these cultures as well as cell free faecal extracts will also degrade mucin glycoproteins (*Variyam* and *Hoskins*, 1981).

Degradation does not occur with initial colonisation in new-borns but is gradually established until 20 months of age when all faecal samples exhibit mucin degradation (*Norin* et al., 1985). A similar pattern also occurs with faecal glycosphingolipid degradation; it most likely occurs around weaning time (*Larson* et al., 1987).

Degradation of mucin glycoproteins by cell free faecal extracts and cell free supernates of anaerobic faecal cultures is due to their containing a multiplicity of highly active bacterial glycosidases required for degradation of the oligosaccharide chains of mucin glycoproteins (*Prizont* and *Koningsberg*, 1981; *Hoskins* and *Boulding*, 1976a; *Hoskins* and *Boulding*, 1981). Although endoglycosidases may be present and may play a role in chain degradation, evidence to date indicates that the major degradation is accomplished by sugar-

specific exoglycosidases acting sequentially to hydrolyse one glycoside linkage at a time beginning with the terminal sugar at the outer, non-reducing end of each chain. Yet, the ability to produce all the glycosidases required for chain degradation is a property that is restricted to a small number of normal commensal bacteria. In man the largest populations of these bacteria comprise a subset that averages 1% of cultivatable faecal bacteria (*Miller* and *Hoskins*, 1981). This was first clearly shown by Salyers and associates (*Salyers* et al., 1977a, 1977b) who made a systematic study of the ability to ferment hog gastric mucin by strains of bacteria isolated from human faeces. Out of a total of 342 tested, representing 8 genera and 32 species, only 8 strains fermented the mucin. These were 2 of 5 strains of *Bifidobacterium bifidum* and 6 of 9 strains of *Ruminococcus torques* (*Salyers* et al., 1977a). My colleagues and I obtained identical results using a different approach (*Hoskins* et al., 1985).

In our studies we tracked down and isolated the strains in fresh faecal samples that were responsible for producing the α-glycosidases which cleave the A, B or H blood group antigenic determinant glycosides from their terminal positions on the oligosaccharide chains of mucin glycoproteins. We found that production of these enzymes was strongly associated with the ability to degrade hog gastric mucin, which has blood group A, and H antigenic determinants. The 5 isolates we obtained were *Ruminococcus torques* strains (2), *Ruminococcus* AB (1), and *Bifidobacterium* species (2) (*Hoskins* et al., 1985). These are Gram-positive, non-sporulating, obligate anaerobes, which are normal inhabitants of the colon in healthy humans (*Moore* and *Holdeman*, 1974; *Holdeman*, *Good* and *Moore*, 1976) (Figure 3). The salient feature of these strains is their production of a variety of glycosidases that include ß-galactosidases and ß-N-acetylhexosaminidases that degrade the backbone of each oligosaccharide chain, sialidases, and one or more α-glycosidases that cleave the ABH and Lewis blood group sugars from the non-reducing end (Table 6). The strains differed from one another in their ability to produce the blood group-degrading α-glycosidases. Thus, all strains produced α-L-fucosidases that cleave the H and Lewis α-L-fucosyl moieties from the chains, but only the 2 *R. torques* strains produced a blood group A-degrading α-N-acetylgalactosaminidase, and only the *Ruminococcus* AB strain produced a blood group B-degrading α-galactosidase. When inoculated in culture medium containing B salivary glycoproteins from a blood group B secretor only the *Ruminococcus* AB strain was able to cleave the B antigenic determinants from the outer end of the chain. But, alone among these isolates, the *Ruminococcus* AB strain did not produce ß -N-acetylhexosaminidases so that its ability to further degrade the chains was limited. However, when this strain was co-cultured in medium containing B-salivary glycoprotein with one of the other strains that produced ß-N-acetyl hexosaminidases, a symbiotic association occurred wherein each supplied a glycosidase the other lacked, resulting in extensive degradation of the blood group B-salivary glycoprotein and greater growth of each than either had achieved when grown alone in the same medium (*Hoskins* et al., 1985).

Degradation of mucin glycoproteins by these strains is likely to be confined to the oligosaccharide moieties and not to the polypeptide core. Using azo-albumin as a general substrate we were unable to demonstrate any protease activity at pH 8.0 in culture supernates and cell sonicates of any of the 5 strains nor at pH 6.5 in one of these.

Table 6: Extracellular glycosidases produced by human faecal mucin-degrading bacteria (adapted from *Hoskins* et al., 1985)*

Strain	BGD activity** vs.			Glycosidase activity			
	A	B	H	Sialidase	ß-galacto-Sidase	ß-N-acetyl-glucosaminidase	ß-N-acetyl galactosaminidase
Ruminococcus torques IX-70	4+	0	2+	3+	4+	4+	2+
Ruminococcus torques VIII-239	4+	0	2+	3+	4+	4+	2+
Ruminococcus AB VI-268	0	4+	2+	2+	1+	0	0
Bifidobacterium bifidum VIII-210	0	0	3+	3+	4+	4+	2+
Bifidobacterium infantis VIII-24-	0	0	3+	2+	4+	4+	1+

* Enzyme activities, originally expressed as units per mg bacterial protein, are expressed here on a scale of 0, 1+ to 4+ for simplicity.
** BGD = Blood group degrading activity.

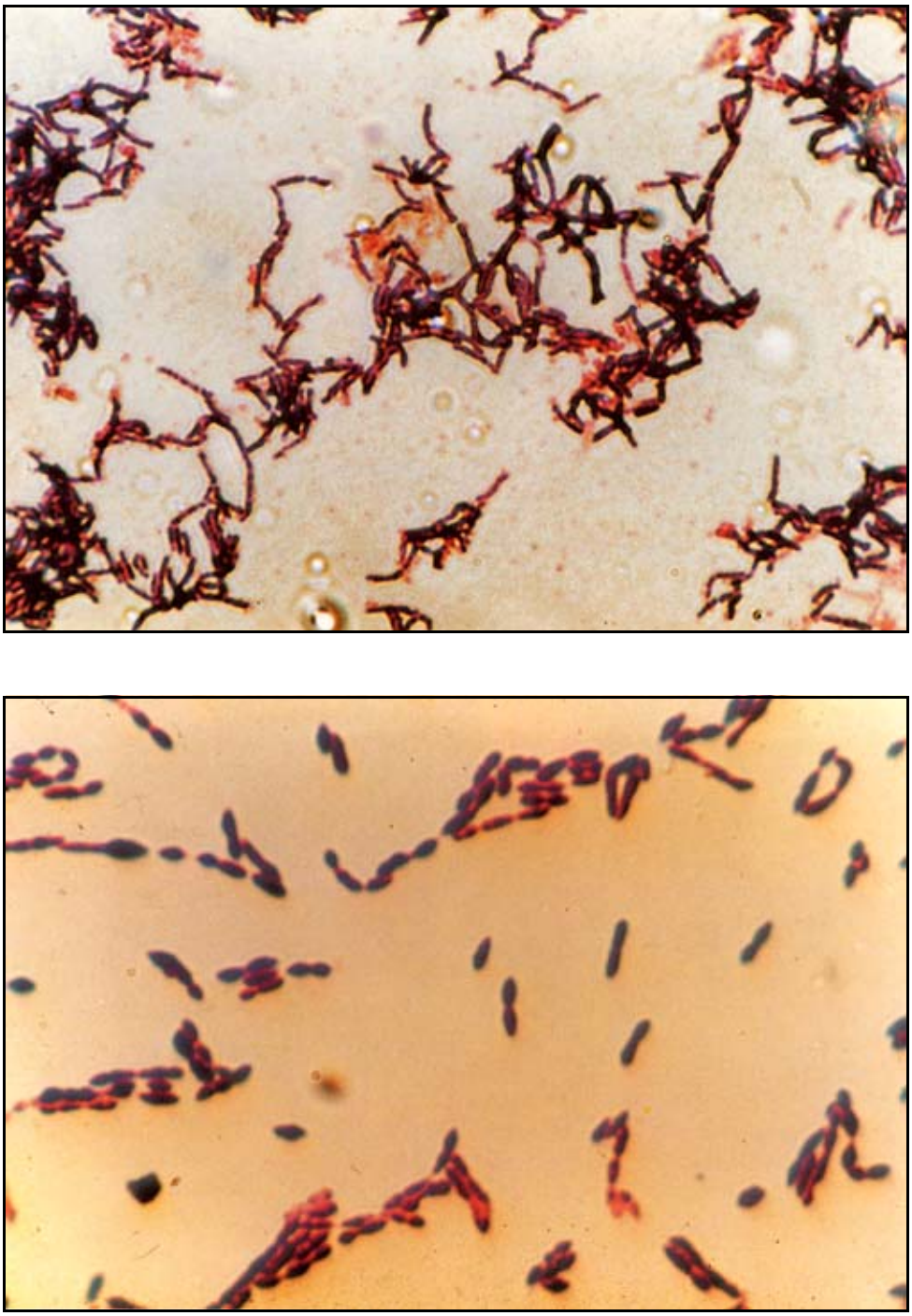

Figure 3: Gram-stained preparations of two mucin-degrading strains of bacteria isolated from human faeces.
Top: *Bifidobacterium bifidus* strain VIII-210. Bottom: *Ruminococcus* AB strain VI-268. Magnification: 500x.

A second feature of glycosidase production by these strains is that it is constitutive and not inducible (*Hoskins* et al., 1985). Competition studies in chemostat cultures (*Dykhuizen* and *Hart*, 1982) suggest that, under conditions where the concentration of nutrient substrate is low, strains that constitutively produce enzymes required for utilising that substrate have a competitive growth advantage over strains whose synthesis of these enzymes is substrate-induced. Such conditions probably apply to the caecum and colon; here the total amount of mucus secreted per 24 hrs is not known, but measurements of ileostomy secretions suggest that not more than 2-3 g of combined mucus secretions from stomach and small intestine enter the caecum every 24 hrs (*Englyst* and *Cummings*, 1986).

A third important feature of glycosidase production by these strains is that the glycosidases are released into the environment as extracellular enzymes (*Hoskins* and *Boulding*, 1981; *Hoskins* et al., 1985). Therefore they can act on a variety of complex glycoconjugates which may not be in the immediate vicinity of the bacterial cell that produces them.

These properties of glycosidase production by mucin-degrading bacteria distinguish them from most other enteric bacterial populations. Thus, some strains of *Bacteroides* and *Bifidobacterium* outnumber mucin-degraders by about 100:1 and dominate the enteric microbial ecosystem (*Moore* and *Holdeman*, 1974; *Holdeman*, *Good* and *Moore*, 1976). When we tested them we found that these strains possessed cell-bound sialidase, ß-galactosidase and ß-N-acetylglucosaminidase activities, but they lacked the α-glycosidases required to cleave the terminal ABH and Lewis blood group glycosides on the outer, non-reducing end of the mucin oligosaccharide chains and therefore could not make the initial cleavage to degrade such chains (*Hoskins* et al., 1985). These strains had a limited ability to degrade gut mucin glycoproteins and display little or no growth in anaerobic culture media containing intact mucin glycoprotein as the sole carbohydrate source (*Harouny* and *Hoskins*, 1983). It is presently unclear whether their lack of producing the α-glycosidases required for initial cleavage, or the cell-bound nature of their other glycosidases, or both factors, is the more important limiting factor in their ability to degrade mucins. We are currently performing experiments to clarify this point.

It is important to emphasise that the subset of mucus-degrading bacteria described above may not be the only bacteria capable of degrading mucus in human colon contents. Our quantitative studies suggest, however, that they constitute the largest populations of mucus-degrading bacteria in human faeces which produce the requisite glycosidases as extracellular enzymes (*Hoskins* and *Boulding*, 1981; *Hoskins* et al., 1972). Other incompletely characterised strains including *Bacteroides* sp. have been reported to ferment mucin (*Bayliss* and *Houston*, 1986) while some strains of *Clostridium perfringens*, which are recovered in low and variable numbers from human faeces, produce the requisite glycosidases for oligosaccharide chain degradation (*McGuire*, *Chipowsky* and *Roseman*, 1972; *Aminoff*, 1972). Furthermore, host species differences may influence the type of enteric bacteria degrading mucus glycoproteins. Thus, a mucin-degrading *Peptostreptococcus* was recently isolated from rat faeces (*Carlstedt-Duke* et al., 1986) while some *Bacteroides* strains isolated from swine colons have mucin-degrading properties (*Stanley* et al., 1986) (Table 7).

Table 7: Faecal bacteria that degrade mucin oligosaccharides

Source	Species	Reference
I. Human	Ruminococcus torques strains Ruminococcus AB strain Bifidobacterium strains	Saylers et al., 1977a; Hoskins et al., 1985; Bayliss and Houston, 1986
	"Unidentified Gram-pos. rods"	Bayliss and Houston, 1986
II. Pig	Clostridium perfringens A strains Bacteroides strains	Stanley et al., 1986
III. Rat	Peptostreptococcus strain	Carlstedt-Duke et al., 1986

Nutritional Consequences of Mucus Degradation for Enteric Bacteria

With few exceptions the great majority of enteric bacteria are saccharolytic i.e. utilise monosaccharides as carbon and energy sources. Monosaccharides of the types comprising the oligosaccharides of mucin glycoproteins and glycolipids are readily utilised by enteric bacteria *in vivo* and *in vitro*. Thus, only small amounts of these monosaccharides were recovered in the dialysable constituents of faeces from conventional rats in whom bacterial degradation of gut mucus is extensive (*Hoskins* and *Zamcheck*, 1968), and utilisation of galactose and other monosaccharides by several numerically dominant enteric species has been demonstrated during growth in pure cultures (*Hoskins* and *Boulding*, 1976a; *Miller* and *Hoskins*, 1981).

A study performed by Victor Harouny in the laboratory of Hoskins demonstrated how degradation of mucin glycoproteins by the subset of mucin degrader strains could have a nutritional support role for other enteric bacteria *in vivo* (*Harouny* and *Hoskins*, 1983). Fresh faeces from 5 healthy subjects were serially diluted and 10^{-10} g were inoculated into

a. anaerobic medium without any carbohydrate,
b. same as a. but containing intact hog gastric mucin as the sole carbohydrate source,
c. same as a. but containing a mixture of galactose, fucose and N-acetylhexosamine monosaccharides in the same amount as were present in the chains of intact mucin, and
d. same as b. but with the mucin preincubated for 24 hrs with the sterile culture supernate of the mucin-degrader strain R. torques IX-70.

This supernate contained the glycosidases that extensively degraded the oligosaccharide chains of the mucin prior to introducing the faecal inoculum. At 10^{-10} g faecal inoculum the mucin degrader strains were diluted out and only the larger dominant strains of enteric bacteria remained in the inoculum. Bacterial growth failed to occur in the anaerobic medium lacking any carbohydrate and was slight in the medium containing intact mucin. By contrast, there was marked and comparable growth in the media containing either the monosaccharide constituents of the mucin or mucin pre-degraded with the glycosidases produced by the mucin-degrader strain IX-70. *In vivo*, the extracellular glycosidases produced by this much smaller mucin-degrader population presumably would perform a similar function. Larger populations of enteric bacteria could be sustained by these means under conditions such as host fasting when fermentable dietary carbohydrates are not entering the

caecum. It has been suggested that about 70 g of fermentable carbohydrate are required daily by enteric bacteria in order to sustain their colonic biomass (*Smith* and *Bryant*, 1979). This is far in excess of the 1-2 g mucus recovered daily in ileostomy effluent (*Englyst* and *Cummings*, 1986). Therefore, it is very doubtful that the nutritional needs of enteric bacteria could be met by gut mucus secretions alone. Nevertheless, the continuous secretion of mucin into the mucus layer overlying the mucosa probably sustain focal bacterial populations in the micro-environment of the mucus layer with the help of glycosidases from mucin degrader strains.

The amount of carbohydrate in epithelial cell glycosphingolipids shed daily into the gut lumen is negligible compared to the normal carbohydrate consumption of enteric bacteria. For example, only 1-2 mg is excreted per day in faeces of germfree rats (*Gustafsson* et al., 1986). In man, one can estimate the amount of mucosal glycolipids entering the lumen daily from measurements of epithelial shedding rates. About 6-12 g of small intestinal epithelial protein is lost by epithelial cell shedding each day (*Croft* and *Cotton*, 1973). With the glycosphingolipid concentration of intestinal epithelium at 8-10 mg per gram protein (*Bjork* et al., 1987), this is equivalent to a daily shedding of 50 to 120 mg of glycosphingolipids.

Another feature of oligosaccharide chain degradation pertinent to microbial nutrition is that it enhances the susceptibility of the polypeptide core of mucus glycoproteins to proteolytic degradation. Colonic contents exhibit strong protease activity (*Hoskins* and *Boulding*, 1976a; *Variyam* and *Hoskins*, 1983). While the intact multimeric mucin glycoprotein molecule has unglycosylated regions of the polypeptide core that are susceptible to cleavage by proteases, the intervening highly glycosylated subunits resist proteolysis, probably because of steric hindrance by the numerous oligosaccharide chains linked to the core polypeptide in these subunits. We found that if the oligosaccharide chains were first degraded from these subunits by incubation with the glycosidases in cell free culture supernates of mucin degraders, the underlying polypeptide core became highly susceptible to pancreatic proteases (*Variyam* and *Hoskins*, 1983). Although ammonia is readily utilised as a nitrogen source by many enteric bacterial strains (*Bryant*, 1974), others appear to require amino acids, and some seem to utilise peptides better for growth than mixtures of amino acids (*Bryant*, 1974; *Russell*, 1983). The gut mucin polypeptide core is highly enriched in serine, threonine, and proline (*Allen* and *Hoskins*, 1988). Hence, it would be of ecological interest to determine whether enteric bacteria might obtain special growth advantages from utilising fragments of this mucin core polypeptide obtained by proteolytic degradation of the deglycosylated subunits.

Other Actions of Glycosidases from Mucin Degraders in the Gut Lumen

The extracellular glycosidases produced by mucin degrader strains of enteric bacteria act not only on the oligosaccharide chains of mucin glycoproteins but also on related oligosaccharides on the surface of enteric bacteria. Thus, faecal extracts and the cell free supernates of anaerobic faecal cultures will degrade the blood group B-like antigen from the surface of *E. coli* 086, releasing the antigenic determinant α-D-galactose from the surface structures (*Cromwell* and *Hoskins*, 1977). Such actions *in vivo* may markedly alter the spectrum of bacterial surface antigens presented to the host's mucosal barrier from that found in pure cultures of the

same bacteria. Immune responses of the host to enteric bacteria which lead to autoimmune tissue damage have been implicated in the pathogenesis of idiopathic ulcerative colitis, some forms of arthritis, and more recently Graves' disease (*Fiocchi* and *Farmer*, 1987; *Inman*, 1987; *Heyma*, *Harrison* and *Robins-Browne*, 1986). Degradation of bacterial antigens in the gut lumen by bacterial glycosidases from mucin degraders and by intraluminal proteases may ordinarily protect against such immune responses.

Extracellular glycosidases from mucin degrading bacteria also act on glycosphingolipids extracted from the gut mucosa. Recently, *Larson*, *Falk* and *Hoskins* (1988) showed that glycosidases from cell free culture supernates of mucin degrader strains rapidly degraded the oligosaccharide of chains of GM3 and the blood group active fucoglycolipids extracted from human meconium and rat intestinal mucosa. While the amount of mucosal glycosphingolipids entering the colonic contents from sloughed epithelium is too small to be a major source of nutrient carbohydrate for enteric bacteria, the significance of their degradation may be on its effects on the functions of glycosphingolipids on the luminal membranes of colonic mucosal cells. There is considerable evidence to indicate that the oligosaccharide moieties of cell surface glycolipids have an important biological role as receptors for macromolecules like cholera toxin as well as binding sites for bacterial adherence (*Curatolo*, 1987; *Holgersson* et al., 1985; *Bock* et al., 1985). One of the latter, lactosylceramide, preferentially binds strains of several genera of enteric bacteria when these are overlain onto developed thin layer chromatograms of mucosal glycolipids (*Holgersson* et al., 1985; *Bock* et al., 1985; *Hansson* et al., 1983). We found that lactosylceramide was the principal product accumulating during degradation of more complex glycosphingolipids by glycosidases from 4 of the 5 mucin degrader strains (*Larson*, *Falk* and *Hoskins*, 1988). Thus, glycosidases from mucin degrader strains may have another important ecological role in the gut lumen: they may promote adherence of normal commensal bacterial populations by degrading mucosal glycolipids to simpler structures like lactosylceramide.

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4. The Role of Lectins in the Organisation of the Mucus Blanket

As mentioned in previous paragraphs, the intestine is covered by a very thick mucus blanket of up to 200 microns. This makes it very difficult to process for electron microscopy. However, by stabilising the mucus layer using antibodies developed against the mucus of the host species concerned (*Costerton*, *Rozee* and *Cheng*, 1983), it is possible to hold the mucus blanket on the tissues during careful processing (Figure 4).

In the mucus layer covering the mucosa of adult host individuals, a large bacterial population can be seen (Figure 5). In addition, in some, protozoan species have been observed (Figure 6). In the bottom layer of the mucus very few types of bacteria appear to be attached. In fact, often as few as only two types of organisms may be found in large numbers associated with the intestinal surface. The majority of the bacterial population of the mucus is not actually in contact with the mucosal surface but inhabits the overlying mucus (Figure 7).

To obtain better insight in the role of the mucus in determining the colonisa-

tion pattern of the gut, new-born animals have been studied where the mucus structure is not fully set up. In four-hour-old calves electron-microscopic sections revealed villi with goblet cells starting to produce fairly large amounts of mucus (Figure 8) and large amounts of bacteria associated with these pockets of mucus. The general, yet uncovered, microvilli were not associated with large numbers of organisms. In case of neonatal diarrhoea in the calve, like in *E. coli* K99 diarrhoea, this leaves a 'window' of uncovered tissue. The intestine is relatively bare of mucus at this stage so that the *E. coli* K99 can adhere to the surface using their pili, as was seen when monoclonal antibodies were used specific for these pili (Figure 9). This experiment of nature shows that when there is not a full and complete mucus blanket over the tissue, the intestine is very vulnerable to infection.

In calves of six days, the surface of the microvilli is completely covered (Figure 10). At that time, the mucus is nicely continuous and a large 'natural bacterial population' in this mucus surface renders these animals not longer susceptible to neonatal diarrhoea. It could therefore be interesting to the agricultural science to put in a large number of 'natural' (bovine) bacteria immediately after birth in order to initiate rapid colonisation as soon as mucus secretion starts. By inoculating calves orally with large numbers of organisms of 29 different species isolated from the cow, a better weight gain has been obtained in an experimental station (Table 8). In addition, it was found absolutely impossible to implant the enterotoxin producing *E. coli* K99.

Other elucidating studies have been done in the rumen of calves. For colonisation of the roots of plants, lectins - proteins with affinity for specific carbohydrates - are extremely important. This effect of lectins is not confined to plants. In the rumen, the cow makes a lectin with affinity for certain organisms, the great majority of which is Gram-positive (Figure 11). The presence of relevant lectins is important for proper colonisation of the rumen. This may explain why natural colonisation in the rumen of the calf is fairly complete at four days after birth and absolutely complete at ten days (*Cheng*, *Irvin* and *Costerton*, 1981). This ruminal population stays with the animal throughout its life. Obviously, serious physiologic disturbance may occur when this bacterial population is affected by antibiotic therapy.

Investigations in the neonatal gut of the rat have been performed to study the hypothesis that endogenous lectins call in and mediate with a specific beneficial population of bacteria. The approach was indirect by adding exogenous lectin - phytohaemagglutinin (PHA) - to the system (*Banwell* et al., 1985). This had a specific effect as instead of the 'clean intestinal surface' with a very few adherent bacteria of the normal untreated rat, overgrowth was observed by cocci as well as rods (Figure 12). These organisms colonised down the microvillous surface, which affected the tissue very profoundly. Influx of leukocytes (lymphocytes) was found associated with a great thinning of the mucus. Scanning electron microscopy provided the same picture: Large numbers of cocci and rods at the surface of the villi and far less mucus than there would normally be present (Figure 13).

The explanation for the dramatic effect of PHA on the type of mucosal colonisation could be as follows: when the mucus is excreted by goblet cells, it shows an affinity for neighbouring tissue. It would roll over in a highly structured way (Figure 14). In this manner the thick layer of complex structured mucus, as is seen in older animals, is built up; a process in which

Table 8: Effect of inoculation on weight gain

Animals	Weight (kg) at slaughter	% Increase over control	Digestive tract (kg)	% Increase over control
Control	26.5	-	2.1	-
Treated	32.4	23	2.6	24

endogenous lectins play an important role. There are two possible ways in which PHA could have deranged the system. The first possible explanation is that PHA could have affinity for the bacteria and take them to the surface of the microvilli. The second possible explanation to be considered is that PHA attaches to the villi and thus prevents normal spread and rolling over of endogenous lectins so that bacteria can attach directly to the microvillous surface (Figure 15).

Bacteria, isolated from the overgrowing population in PHA treated newborn rats, did not show affinity to PHA *in vitro*. Therefore, the second hypothesis is the most likely, e.g. if PHA is an antagonist of endogenous lectins and thus interferes with the function of the endogenous lectin, then, rather than having a well structured mucus over the surface of the microvilli, there may be an aperture where the tissue is exposed to luminal bacteria (Figure 15). Then, bacteria that would normally not or only sparsely grow in the mucus blanket would be able to express their very high affinity for tissue and colonise it to cause the damage reported above.

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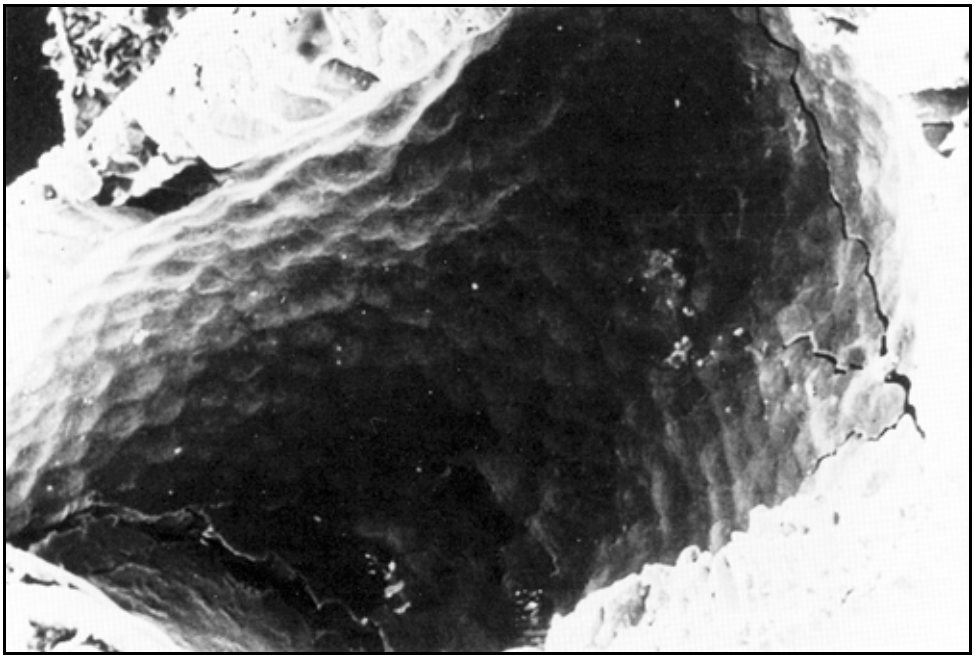

Figure 4: SEM of the surface of rat intestine showing the complete retention of the thick mucus layer.

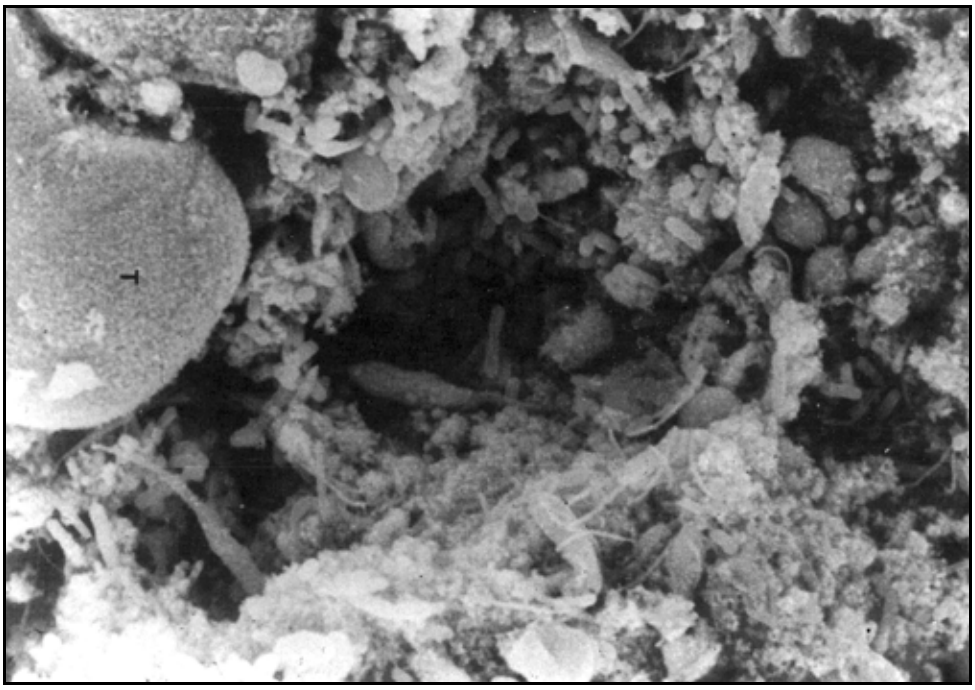

Figure 5: Higher magnification of the same SEM showing large bacterial populations within the mucus blanket.

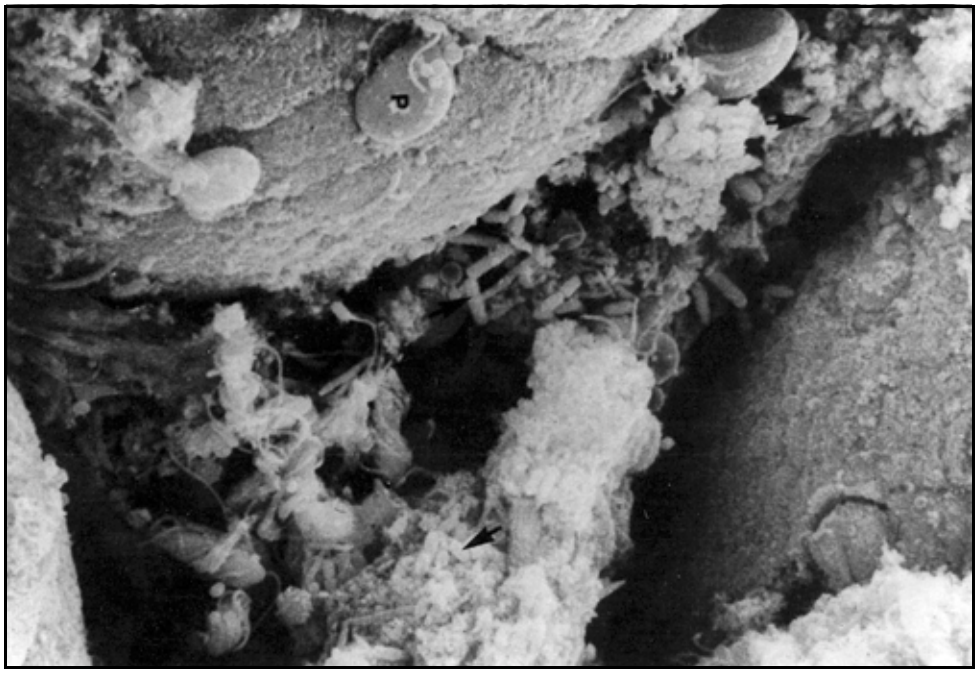

Figure 6: High magnification SEM of the same tissue surface showing adherent protozoa (*Giardia*) in the mucus blanket of the normal intestine.

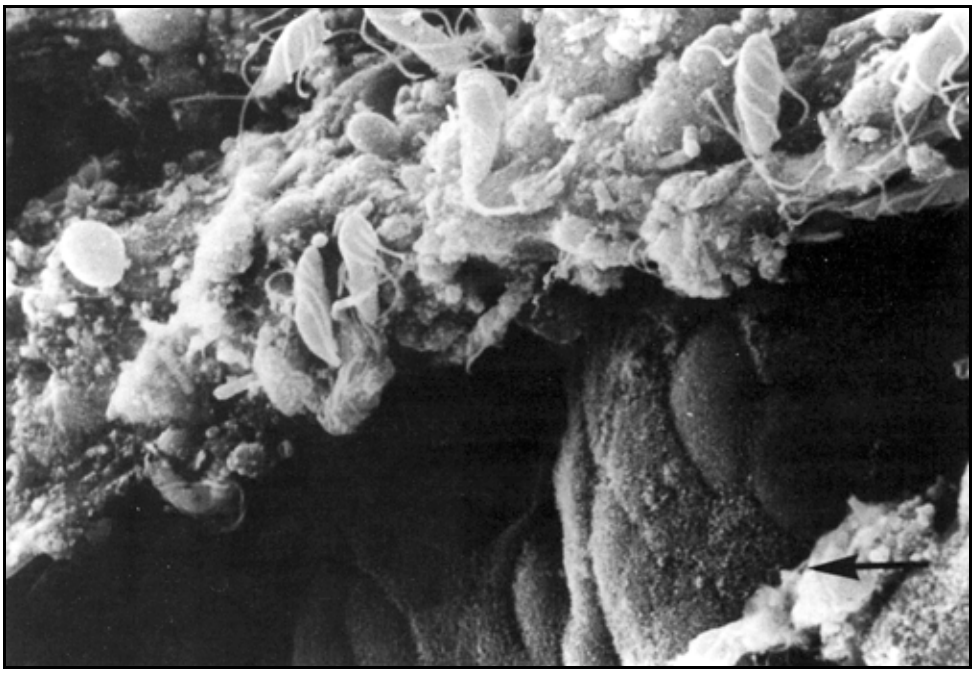

Figure 7: SEM of the intestinal surface of a different rat showing the association of bacteria and protozoa with the mucus layer and their absence from the microvillar surface.

Figure 8: SEM of the intestinal surface of a four-hour-old calf showing the production of mucus masses by individual goblet cells.

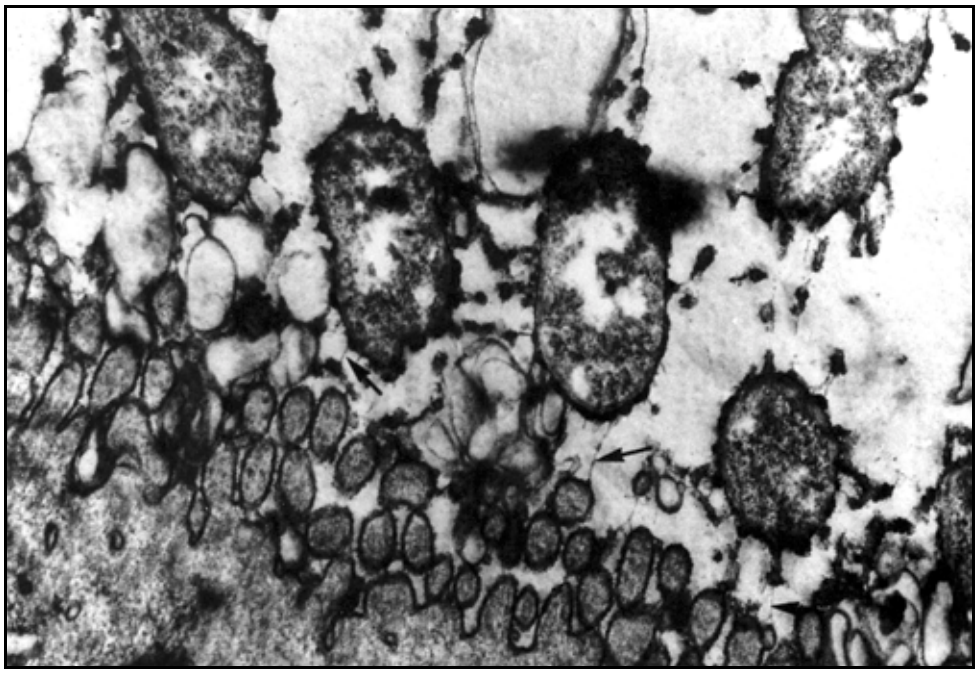

Figure 9: TEM of the surface of the intestine of a new-born calf infected by a K99 strain of *E. coli* and treated with specific anti-K99 pilus antibody in order to visualise these pilus structures (arrows).

Figure 10: SEM of the intestinal surface of a six-day-old calf showing the complete occlusion of the tissue surface by a mucus blanket colonised by bacteria.

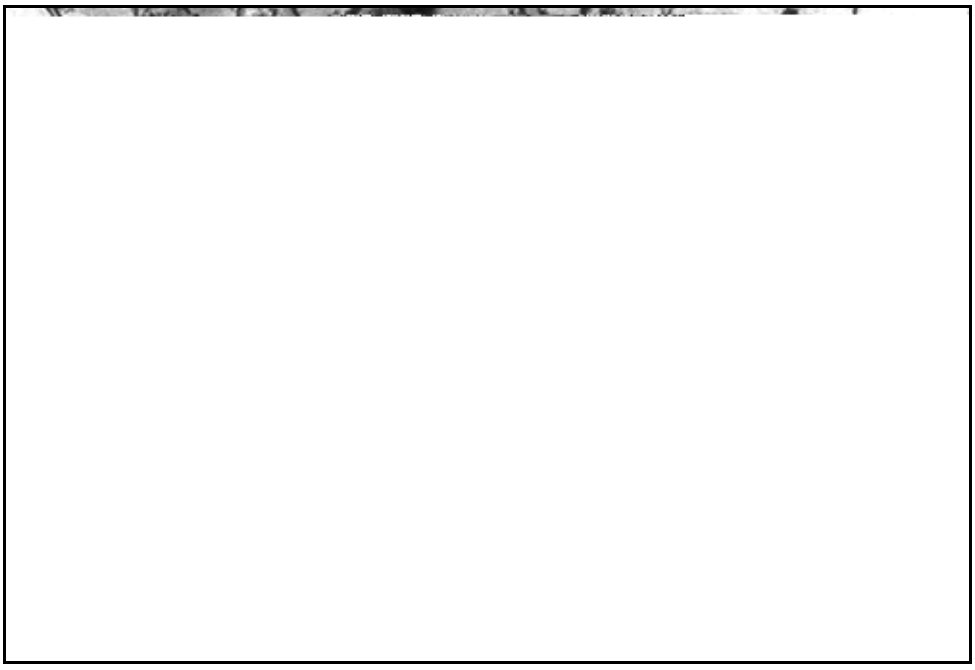

Figure 11: TEM of the surface of the bovine rumen showing a preponderance of Gram-positive bacteria next tot he tissue in a complex layer of adherent bacteria.

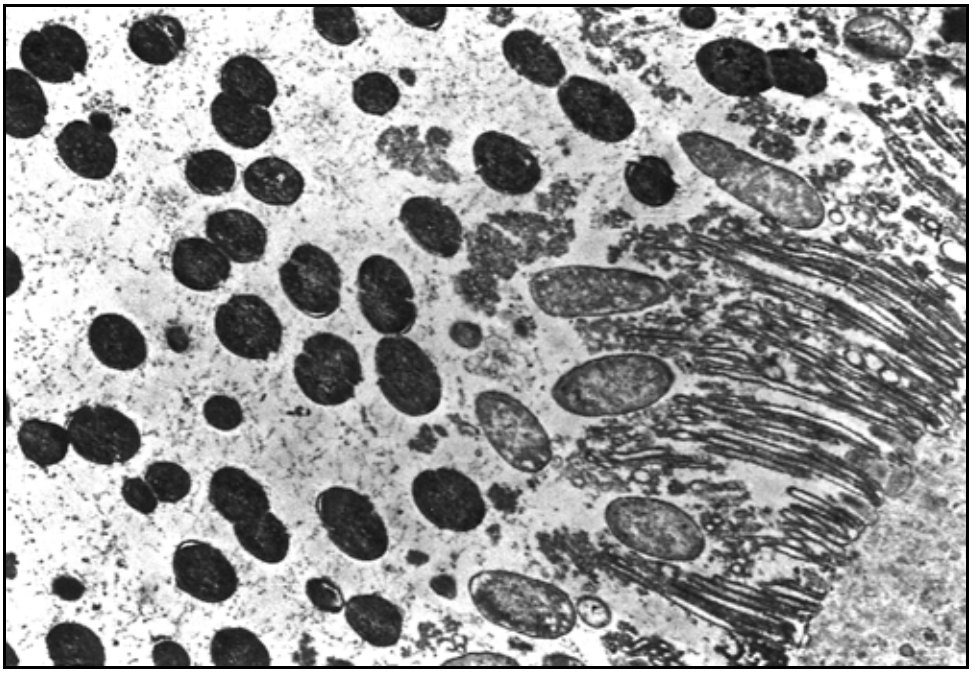

Figure 12: TEM of the surface of the intestine of a PHA-fed rat in which the microvillar surface was heavily colonised by Gram-negative rods and Gram-positive cocci.

Figure 13: SEM of the intestinal surface of the same animal seen in Figure 9 showing both rods and cocci embedded in the microvillar layer of the colonised intestine.

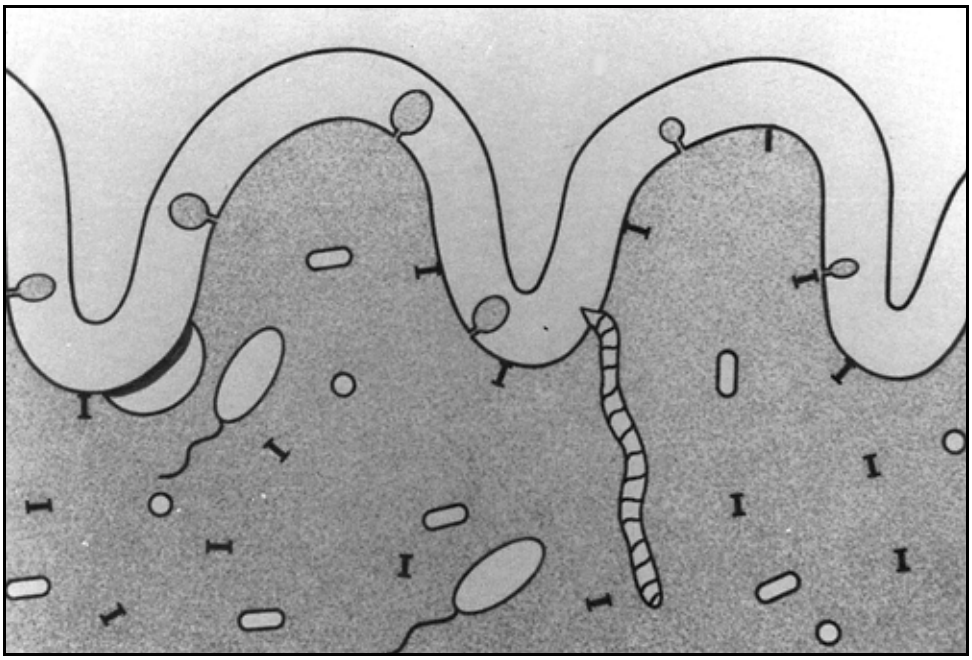

Figure 14: Diagrammatic representation of mucus secretion in the normal intestine in which mucus is produced in goblet cells and forms an even layer because of the affinity of natural lectins (⊢) from both the tissue surface and the mucus.

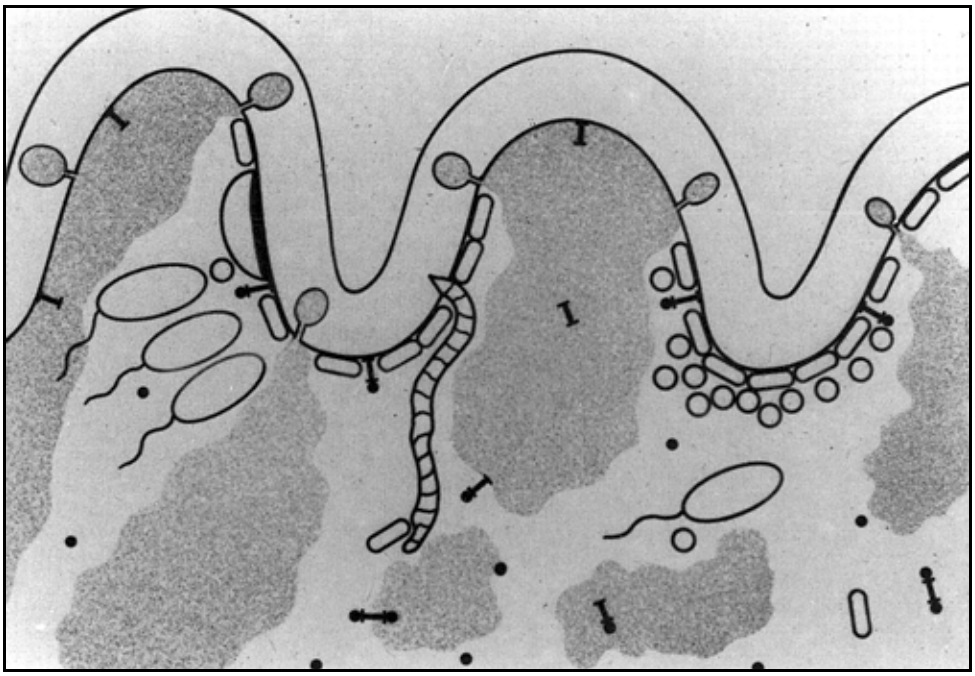

Figure 15: Diagrammatic representation of mucus secretion in the intestine of a PHA-treated animal showing the disruption of mucus structure caused by the association of PHA (●) with the natural lectin (⊢) and the consequent colonisation of the unprotected gut surface by bacteria and protozoa.

5. The Mucus Blanket as a Protective and Chemotactic Factor at the Epithelial Surface

The consequences of microbial interactions in the intestine are a titration of various host factors against virulence characteristics of the organism. These consequences have profound medical implications in both the developed and the Third World. In addition to relieving human suffering, an understanding of and ability to control disturbances of intestinal microecology will have tremendous economic, social and political impact. An indication of the variety of processes responsible for the microecology of the intestine which must be understood is provided in Tables 9 and 10.

Walker and colleagues have studied some factors which contribute to microbial partition in the gut. Specifically, they examined host-microbial events responsible for infection with *Campylobacter jejuni*, a major enteric pathogen and also those associated with the development of opportunistic infections in immunocompromised rodents.

Enteric Disease Studies

Infections similar to clinical *Campylobacter* diarrhoea can be induced in rabbits with the Removable Intestinal Tie Adult Rabbit Diarrhoea (RITARD) model (*Spira* et al., 1981; *Caldwell* et al., 1983; *Caldwell* et al., 1986). This procedure ensures an experimental ileus as it inhibits normal peristalsis for several hours so that the bacterial inoculum can establish sufficiently to cause a mucoid diarrhoea associated with bacter-

Table 9: Factors responsible for determining intestinal microecology

Indigenous population stabilisers

1. Gel embedding (entrapment)
2. Gel attachment (chemical bond)
3. Epithelial attachment sites
4. Unique niche fitting (*C. pylori*)
5. Favourable nutrient environment
6. Multiplication rate to match population size vs. environment
7. Immunologic tolerance to indigenous flora

Exogenous population inhibitors

1. Immobilisers - antibody - mucus glycoprotein
2. Multiplication reduction
   - colonisation resistance factors (largely unknown)
3. Physical barriers/removers
   - viscosity of mucus
   - peristalsis
   - mucus secretion
   - fluid secretion
   - cell sloughing

aemia after a few days. Many animals die as a consequence of the infection. If the rabbit is fed the organism without the RITARD procedure, colonisation for two to three weeks takes place, but no diarrhoea occurs. Rechallenge of these transiently colonised animals shows immunity with no disease, no bacteraemia, and rapid gut clearance of the organism, usually in less than 24 hours (*Burr* et al., 1988).

In vitro studies were conducted to better understand control mechanisms involved in *Campylobacter* infection and immunity in the rabbit. These studies used adherence assays not only to epithelial cells (INT 407), but also to rabbit intestinal mucus (*McSweegan* and *Walker*, 1986; *McSweegan*, *Burr* and *Walker*, 1987) as the organism must interact first with the latter substrate. Strains of *C. jejuni* used varied greatly in their ability to adhere to either the cell or mucus substrate (*McSweegan* and *Walker*, 1986). Lipopolysaccharide was an adhesin which bound both substrates, but flagella, another adhesin, bound only to cells. Binding to epithelial cells *in vivo* may not occur, but *in vitro* mucus as well as other defences are removed. *Lee* et al. (1986) found that *C. jejuni* colonises crypts, but does not seem to attach to or penetrate epithelial cells. The spiral morphology, motility, mucinase production and chemotaxis to mucin are all bacterial characteristics, which would enhance this process.

A more realistic *in vitro* system for *C. jejuni* was created by overlaying INT 407 cells with mucus. Bacteria were placed on top of the mucus and penetration and subsequent attachment to epithelium were quantitated. Mucus reduces adherence to cells by about 50%. If the rabbits were immune to *C. jejuni*, their mucus totally blocked adherence to epithelial cells in this assay (*McSweegan*, *Burr* and *Walker*, 1987).

The immune mucus phenomenon de scribed above could be associated with titres of SIgA in the mucus. Whole eventually provide secretory IgA to mucosal surfaces (Figure 16). It is of

Table 10: Factors responsible for a disturbed ecosystem

Exogenous population colonisation mechanism

1. Motility
2. Mucinase (protease)
3. Morphology
4. Invasive properties
5. Numbers
6. Metabolic ability to exploit altered environment

Compromised states

1. Traumatic injury/radiation
   - peristalsis impaired
   - mucus blanket disruption
   - loss of cell surface fibronectin
   - crypt cell damage
   - immunosuppression
2. Viral enteritis
   - virus destroys enterocytes
   - bacteria adhere to lesion created by virus
3. *Clostridium diffcile*
   - antibiotic impairment of colonisation resistance
   - age associated immunity reduction
4. Autoimmune dysfunction (Ulcerative colitis; Ankylosing spondylitis)
   - genetic material from bacteria transferred to host cell
   - viral antigen attached to self-antigen
5. Carcinogenesis
   - substrates available for bacterial carcinogen production
6. Blind loop syndrome
   - intestinal stasis
7. Hepatic encephalopathy
   - metabolic derangement
8. Abnormal drug metabolism due to unique flora

interest that these antigen-sampling organs are relatively free of mucus due to bacterial cells but not flagella or *E. coli* could absorb the SIgA from the mucus and loss of SIgA titre eliminated the immune effect. SIgA alone, collected by lavage (*Burr* et al., 1987), did not inhibit adherence to cells. When added to non-immune mucus, however, lavage fluid from immune animals caused a total reduction in bacterial attachment to INT 407 cells. This suggests that the antibody and mucus must work together to effect inhibition of bacterial passage.

Mucus and its flow due to secretion by goblet cells and peristalsis is, thus, a major component of host defence which can be augmented significantly by specific antibody. This interaction of immune and non-immune components suggested that the intestinal lymphoid tissue response to *Campylobacter* should also be examined. This system is scattered throughout the gut, but can be found concentrated in Peyer's patches where specialised epithelial cells, called M cells, cover follicles containing lymphocytes and macrophages (*Owen*, 1982; *Owen* et al., 1986; *Sneller* and *Strober*, 1986). M cells take up antigen and pass it apparently unchanged into the follicle where it is processed to scarcity of goblet cells in the adjacent area.

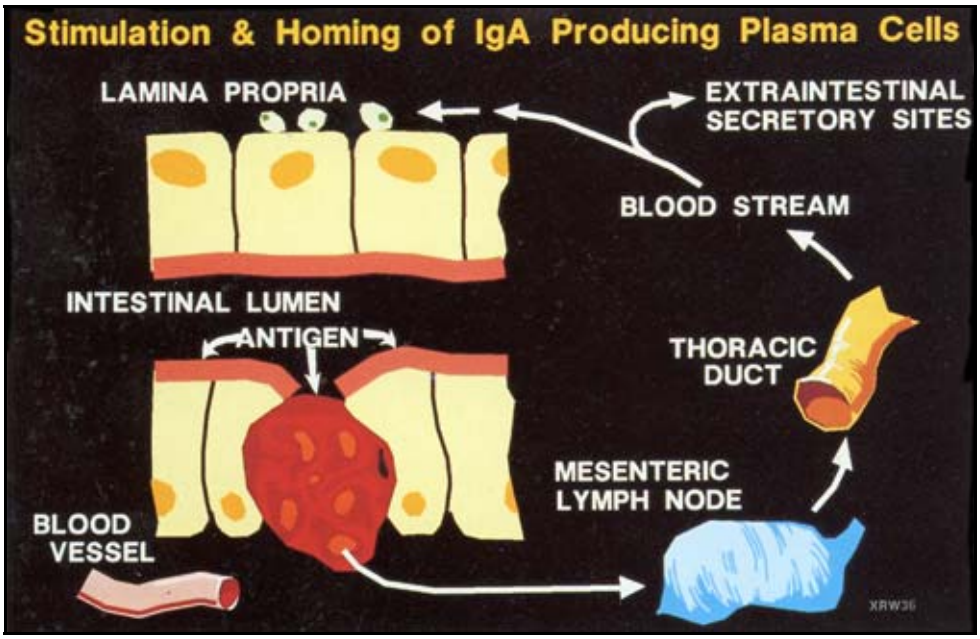

Figure 16: Antigen in the intestinal lumen interacts with lymphoid tissue sites to produce IgA producing plasma cells. These cells move systemically via the lymphatic and blood circulatory systems to extra-intestinal mucosal surfaces.

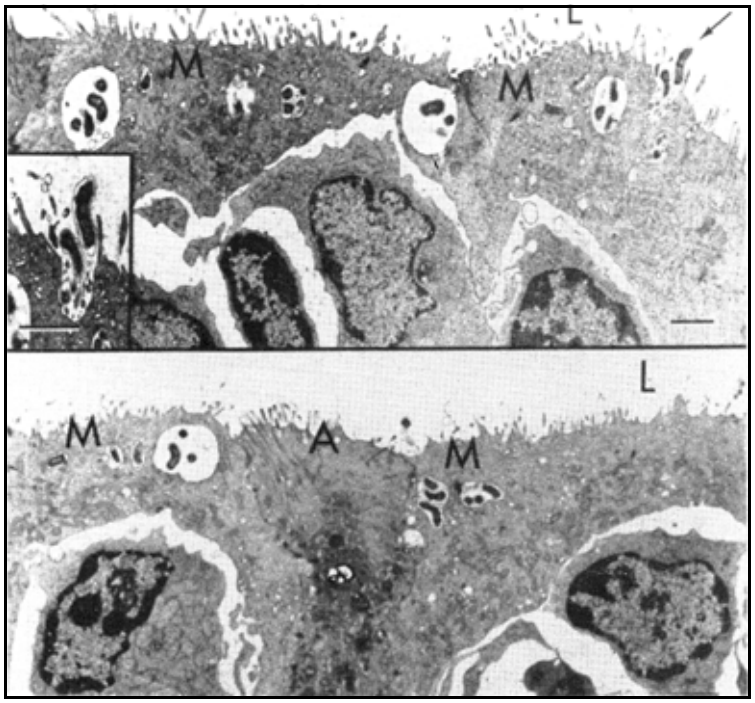

Figure 17: Endocytosis of *C. jejuni* by rabbit M cells (M). Bacteria are seen in vacuoles within the M cells. Inset in top photograph shows bacteria being endocytosed from intestinal lumen (arrow). Relationship between M cells and absorptive epithelial cells (A) lining the lumen (L) is shown in bottom photo. Space bar represents 2 micrometers.

Walker et al. (unpublished data) found that *Campylobacter* preferentially binds to and is transported through M cells (Figure 17), but not to other intestinal epithelial cells (enterocytes). This may be a portal for translocation of the campylobacters which could contribute to the disease process (*Walker* et al., 1986) as well as cause immunity. The fact that bacteria could be found free in the M cell follicle, suggests that this could also be a route for translocation by opportunistic pathogens in immunocompromised individuals.

Opportunistic Infection Studies

Although the M cell still needs to be studied as a means for translocation of microorganisms across the mucosa in immunocompromised subjects, other studies already completed provide some insight into non-specific factors affecting microecology of the gut in the immunocompromised host.

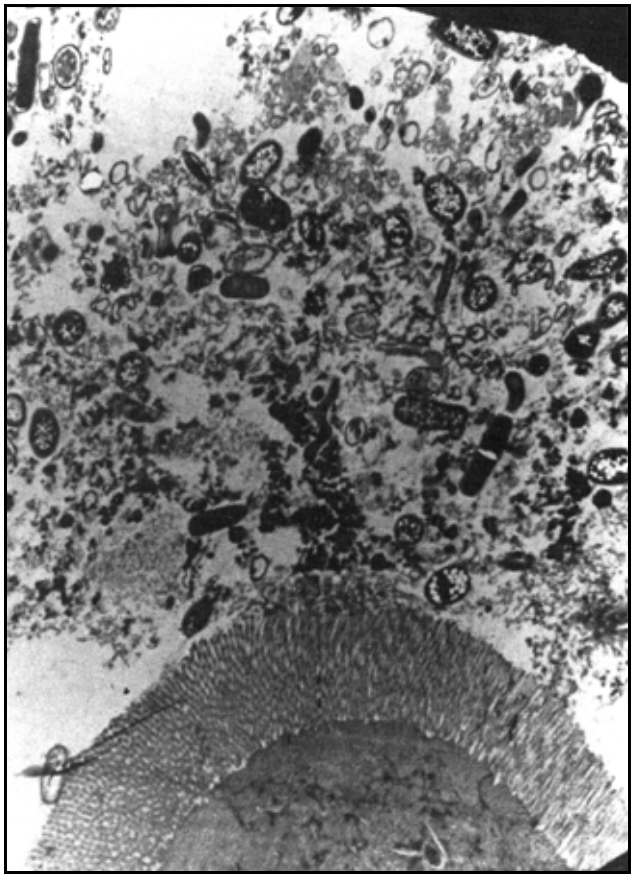

Figure 18: The mucus gel in a rabbit ileum was stabilised with specific antiserum to show relation of gel to the brush border of an epithelial cell. Note the numerous microorganisms seen in the gel layer.

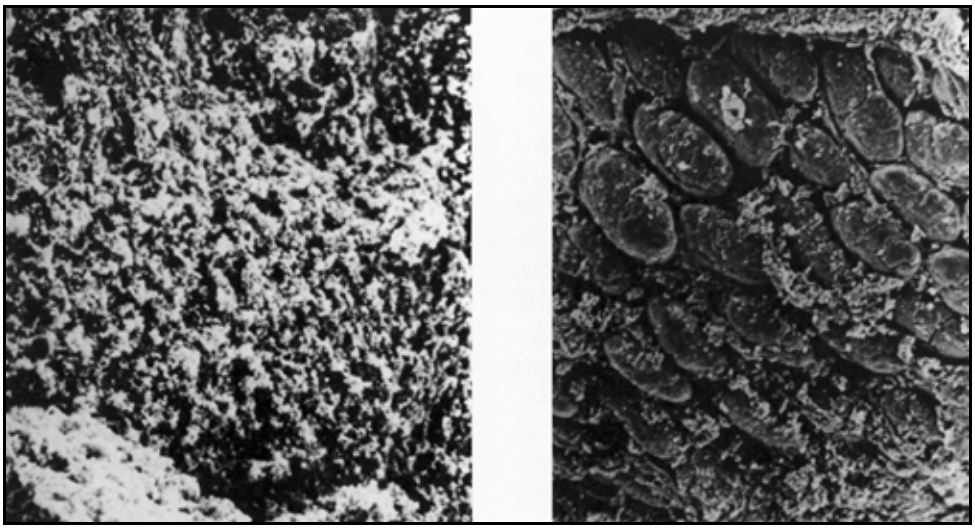

Figure 19: Scanning electron micrograph of surface of mouse ileum in which mucus gel has been stabilised with anti-mucus antibody. The mucus material in the sample from a normal mouse is seen to virtually occlude the mucosal surface (left hand side of figure). The blanket is reduced to patchy accretions and the villi are clearly seen through this discontinuous layer 3 days after exposure of mice to 10 Gy of gamma radiation (right hand panel). Space bars represent 50 micrometers.

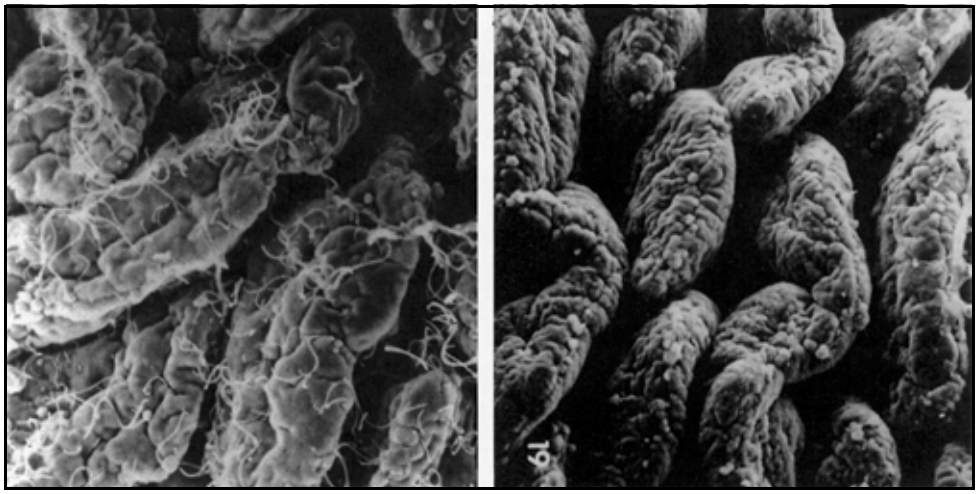

Figure 20: The normal rat ileum is healthy colonised with segmented filamentous microflora (SFM) which are inserted at one end into the intestinal epithelial cell (left hand panel). After sublethal (5 Gy) or lethal (10 Gy) gamma radiation the SFM are significantly reduced in number and, in contrast, to rats given sublethal radiation, they never returned in the lethally irradiated animals (right hand figure).

Disruption of tight junctions between goblet cells and adjacent absorptive epithelial cells following wounds and/or irradiation not only provide a route for escape of endotoxin from the gut, but also indicates activity by the goblet cells (*Walker* and *Porvaznik*, 1983). Also, intestinal stasis may permit host proteases to accumulate in the intestine. These events suggest that the mucus blanket could be changed following injury. Since most microorganisms are found in the mucus (Figure 18), this could be very significant.

Walker and colleagues (1985) have looked at the normal mucus blanket and at the mucus blanket on days 1 to 3 after lethal irradiation of mice (Figure 19). They saw progressive disruption of blanket integrity so that exposed villi were visible.

Segmented filamentous microflora (SFM) were examined in rats by scanning electron microscopy. These organisms were used as an indicator of disruption of the gut ecosystem and possible loss of colonisation resistance (*Walker* and *Porvaznik*, 1983). Following sublethal radiation SFM decreased but returned to normal levels by day 3. After lethal radiation they never returned (Figure 20). When rats were exposed to sublethal radiation facultative flora decreased and then returned to normal levels after the SFM returned. This was in contrast to a more rapid return and overgrowth of facultative flora in lethally exposed animals. This event is associated with translocation and lethal systemic infections (*Walker* and *Porvaznik*, 1983). The importance of colonisation resistant flora is shown by

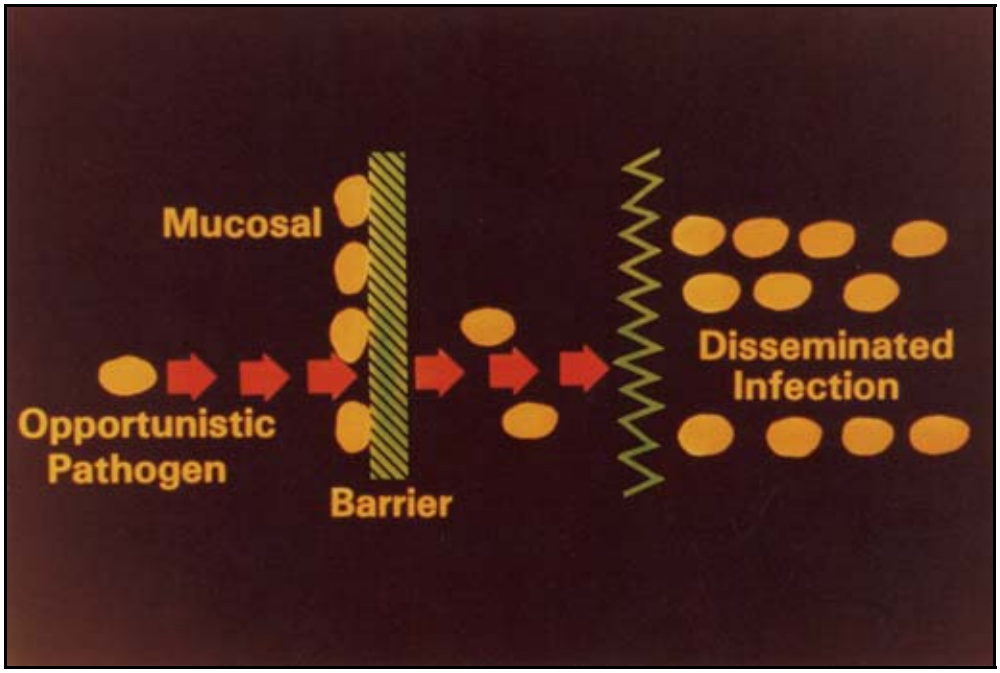

Figure 21: Opportunistic pathogens in the lumen of the intestine must overcome a series of defence systems to colonise the epithelial surface of the mucosal barrier. If sufficient organisms colonise this surface, some may translocate into normally sterile tissues. If normal systemic defences (jagged line) are lost due to radiation or other immunocompromising injuries, disseminated infection can occur.

experiments with metronidazole. If mice are treated with systemic metronidazole, the decrease in anaerobes is accelerated and lethal opportunistic infections occur earlier (*Brook*, *MacVittie* and *Walker*, 1988).

Figure 21 illustrates diagrammatically how breakdown of various mucosal defences such as described above could permit colonisation of the mucosal surface by opportunistic pathogens. If sufficient numbers of these organisms translocate through the mucosal barrier, they can cause disseminated infections if normal defences such as granulocytes (illustrated by jagged line) are not present.

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IV. DIGESTIVE TRACT BACTERIA

1. Microbial Ecology of the Human Oral Cavity

The teeth and oral epithelial surfaces are colonised by a great variety of bacterial species. A partial list of indigenous (autochthonous) organisms found in the adult human mouth is shown in Table 11 (*Gibbons* and *van Houte*, 1975; *Gibbons* and *van Houte*, 1978; *Rosebury*, 1962; *Socransky* and *Haffajee*, 1988; *van Houte*, 1980). Characteristically, such indigenous bacteria are well adjusted to their environment and can successfully colonise the mouth for appreciable periods of time or throughout life. Their relationship with the host is neither a purely mutually beneficial one (symbiosis) nor are oral bacteria obligately pathogenic (antibiosis) (*Rosebury*, 1962).

Table 11: Oral flora

Gram$^+$ cocci	Gram$^-$ cocci	Gram$^+$ rods	Gram$^-$ rods
Staphylococcus sp.	*Veillonella* sp.	*Actinomyces* sp.	*Actinobacillus actinomycetemcomitans*
Streptococcus sp.	*Neisseria* sp.	*A. viscosus*	
S. mutans group*		*A. naeslundii*	
S. mutans		*A. odontolyticus*	*Bacteroides* sp.
S. sobrinus		*A. israelii*	*B. forsythus*
			B. gingivalis
S. milleri		*Arachnia*	*B. intermedius*
		Bacterionema	*Campylobacter*
S. mitior			
		Bifidobacterium	*Capnocytophaga* sp.
S. salivarius			*C. gingivalis*
		Clostridium	*C. ochracea*
S. sanguis			
		Corynebacterium	*Eikenella corrodens*
Enterococci			
		Eubacterium	*Fusobacterium*
Anaerobic strep.			
		Lactobacillus sp.	*Haemophilus*
		L. casei	
		L. plantarium	*Leptotrichia*
		L. acidophilus	
		L. fermenti	*Selenomonas sputigena*
		L. brevis	
		Nocardia	Spirochetes
		Propionibacterium	*Wolinella recta*
		Rothia	

* Referred to as *S. mutans* in text.

The term amphibiosis has been suggested for this situation by *Rosebury* (1962) to signify a spectrum of relationships between these two extremes, which may be more or less beneficial, or harmful depending on the conditions. Beneficial effects of the oral flora include its general contribution to host immunity by means of antibodies that cross-react with overt pathogens, the specific suppression of organisms such as *Candida albicans*, or the production of vitamins. The pathogenic potential of the oral flora is illustrated by many infectious processes such as dental caries, periodontal diseases, endodontic infections, sub-acute bacterial endocarditis, actinomycosis, bite wounds, etc., which require a variety of predisposing factors. For dental caries, the frequent consumption of dietary carbohydrates is required to permit expression of the cariogenic potential of dental plaque bacteria through their ability to produce high concentrations of organic acids which are responsible for the demineralisation of the underlying tooth surface (*Gibbons* and *van Houte*, 1978; *van Houte*, 1980). Sub-acute endocarditis reflects a combination of the dissemination of oral organisms such as *S. mutans* and *S. sanguis* and previously damaged heart tissue (*van Houte*, *Jordan* and *Bellack*, 1971).

Acquisition of the Oral Flora

Exposure of the oral tissues to bacteria during and directly after birth leads rapidly to the development of an oral flora (*Gibbons* and *van Houte*, 1975; *Gibbons* and *van Houte*, 1978; *Socransky* and *Haffajee*, 1988). The selection of bacteria from the outer environment is based on their ability to adhere to oral surfaces and to grow under the prevailing conditions; the "cleansing" activity of saliva plays an important role in bacterial disposal. The evidence indicates that the mouth harbours a unique flora, which meets very specific requirements for adherence and growth. This indicates that the new-born acquires its characteristic flora from other human beings. Indeed, bacteriocin-typing studies with *S. mutans*, demonstrating the transmission of this organism from parents to their children, directly support this contention (*Berkowitz*, *Turner* and *Creen*, 1981). Furthermore, transmission of *S. mutans* appears to require generally direct contact (e.g. contaminated food, kissing) and is influenced by the cell numbers transferred; its emergence in children of parents with low cell numbers may be greatly delayed (*Berkowitz*, *Turner* and *Green*, 1981).

Initially, many different organisms can be found in the infant's edentulous mouth reflecting contact with a variety of sources (*Gibbons* and *van Houte*, 1978; *Socransky* and *Haffajee*, 1988). However, the presence of many of these organisms is transient. Indigenous organisms capable of colonising the epithelial surfaces e.g. *S. salivarius* can be isolated consistently already within a few weeks or even days and the spectrum of bacterial species and their number gradually increases with time. In this early phase of bacterial colonisation, streptococci, actinomyces, neisseriae, and veillonellae can be regularly isolated. Most of these organisms are facultative with respect to oxygen and many overtly anaerobic species are still absent; bacterial oxygen utilisation may permit the colonisation of some anaerobic organisms such as veillonellae (*Ritz*, 1967).

The eruption of teeth permits a major addition to the already complex flora and is indispensable for the colonisation of organisms such as *S. mutans* and *S. sanguis* and *Lactobacillus* species; in this regard, teeth may provide a specific non-shedding surface which enables their attachment, or specific sites e.g.

deep fissures which are relatively sheltered from the oral cleansing forces and which may further facilitate their colonisation (*Gibbons* and *van Houte*, 1975; *Gibbons* and *van Houte*, 1978; *Socransky* and *Haffajee*, 1988). Tooth eruption also leads to the development of the gingival sulcus around the teeth and the emergence of a major anaerobic flora, particularly Gram-negative rods (Table 11) that are associated with different types of periodontal disease (*Socransky* and *Haffajee*, 1988). The low oxidation-reduction potential prevailing within the sulcus plays an important role in this regard; specific nutrients may also promote bacterial growth e.g. host-derived haemin or hormones and vitamin K analogues, produced by other bacteria, for growth of *B. gingivalis* (*Loesche*, 1968). The preferential habitat of some oral organisms, based on adherence and growth factors, is shown in Table 12. Such organisms can also be found, albeit less frequently and in lower concentrations, in other sites of the dentulous adult mouth. Furthermore, the indicated sites are probably in all cases indispensable for their persistent colonisation.

The most densely-populated surfaces are generally the teeth (*Gibbons* and *van Houte*, 1975; *Gibbons* and *van Houte*, 1978). Here tightly adherent masses of bacteria, termed dental plaque, can reach concentrations of 2×10^{11} cells (viable and non-viable) per gram wet weight. The concentrations on the tongue dorsum may average 100 bacteria per cell whereas those on the buccal mucosa average about 10-20 bacteria per cell. The bacterial concentrations in saliva are generally between 10^7 and 10^8 per ml. The "flora" of saliva originates from bacterial cells dislodged from the teeth and epithelial surfaces. Bacterial growth on the oral surfaces, particularly when the cell density is high, is relatively low and it has been estimated that the biomass doubles only a few times per day. The high cell density in dental plaque limits the inward diffusion of nutrients. Nutrient limitation is responsible for a bacterial growth rate far below its optimal level, particularly in the deeper parts of plaque (*Critchley*, 1969).

Dental Plaque Formation and Ecology

Dental plaques form on the enamel covering the crowns of teeth, on enamel near or in the healthy gingival sulcus (about 1 mm deep), on cementum or underlying dentin of roots of teeth exposed to the oral environment by periodontal disease, or in the pathologically deepened gingival sulcus ("pocket") associated with loss of tooth-supporting

Table 12: Preferential colonisation sites of some oral bacteria

Organism	Coronal plaque	Gingival sulcus	Tongue dorsum
S. mutans	+		
S. sanguis	+		
S. salivarius			+
A. viscosus	+		
A. naeslundii			+
Lactobacillus sp.	+*		
Anaerobes (e.g. Bacteroides, Fusobacteria, Spirochetes)		+	

* Particularly caries lesions

bone (periodontal disease) (*Gibbons* and *van Houte*, 1975; *Gibbons* and *van Houte*, 1978; *Socransky* and *Haffajee*, 1988; *van Houte*, 1980). In view of the powerful oral cleansing forces, pre-selection sites for plaque are the relatively protected tooth fissures, areas between teeth, and areas near the gingival sulcus. Exposed enamel, or cementum or dentin on the roots of teeth, are covered by an acquired pellicle on which plaque can form. Plaque formation under natural conditions may involve bacterial colonisation of this pellicle when all plaque has been removed (e.g. oral hygiene) as well as continuous replacement of lost cell mass not involving an exposed pellicle.

Plaque formation on the pellicle entails initial attachment of bacteria that are present in saliva or on contacting surfaces. This adhesion is bacterium-specific and is based on the interaction with different pellicle components such as various salivary glycoproteins or enzymes (*Gibbons* and *van Houte*, 1975; *Gibbons* and *van Houte*, 1978; *van Houte*, 1982). Some of these interactions may involve lectin-like (carbohydrate-protein) binding between specific bacterial adhesins and pellicle receptors. Initial bacterial adhesion is probably sub-optimal because components in saliva to which cells available for attachment are exposed, will bind to and block the specific bacterial adhesins which mediate bacterial attachment to the pellicle and will also cause bacterial aggregation (*Gibbons* and *van Houte*, 1975).

Further increase in the size of plaque involves *in situ* proliferation of bacteria and additional adherence. Cell accumulation involves adhesion between bacteria and the interbacterial matrix, which contains salivary glycoproteins, and extracellular polymers synthesised by bacteria as well as direct cell-to-cell binding. These interactions, possibly together with mechanical entrapment of bacteria in matrix components, are responsible for the structural integrity of plaque. The adhesive interactions between matrix components and different bacteria as well as cell-to-cell binding are also highly specific and may also involve lectin-like binding mechanisms (*Gibbons* and *van Houte*, 1975; *Gibbons* and *van Houte*, 1978; *van Houte*, 1982).

The relative significance of bacterial adhesion and growth during plaque formation may be summarised as follows:

1. The initial composition of dental plaque is governed by bacterium-specific interactions with the pellicle. In fact, these interactions and bacterial adhesive interactions with oral epithelial surfaces constitute an important ecological determinant (*Gibbons* and *van Houte*, 1975; *Gibbons* and *van Houte*, 1978; *van Houte*, 1982) and form the basis for the preferential colonisation of different oral bacteria in different oral sites (Table 12),
2. depending on the conditions, factors influencing bacterial growth may cause shifts in bacterial composition during plaque development (see below), and
3. the total biomass of dental plaque, allowed to develop for a significant period of time, is mainly determined by *in situ* bacterial proliferation rather than bacterial adhesion. With respect to the composition of the flora on epithelial surfaces, bacterial adhesion appears as the most significant determinant since maintenance of the flora requires continuous bacterial attachment due to the constant shedding of epithelial cells (*Gibbons* and *van Houte*, 1975; *Gibbons* and *van Houte*, 1978; *van Houte*, 1982).

The growth of oral indigenous bacteria is influenced by a vast array of factors.

They include oxygen tension, dietary carbohydrates, bacterial acids (pH), bacterial toxins (e.g. bacteriocins) and a variety of enzymes (*Gibbons* and *van Houte*, 1975; *Gibbons* and *van Houte*, 1978; *Loesche*, 1968; *Ritz*, 1967; *Socransky* and *Haffajee*, 1988). Many of these factors have been studied particularly in their relation to dental plaque. The presence of oxygen may play a role in the gingival sulcus (see earlier) but also in the deep layers of plaque (*Ritz*, 1967). In view of their significance for dental caries development, much is known about the effect of dietary carbohydrates. Many oral bacteria are fastidious and require carbohydrates for growth and absence of carbohydrate consumption causes major shifts in the oral flora. Especially organisms such as *S. mutans* and lactobacilli are very adversely affected; other organisms may be less dependent upon dietary carbohydrate by utilising host sources of carbohydrate or by the metabolism of other substrate types (*van Houte*, 1980).

Bacterial metabolism of dietary carbohydrates, comprised mainly of sucrose and starches with lesser amounts of lactose, fructose, glucose and some other sugars, may lead to a variety of products with varying ecological effects (*van Houte*, 1979). These include:

1. A variety of organic acid end products (e.g. lactate, propionate, acetate) from all carbohydrates including starches (prior hydrolysis by host- or bacterium-derived amylase),
2. synthesis and degradation of a variety of extracellular polysaccharides from sucrose, specifically. These include glucans (dextran, rich in 1.6 linkages and mutan rich in 1.3 linkages) synthesised by *S. mutans* or *S. sanguis*, fructose (levan) synthesised by *S. salivarius* and *A. viscosus*, and heteropolysaccharides synthesised from other sugars as well. Examples of the latter are a polymer consisting of N-acetyl-glucosamine, glucose and galactose synthesised by *A. viscosus* and a glucose-rhamnose "capsule" synthesized by *L. casei*,
3. synthesis of intracellular glycogen-type polysaccharides by a wide variety of bacteria from all carbohydrates.

Bacterial acidogenesis in dental plaque constitutes an important ecological force in view of the widely-varying pH tolerance of plaque bacteria (*Gibbons* and *van Houte*, 1978; *van Houte*, 1980). Extracellular glucans, particularly insoluble mutan, are important matrix components of plaque exposed regularly to sucrose. They contribute to voluminous plaque formation by promoting the structural integrity of plaque through intercellular binding or, possibly, cell entrapment; glucans are not readily degraded by enzymatic action. Levan as well as glycogen and the glucose-rhamnose capsule of *L. casei* are rapidly degraded to acids when the environmental carbohydrate supply is depleted (*Gibbons* and *van Houte*, 1975; *Gibbons* and *van Houte*, 1978; *van Houte*, 1982). Glycogen metabolism has been implicated in caries aetiology by causing prolonged acid production in plaque during its degradation and an enhanced acid production during its synthesis; it may also promote bacterial survival (*van Houte*, 1979).

In view of the above, the earlier mentioned effect of dietary carbohydrate on the oral populations of *S. mutans* and lactobacilli appear to be due to:

1. their particular dependence on dietary carbohydrates for growth,
2. their high acid tolerance providing them with a selective advantage over other organisms in acidic environments; this correlates with the uniquely high proportions of lactobacilli in caries lesions, exhibiting

often a low pH, and the proportions of *S. mutans* in caries-associated and frequently acidic plaques, and
3. extracellular glucan synthesis in the case of *S. mutans* which promotes cellular adhesion and thereby an increase of its plaque proportions.

Ecological Aspects of Dental Caries

Dental plaque is a prerequisite for the development of dental caries, periodontal diseases, and dental calculus (*Gibbons* and *van Houte*, 1978; *Socransky* and *Haffajee*, 1988; *van Houte*, 1980). Another prerequisite factor for dental caries is dietary carbohydrate. The pathogenesis reflects essentially a disturbance of the equilibrium between the tooth surface and protective saliva on the one hand and dietary carbohydrate and dental plaque on the other. Acid production by plaque bacteria from carbohydrate leads to an increased H^+-concentration in the plaque milieu (lower pH). This may lead to undersaturation of calcium and phosphate ions with respect to tooth mineral (hydroxyapatite, $Ca_{10}(PO_4)6(OH)_2$) and loss of Ca and P from the tooth surface (demineralisation); this process is reversible with increasing plaque pH (remineralisation).

In view of the pivotal role of plaque pH in dental caries development, a primary aetiological role is restricted to acidogenic organisms; this is true not only for enamel caries but probably also for root surface caries (*Jordan* 1986). Dental caries may be defined as a dietary-modified bacterial infectious disease. This concept implies a dynamic interaction between dietary carbohydrate and the plaque flora composition i.e. a higher carbohydrate intake induces an ecological shift towards higher proportions of acid-tolerant, acidogenic bacteria which is accompanied by a higher pH-lowering and cariogenic potential of plaque (*van Houte*, 1980). This concept is supported, among others, by the following observations:
1. plaques associated with caries activity on enamel, when exposed to carbohydrate, exhibit a pH profile which is in a lower pH range than that of plaques associated with caries inactivity,
2. the flora of the former is characterised by increased proportions of *S. mutans* and, less frequently and mostly in fissures, of lactobacilli, whereas the proportions of other predominant acidogenic organisms, mainly streptococci and actinomyces, remain unchanged or are decreased, and
3. *S. mutans* and lactobacilli are among the most acid-tolerant plaque organisms. This trait is of special significance because the lower the pH reached in plaque during carbohydrate utilisation, the greater is the probability and extent of tooth surface demineralisation.

Differences among plaque bacteria with respect to acidogenicity and acid tolerance are a matter of degree. Nevertheless, there is considerable evidence that quantitative differences with respect to these traits are important. Consequently, the aetiology of dental caries in enamel appears to entail considerably bacterial specificity. *S. mutans* is presently considered as a prime aetiologic agent; lactobacilli probably play a much lesser role whereas the significance of other acidogenic organisms requires further clarification. The spectrum of aetiologically significant plaque organisms is probably larger in the case of root surface caries which requires less stringent acidic plaque conditions (*Jordan*, 1986).

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2. Stomach Microbial Ecology

Traditionally, the normal stomach with its acidic gastric juice has been considered a sterile organ protecting the upper gut so that only transient oral bacteria occur following ingestion of food. With a reduction in gastric acid, different bacteria can colonise the stomach. Typically, this is seen in patients with pernicious anaemia. With pH 4 to 5 only acid resistant lactobacilli plus streptococci can survive but when the pH becomes greater than 5, many oral and faecal organisms can be isolated (*Hill*, 1983).

In 1983 and 1984, *Marshal*, and *Marshal* and *Warren*, respectively reported the isolation of a micro-aerophilic spiral organism from the mucosa of the majority of patients with chronic gastritis. This association has been confirmed world-wide. The organism is now called *Helicobacter pylori*; it is motile by multiple sheathed flagellae and adheres to gastric epithelium beneath the mucus gel. The organism is poorly seen on Haematoxilin-Eosin-stained sections but can be easily seen with a number of stains such as silver or a modified Giemsa (*Rathbone*, *Wyatt* and *Heatly*, 1986a). Colonisation has never been seen in an entirely normal stomach (antrum and corpus) and colonisation of the intestinal epithelium has also not yet been observed. The only time the organism is seen in the duodenum was in association with gastric metaplasia. Ultrastructurally, the organism adheres mostly in the intercellular gutters where they are associated with microvillous depletion and epithelial mucus depletion. Biochemically, a marked feature of the organism is its strong urease activity.

Helicobacter pylori is associated with both a systemic as well as a local immune response (*Rathbone* et al., 1986b). Studies looking at antibody coating of the bacteria demonstrate IgG, IgA and IgM coating on the bacteria. What was noted in these investigations was that in all subjects studied the bacteria deep in the gastric pits were uncoated (*Wyatt*, *Rathbone* and *Heatly*, 1986). Mucosal culture studies demonstrate that the gastric plasma cells are producing *Helicobacter pylori*-specific antibodies. Gastric T cell studies show increased stimulated T helper cells in the patients with *Helicobacter pylori*-associated gastritis.

There is evidence for acute infection from reported episodes of 'epidemic' gastritis with deliberate human ingestion of *Helicobacter pylori* (*Rathbone* et al. 1986c). Treatment studies have demonstrated that clearance of the organisms is associated with an improvement in the histological picture (*McNulty* et al., 1986). Recolonisation occurs and has been demonstrated to be with the same strain (*Langenberg* et al., 1986).

In patients with normal acid secretion, *Helicobacter pylori* is the only organism colonising the human stomach. This colonisation appears stable and long term. The organism is well adapted to living in its niche, protected from gastric acid by the mucus bicarbonate barrier The histological entity we call chronic B-gastritis, would appear at least in part to be the gastric immune reaction to *Helicobacter pylori*. Interestingly, the inflammation and colonisation is long term with the immune reaction failing to eliminate the organism. One presumes this highly specialised organism living in its unique niche derives benefit from the chronic inflammatory reaction.

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3. Sequential Development of the Human Intestinal Microbial Flora

Sources of Neonatal Bacterial Colonisation

Before Birth:

As long as the amniotic membrane remains intact, the normal foetus is sterile until shortly before birth.

During Birth:

As a result of passing through the vagina the gastrointestinal tract of the neonate is seeded with a wide variety of microorganisms originating from both the maternal microbial flora and the environment. For example, studies examining *Escherichia coli* from maternal and infant stool have shown that many babies acquire strains of *E. coli* identical to strains isolated from their mothers (*Gothefors* et al., 1976). Organisms best suited for survival in the intestinal environment become established by a process of natural selection. Many of the microorganisms are not able to colonise habitats in the neonatal gastrointestinal tract and disappear from it soon after birth.

After Birth:

Factors other than the maternal microbial flora are also undoubtedly important in the development of the neonate intestinal flora since infants are not always colonised with strains of bacteria from their mothers. The external environment (e.g., air and hospital personnel) is major source of colonising bacteria. In addition, external forces, such as food source and antimicrobial agents, can have dramatic influences on bacterial colonisation of the infant intestinal tract.

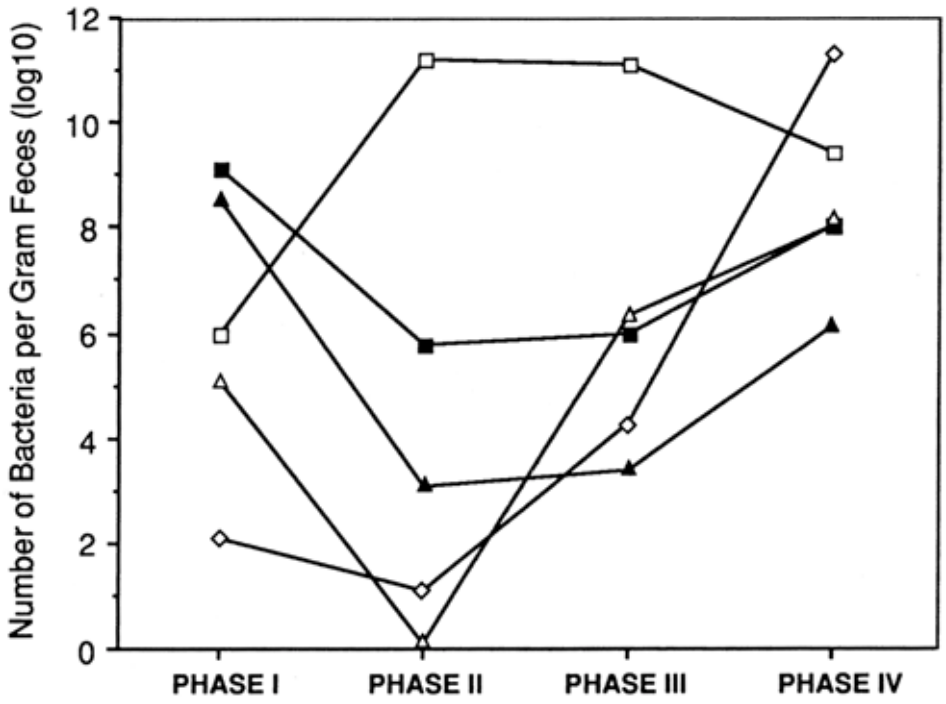

Figure 22: Sequential development of the normal intestinal flora in breast-fed infants.
■: *Enterobacteriaceae*; □: *Bifidobacterium*; ▲: *Streptococcus*; △: *Clostridium*; ↕: other anaerobes

Succession of Microbial Colonisation of the Infant Large Intestine

Despite the wide variety of bacteria constantly infiltrating the intestinal tract of infants, the pattern of colonisation is predictable and dependent upon complex regulatory mechanisms. For purposes of discussion the sequential microbial colonisation of the infant intestinal tract can be divided into four major phases:

Phase I (0-2 Weeks):

Phase I is the initial period of bacterial colonisation which occurs during the first two weeks of life. The intestinal flora is very unstable at this time and numerous changes in bacterial populations are occurring.

Phase II (2 Weeks-Preweaning):

Phase II is the remaining period during which breast milk and/or formula milk is the exclusive form of nutrition. The intestinal flora stabilises during this period but the actual composition of the flora is dependent on the food source.

Phase III (Weaning):

Phase III is initiated, in breast-fed infants, at the time that dietary supplements such as formula or cereal are introduced into the infants diet. The introduction of alternate food sources to the breast-fed infant causes major perturbations in the intestinal ecosystem.

Phase IV (Post-Weaning):

Phase IV is the period after weaning is completed. The intestinal bacterial flora of breast-fed and formula-fed infants approaches that of adults during this phase.

Sequential Development of the Normal Intestinal Flora in Breast-Fed Infants

Regardless of the animal species, Enterobacteriaceae and enteric strepto

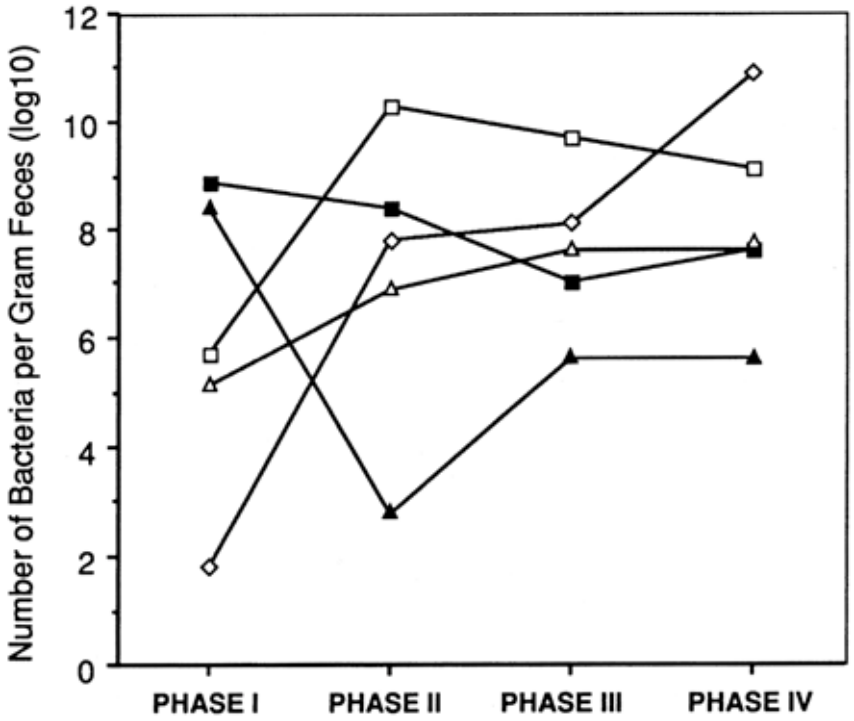

Figure 23: Sequential development of the normal intestinal flora in formula-fed infants.
■: *Enterobacteriaceae*; □: *Bifidobacterium*; ▲: *Streptococcus*; △: *Clostridium*; ↕: other anaerobes

cocci are almost universally the first microorganisms to appear in the colon of the new-born (Figure 22). *Bifidobacterium* usually appear in the intestinal tract shortly after *Enterobacteriaceae* and achieve levels of approximately 10^9 CFU/gram of faeces by the end of the second week of life (Phase I). In many animal species, transient colonisation of the colon with a variety of bacteria, including clostridia and other anaerobes, may occur during the first few days of life.

From two weeks of age until solid foods are given (Phase II), breast-fed infants have a very simple flora consisting primarily of high numbers of *Bifidobacterium* (10^9 CFU/g faeces) and lower numbers of *Enterobacteriaceae* and *Streptococcus*. Colonisation with *Bacteroides* and *Clostridium*, which may occur during the first week of life, decrease to very low numbers during the period of exclusive breast-feeding.

The introduction of solid food to breast-fed infants causes major disturbances in the microbial ecology of the colon (Phase III). The numbers of *Enterobacteriaceae* and streptococci increase while colonisation by *Bacteroides*, *Clostridium* and other anaerobes takes place.

By the second year of life, the major bacterial populations of the intestinal flora resemble those of adults (Phase IV). During this time the numbers of *Bifidobacterium* decrease while the numbers of *Enterobacteriaceae*, *Streptococcus*, *Clostridium* and other anaerobes continue to increase. The typical adult microbial intestinal flora consists of over 300 different species of aerobic, facultative and anaerobic bacteria.

Sequential Development of the Normal Intestinal Flora in Formula-Fed Infants

The intestinal flora of infants fed a diet based on cow's milk differs markedly from the intestinal flora of breast-

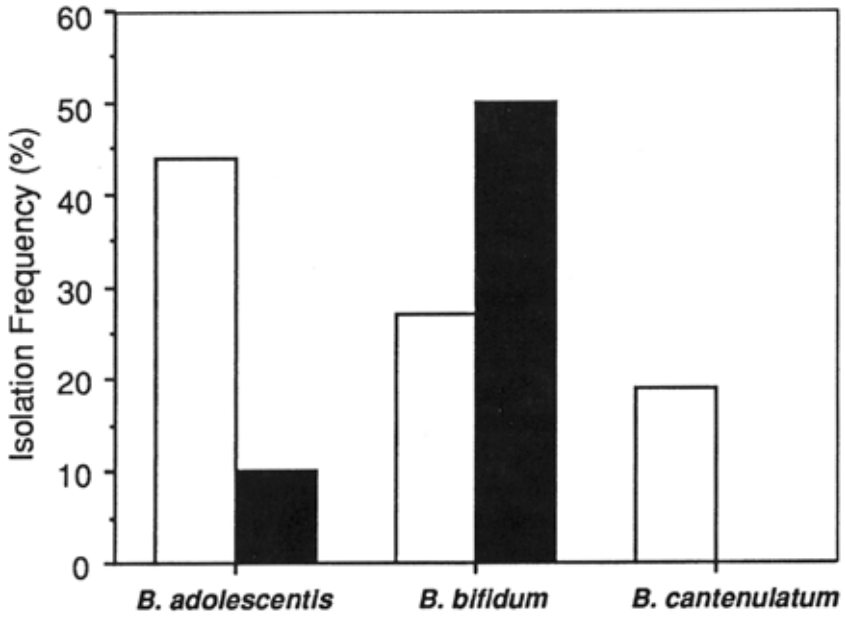

Figure 24: Isolation frequency of Bifidobacterium spp. from faecal samples of breast-fed (□) and formula-fed (■) infants (Modified from: *Mevissen-Verhage* et al., 1987).

fed infants (Figure 23). In in fants, unlike adults, relatively small changes in diet will have major effects on the intestinal flora.

In phase I, the sequence of bacterial colonisation in infants fed exclusively formula milk is quite similar to that of breast-fed neonates. Both groups are rapidly colonised with *Enterobacteriaceae* and *Streptococcus.*

During phase II, the intestinal flora of formula-fed infants is much more complex than that of breast-fed infants. The number of aerobic bacteria, such as *Enterobacteriaceae* and *Streptococcus* is typically higher in the faeces of formula-fed neonates than in the faeces of breastfed neonates. *Bifidobacterium* species are the predominant faecal bacteria in both groups.

However, the total counts of other anaerobic bacteria, such as *Bacteroides, Eubacterium, Peptostreptococcus, Veillonella* and *Clostridium* are significantly higher in the formula-fed group than in the breast-fed group. During phase II, relatively small amounts of formula milk supplementing otherwise exclusive breast-feeding usually results in shifts of the intestinal flora toward formula-fed patterns. The intestinal flora may not return to its original composition for a period of up to four weeks following the resumption of exclusive breast-feeding.

It is during phase III that the bacterial populations of the large intestine of breast-fed and formula-fed infants are beginning to resemble each other in both number and composition of bacteria. This is primarily a result of alterations in the anaerobic intestinal flora of breastfed neonates since the introduction of solid foods has little effect on the composition of the intestinal flora of formula-fed neonates.

Finally, during phase IV, the aerobic and anaerobic intestinal flora of for-

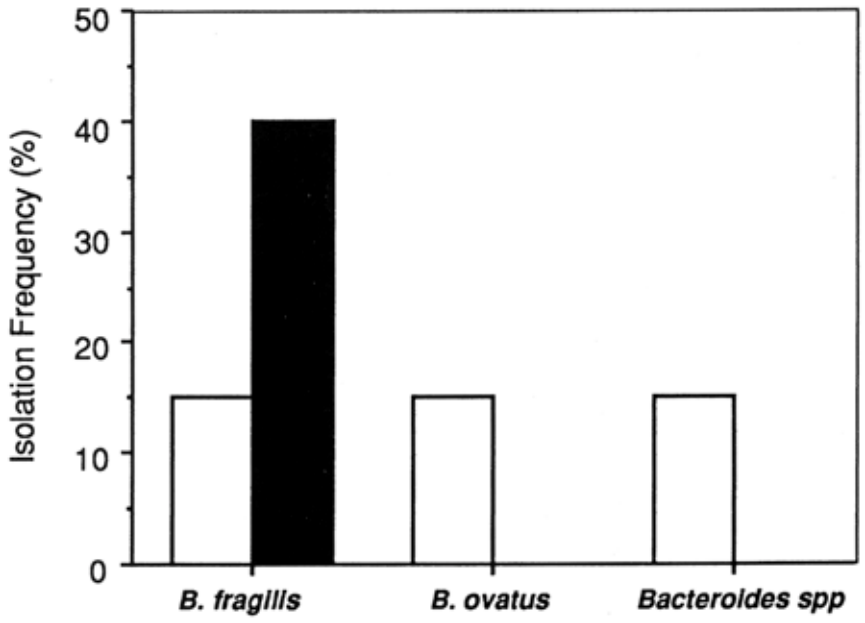

Figure 25: Isolation frequency of Bacteroides spp. from faecal samples of breast-fed (□) and formula-fed (■) infants (Modified from: *Mevissen-Verhage* et al., 1987).

mula-fed and breast-fed infants are essentially identical and resemble the intestinal flora of the adult.

Other Differences in the Development of the Intestinal Flora of Formula-Fed and Breast-Fed Infants

When comparing the intestinal flora of breast-fed and formula-fed neonates most studies have identified bacteria just to the genus level.

However, differences can also be demonstrated between species within a genus in the two groups of infants. As described above, *Bifidobacterium* species are the predominant bacteria in both formula-fed and breast-fed infants.

However, the actual species of *Bifidobacterium* in infants fed exclusively breast milk have been shown to be quite different from the *Bifidobacterium* species found in the intestinal tracts of formula-fed infants. *Mevissen-Verhage* and co-workers (1987) have shown that the *Bifidobacterium* species most frequently isolated from infants are *B. adolescentis*, *B. bifidum* and *B. catenulatum* (Figure 24). Breast-fed infants have all three species in their intestine with *B. adolescentis* being the most common. On the other hand, bottle-fed infants have only B. *adolescentis* and *B. bifidum*, with the latter being isolated most frequently. These investigators found similar results with species of *Bacteroides* (Figure 25). Formula-fed infants have only *B. fragilis* in their faeces while breast-fed infants have *B. fragilis* and *B. ovatus*, as well as other species of *Bacteroides* in their intestine.

In addition to the actual species of bacteria differing between breast-fed and formula-fed neonates, the physicochemical characteristics of a particular species may also differ between the two groups. For example, fewer *E. coli* serogroups are found in the faeces of breast-fed infants than in the faeces of formula-fed infants (*Braun*, 1981). This suggests that the *E. coli* flora in breast-fed neonates is more homogeneous than in formula-fed infants. It has also been reported that *E. coli* serotypes containing the K1 antigen (known to cause meningitis in neonates) are isolated more often in the faeces of formula-fed neonates than breast-fed neonates (*Orskov* and *Sorensen*, 1975). *Braun* (1991) has shown that *E. coli* isolated from the faeces of breast-fed neonates are more influenced by the bactericidal activity of human serum than those isolated from the faeces of formula-fed neonates (Figure 26).

Additional Factors Influencing the Sequential Development of the Intestinal Flora

There are many factors other than diet which influence the sequential development of the intestinal flora. Some of these factors, which will be described in greater detail, include:

- Oxidation-Reduction Potential
- Volatile Fatty Acids
- pH
- Caesarean Section
- Receptors on Intestinal Mucosa
- Antimicrobial Agents
- Geographical Setting
- Intestinal Abnormalities

<u>Oxidation-Reduction Potential</u>

The oxidation-reduction potential of the gastrointestinal tract immediately after birth is positive (*Grütte, Horn* and *Haenel*, 1965). However, within the first few days of life facultative bacteria colonise the intestinal tract and create a reduced environment favourable to the subsequent appearance of anaerobic bacteria (Figure 27). The oxidation-reduction potential of the intestinal tract then continues to decline to the extremely reduced levels characteristic of adults. This phenomenon has also been demonstrated in mice, where the oxidation-reduction potential of intestinal

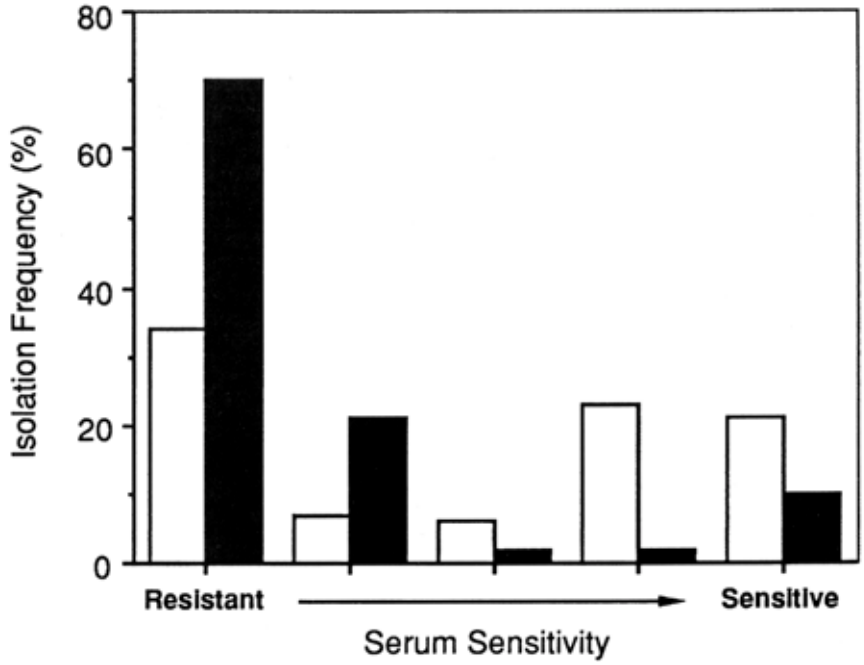

Figure 26: Bactericidal effect of human serum on strains of Escherichia coli isolated from the faeces of breast-fed (□) and formula-fed (■) infants (Modified from: *Braun*, 1981).

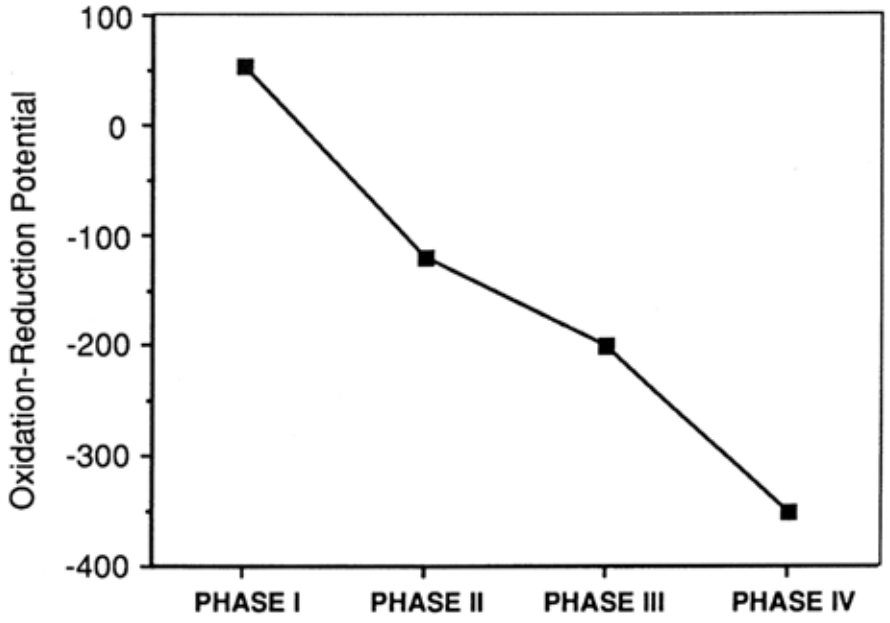

Figure 27: Oxidation-reduction potential in relation to the sequential development of the intestinal flora. (Modified from: *Grütte*, *Horn* and *Haenel*, 1965)

contents is considerably higher in germ-free mice than in gnotobiotic mice colonised by facultative bacteria (*Celesk, Asano* and *Wagner*, 1976).

Volatile Fatty Acids

Volatile fatty acids also play an important role in the sequential development of the neonate intestinal flora. Acetic acid is the major, and often the only, fatty acid in stools of breast-fed neonates during the first few days of life (*Bullen, Tearle* and *Stewart*, 1977) (Figure 28). The presence of acetic acid is presumably due to the predominance of *Bifidobacterium*, a major acetic acid producer. Later in life, some breast-fed infants may also have low levels of propionic and butyric acids in their intestine. On the other hand, a variety of volatile fatty acids are usually present in the intestine of formula-fed infants, including acetic, butyric and propionic acids. Volatile fatty acids have been shown by numerous investigators to be inhibitory to a wide variety of bacteria (*Hentges*, 1983). Interestingly, it is during the period in which the concentrations of volatile fatty acids are increasing that there is a marked decline in the numbers of *E. coli* and streptococci.

pH

The pH of stool obtained from breast-fed neonates stabilises at a mean of about 5.0 to 5.5 after the first week of life and remains at this level as long as the infant receives a diet of only breast milk (*Bullen, Tearle* and *Stewart*, 1977) (Figure 29). In contrast, the mean pH of stool obtained from formula-fed neonates rises over time and may reach values as high as 8.5, suggesting that the intraluminal metabolic

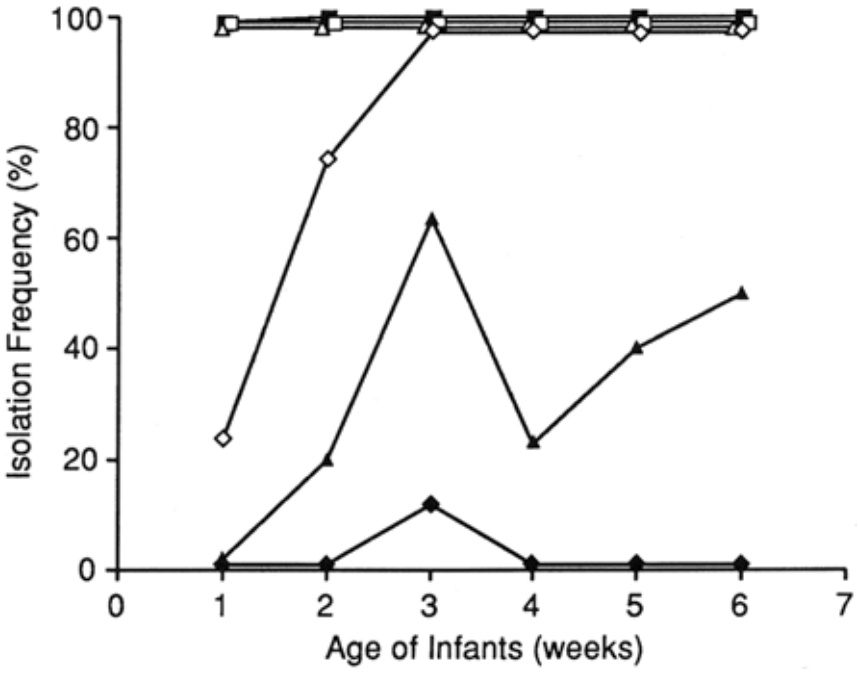

Figure 28: Volatile fatty acids in the faeces of breast-fed and formula-fed infants. ■: Breast-fed, acetic acid; ▲: Breast-fed, propionic acid; ◆: Breast-fed, butyric acid; □: Formula-fed, acetic acid; △: Formula-fed, propionic acid; ↕: Formula-fed, butyric acid (Modified from: *Bullen, Tearle* and *Stewart*, 1977).

events in formula-fed infants differ dramatically from breast-fed infants. The low pH of intestinal contents from breast-fed infants undoubtedly influences the type of bacteria, which can colonise the intestinal mucosa. Interestingly, it has been demonstrated that the pH of stools from breast-fed infants lacking *Bifidobacterium* is just as low or almost as low as the pH of stools from infants with high levels of *Bifidobacterium*. This suggests that factors other than increased acid production by *Bifidobacterium* account for the low pH in breast-fed infants.

Caesarean Section

Babies born by Caesarean section acquire facultative bacteria more slowly and appear to be more prone to acquire nursery strains of bacteria than babies born vaginally. Intestinal colonisation with anaerobic bacteria is delayed to an even greater degree in babies born by Caesarean section, demonstrating the importance of the mother as a source of anaerobic bacteria in infants. For example, *Long* and *Swensson* (1977) found that within 4 to 6 days of age, all full term, formula-fed, vaginally delivered infants are colonised with anaerobic bacteria and of these infants, 61% harboured *B. fragilis* (Figure 30). In contrast, *B. fragilis* is present in only 9% of infants 4 to 6 days of age, which are delivered by Caesarean section.

Receptors on Intestinal Mucosa

The adherence of bacteria to intestinal epithelial cells may also influence the sequential development of the neonatal intestinal flora. In all animal species, the intestine undergoes important modifications during postnatal development. For example, *Cheney* and *Boedekker* (1984) demonstrated that an enteropathogenic strain of *E. coli* does not adhere to brush borders prepared from rabbits 15 days of age or younger (Figure 31).

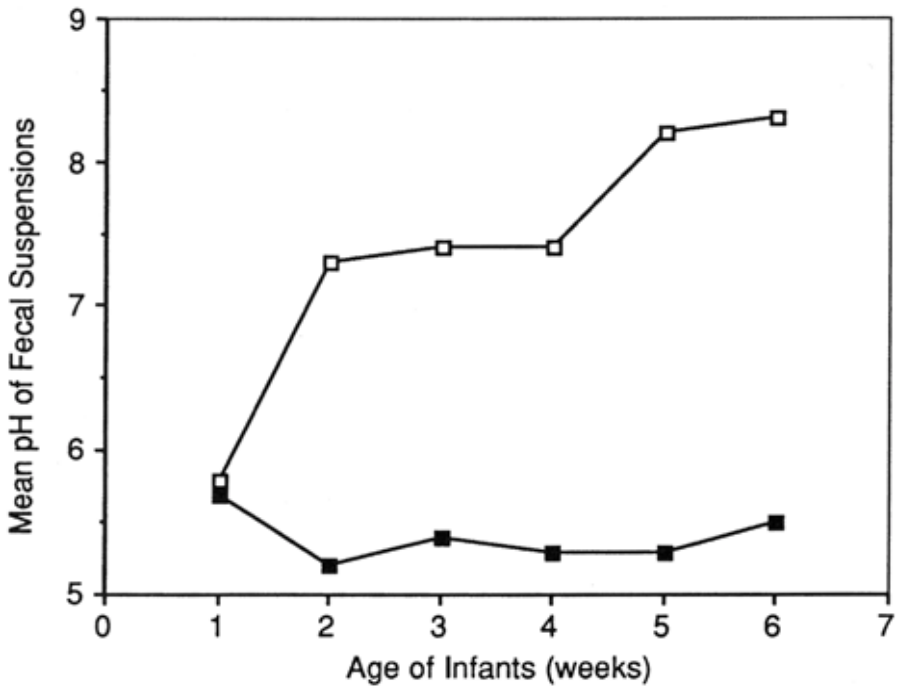

Figure 29: Mean pH of faecal specimens from breast-fed (■) and formula-fed (□) infants (Modified from: *Bullen, Tearle* and *Stewart*, 1977).

Brush border receptors for this strain of *E. coli* were first detected in rabbits 21 days of age and by 35 days of age, the brush border receptor activity had reached adult levels. Examination of brush border lactase activity revealed that the emergence of *E. coli* receptors correlated with the onset of developmental changes associated with weaning.

Antimicrobial Agents

In the presence of broad-spectrum antimicrobial agents, the sequential development of the neonatal intestinal flora can be dramatically altered (*Bennet* et al., 1984). Even when administration of the antimicrobial agent is discontinued, it may take several weeks for the intestinal flora to return to normal.

Geographical Setting

The geographical setting of neonates can also influence the sequential development of the intestinal flora. For example, pre-term neonates hospitalised in Neonatal Intensive Care Units have an abnormal intestinal flora (*Bennet* et al., 1984). It has also been shown that the intestinal flora of infants from different countries may differ (*Simhon* et al., 1982).

Intestinal Abnormalities

Congenital intestinal abnormalities may also influence the sequential development of the intestinal flora in neonates. For example, in neonates with congenital small bowel obstruction the distal bowel remains sterile. This suggests that colonisation of the neonatal intestinal tract occurs per-orally.

Role of the Developing Intestinal Flora in Health of Neonates and lnfants

The intestinal flora plays an extremely important role in the health of neonates and infants. Three examples of

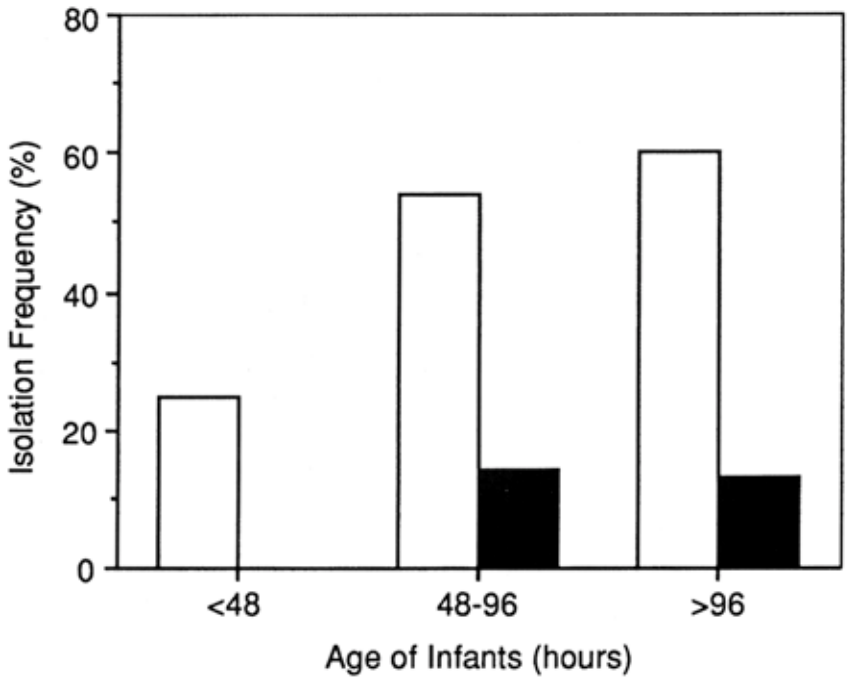

Figure 30: Isolation frequency of *Bacteroides fragilis* from infants born vaginally (□) and by Caesarean section (■) (Modified from: *Long* and *Swensson*, 1977).

the role of the intestinal flora in the health of neonates will be given below.

Vitamin K Deficiency

Vitamin K deficiency has been reported to occur in some breast-fed neonates and infants as well as in adults and infants receiving broad-spectrum antimicrobial agents (*Goldman* and *Deposito*, 1966; *Sutherland, Glueck* and *Gleser*, 1967). Vitamin K deficiency usually does not develop from low intake of vitamin K alone, presumably because of bacterial production of vitamin K in the intestine. The intestinal flora of infants with vitamin K deficiency differs significantly from the intestinal flora of breast-fed and formula-fed infants without a deficiency of vitamin K (*Benno* et al., 1985). It has been postulated that vitamin K deficiency in breast-fed infants is a result of reduced numbers of vitamin K producing bacteria, such as *E. coli* and *Bacteroides*, as well as the low vitamin K content of human milk.

Infant Botulism

Infant botulism is a disease in which *Clostridium botulinum* multiplies in the intestinal tract of infants and produces its potent neurotoxin. A similar type of intestinal infection with *C. botulinum* does not occur in adults. There is experimental evidence in animals, which suggests that variations between the bowel flora of infants and adults may account for differences in their susceptibility to intestinal botulism.

Using infant mice as a model, *Sugiyama* and *Mills* (1978) experimentally reproduced the limited age susceptibility to *C. botulinum* intestinal overgrowth. These investigators showed that *C. botulinum* spores, when injected intracoecally, would germinate in the intestine of mice only between the ages of 7 and 13 days. It is during this time inter-

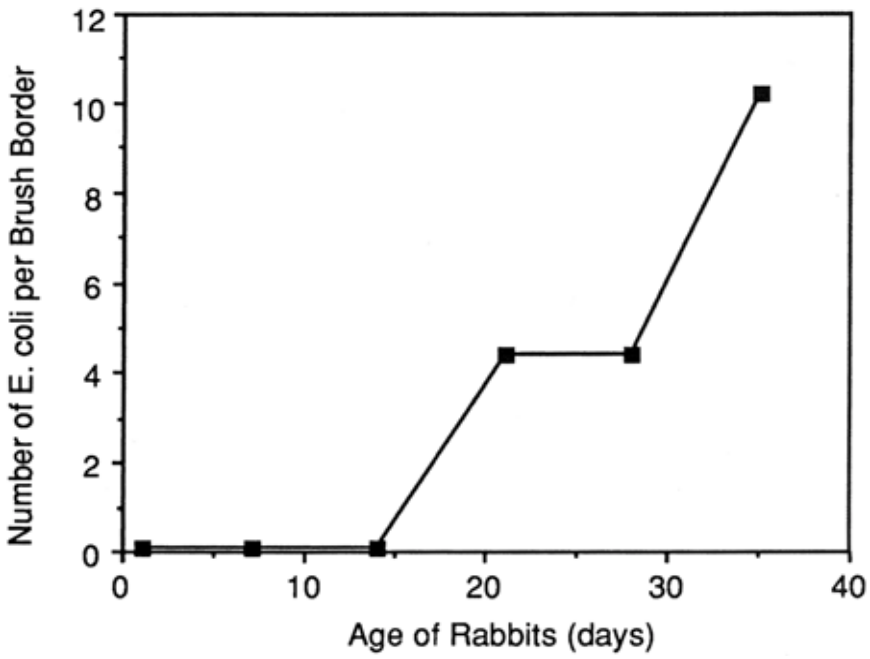

Figure 31: Variation in the adherence of *Escherichia coli* to rabbit intestinal brush borders with respect to age (Modified from: *Cheney* and *Boedeker*, 1984).

val that the intestinal flora of mice undergo dramatic quantitative and qualitative changes.

There is additional evidence, which demonstrates the importance of the intestinal flora in controlling *C. botulinum* intestinal overgrowth. *Moberg* and *Sugiyama* (1978) showed that the intestines of adult germfree mice are colonised with *C. botulinum* when as few as 10 spores of this microorganism are given orally. On the other hand, adult mice with a conventional microbial flora are resistant to *C. botulinum* intestinal colonisation even when 10^5 spores are inoculated orally. When the adult germfree mice are housed with conventional animals, they become resistant to challenge with 10^5 spores. *Burr* and *Sugiyama* (1982) showed that adult mice with a normal microbial flora but treated with large oral doses of two broad spectrum antimicrobial agents are at least 50-fold more susceptible to *C. botulinum* intestinal colonisation than untreated control mice. The increased susceptibility of antimicrobial treated mice to *C. botulinum* intestinal colonisation is abolished by stopping the antibiotics and housing the mice with untreated control mice.

<u>*Clostridium difficile* colonisation of infants</u>

Clostridium difficile is an important aetiologic agent of antimicrobial agent-associated diarrhoeal disease in adults. Asymptomatic adults seldom have toxigenic *C. difficile* in their intestinal tract. On the other hand, up to 90% of infants less than one year of age are asymptomatically colonised with toxigenic *C. difficile*. This suggests that a developmental change in resistance to *C. difficile* intestinal colonisation occurs between infancy and adulthood. How-

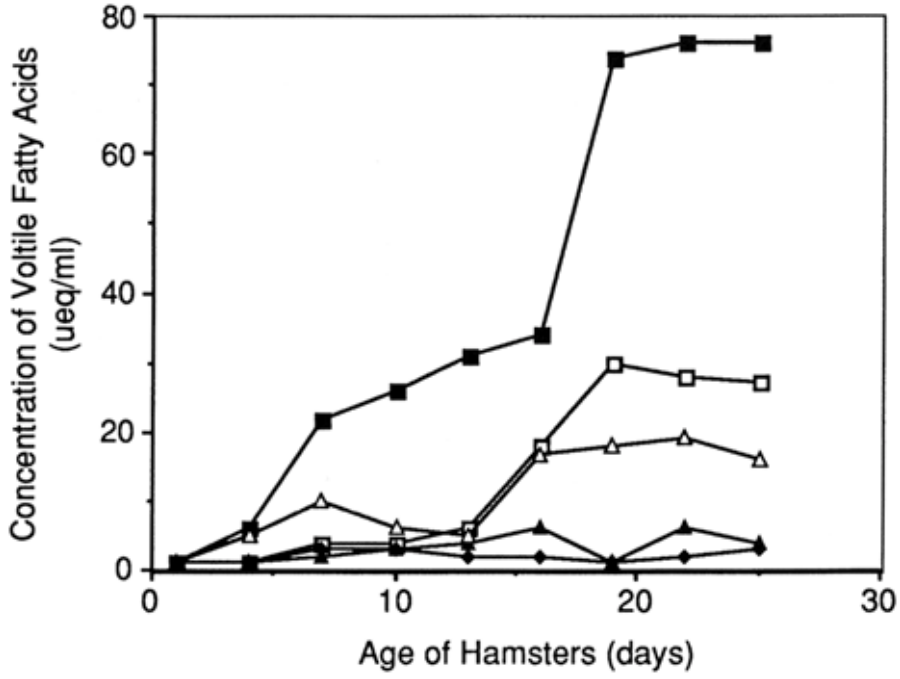

Figure 32: Concentration of volatile fatty acids in the caeca of infant hamsters. ■: Acetic acid; □: Butyric acid; △: Propionic acid; ▲: Valeric acid; ◆: Isovaleric acid (Modified from: *Rolfe*, 1984).

ever, the mechanisms accounting for the resistance to *C. difficile* colonisation in healthy, untreated adults are unknown.

Infant hamsters closely parallel infant humans in their susceptibility to asymptomatic colonisation by toxigenic *C. difficile*. *C. difficile* colonises the intestinal tracts of non-antibiotic treated hamsters between 4 and 11 days of age (*Rolfe* and *Iaconis*, 1983). These hamsters remain healthy even though they possess high titres of *C. difficile* toxin in their intestinal tracts. Hamsters younger and older than 4 to 11 days of age are resistant to *C. difficile* intestinal colonisation unless first treated with an antimicrobial agent. The infant hamster model of asymptomatic *C. difficile* intestinal colonisation has been used to examine the mechanisms responsible for the differences in the susceptibility of infant hamsters and adult hamsters to *C. difficile* intestinal colonisation. One of the parameters which has been examined is the concentration of volatile fatty acids in the intestinal tracts of hamsters of different ages (*Rolfe*, 1984). Figure 32 shows the concentrations of various volatile fatty acids in the intestinal tracts of hamsters from 1 day of age to 25 days of age. Individually, none of these volatile fatty acids inhibited the *in vitro* multiplication of *C. difficile* at concentrations found in the intestinal tracts of hamsters. However, when mixtures of the volatile fatty acids were prepared to correspond to *in vivo* concentrations, the results depicted in Figure 33 were obtained. Volatile fatty acids in hamsters 1 to 10 days of age were not inhibitory to *C. difficile*. Concentrations of volatile fatty acids in hamsters 13 days of age were initially bacteriostatic after which growth of *C. difficile* occurred. Volatile fatty acids in hamsters 16 days of age or older were

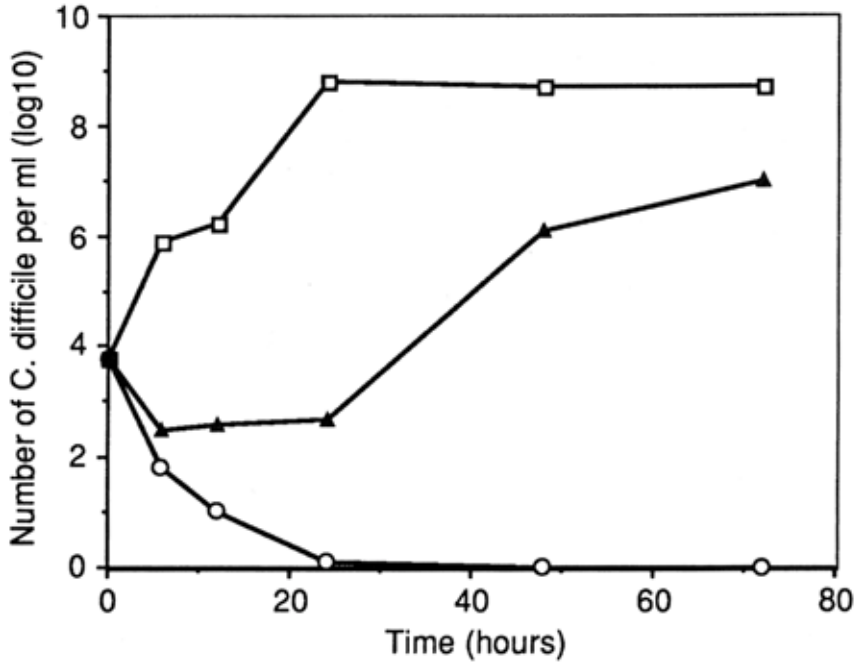

Figure 33: *In vitro* growth of *Clostridium difficile* in the presence of volatile fatty acids. □: Volatile fatty acids in hamsters I to 10 days of age; ▲: Volatile fatty acids in hamsters 10 days of age; ❍: Volatile fatty acids in hamsters 16 days of age and older (Modified from: *Rolfe*, 1984).

bactericidal to *C. difficile*. These results suggest that volatile fatty acids may be one mechanism regulating the growth of *C. difficile in vivo*.

Conclusion

A completely satisfactory understanding of the succession of the normal flora in neonates and infants may not soon be achieved because of the complexity of the ecosystem. However, this is basic information, which must be understood if we ever hope to control intestinal diseases of infancy.

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4. Mechanisms that Predispose to Ecological Stability in the Gut

One of the most remarkable features of the adult indigenous flora of humans and animals is its stability. The populations of microorganisms comprising the flora continually exert strong forces to maintain the community status quo. The practical effect of these activities is the exclusion of invading populations of non-indigenous microorganisms, including pathogens that attempt to colonise the intestinal tract from time to time. Only the most extreme stress situations, such as antibiotic administration, have major effect on the stability of the initial flora.

Factors responsible for the exclusion of non-indigenous organisms from the intestine and therefore maintenance of flora stability have not been identified, although several inhibitory mechanisms have been proposed. They include:

1. competition between the flora and non-indigenous organisms for nutrients present in limited quantities,
2. elaboration of substances by the flora that inhibit multiplication of non-indigenous organisms,
3. competition between the flora and non-indigenous organisms for attachment sites on the intestinal mucosal cells, and
4. establishment of environmental conditions by the flora that adversely affect non-indigenous organisms.

Any one or all may be operative.

Considerable work has been done to identify compounds elaborated by the microflora that inhibit non-indigenous organisms. Although antibiotic substances produced by the flora, such as colicines, interfere with multiplication of non-indigenous organisms *in vitro*, there is no evidence that they function in the intestinal tract. However, there is evidence that volatile fatty acids, elaborated by components of the microflora as metabolic products, play a role in excluding non-indigenous organisms from the intestinal tract. Some time ago, *Meynell* (1963) and *Bohnhoff* and co-investigators (1964a, 1964b) demonstrated that the multiplication of *Salmonella enteritidis*, a non-indigenous pathogen, was inhibited by suspensions of intestinal contents obtained from conventional mice. The contents contained volatile fatty acids in concentrations that prevented multiplication of *S. enteritidis* at low pH and oxidation-reduction potential measured in the intestine.

Treatment of the animals with streptomycin eliminated components of intestinal bacterial flora; this was associated with a decrease in total volatile fatty acids and an accompanying increase in oxidation-reduction potential and pH-producing conditions that favoured the multiplication of *S. enteritidis*. *Maier* and co-investigators (1972) obtained

similar results with *Shigella flexneri*. When conventional mice were infected orally with *Sh. flexneri*, the pathogen failed to multiply in the intestine and persisted at a low population of approximately 10^3 organisms per gram caecal contents. In germfree animals, *Sh. flexneri* multiplied rapidly, attaining populations of about 10^{10} organisms per gram caecal content. Caecal contents from the groups of animals were therefore compared to determine whether volatile fatty acid concentration, oxidation-reduction potential and pH were substantially different. The results showed (Table 13) that the oxidation-reduction potential and the pH in caecal contents of conventional mice were lower and volatile fatty acid concentrations were higher in caecal contents of germfree mice. *Sh. flexneri* multiplied *in vitro* in caecal contents taken from germfree mice but failed to multiply in contents obtained from conventional mice. Therefore, caecal contents obtained from germfree mice were adjusted to stimulate the oxidation-reduction potential and pH as well as volatile fatty acid concentration found in the caecal contents of conventional animals. After adjustment, the bactericidal effect against *Sh. flexneri* was almost identical with the effect observed in caecal contents of conventional animals (Figure 34). Essentially, the same results were obtained when strains of *Shigella sonnei* and *Shigella dysenteriae* were used, indicating that the high concentration of volatile fatty acids, the low pH and oxidation-reduction potential in the intestinal tract of conventional mice are major factors inhibiting the multiplication of *Shigella* and *Salmonella* species *in vivo*.

Several other studies confirmed a role for volatile fatty acids in excluding non-indigenous organisms from the intestinal tract. Population levels of *Clostridium difficile*, for example, appear to be influenced by the concentrations of volatile fatty acids in the intestine (*Rolfe*, 1984). Infant hamsters whose intestines contain low concentrations of volatile fatty acids are readily colonised by *C. difficile*, but adult hamsters with high concentrations of intestinal volatile fatty acids resist colonisation.

Que and colleagues (1986) observed a decline in volatile fatty acid concentrations and an increase in the pH of intestinal contents of mice as a consequence of administering streptomycin (Table 14) resulting in enhanced susceptibility to infections with *Pseudomonas aeruginosa* (*Hentges* et al., 1985) and *Salmonella typhimurium* (*Que* and *Hentges*,

Table 13: Eh, pH and volatile fatty acid values for germfree and conventional mice caecal contents

	Germfree	Conventional
Eh	- 49 mV	- 234 mV
pH	6.80	6.08
Total volatile fatty acids	0.04 N	0.15 N
Concentration of Acetic acid	0.04 N	0.10 N
Concentration of Propionic acid	0	0.005 N
Concentration of Butyric acid	0	0.045 N

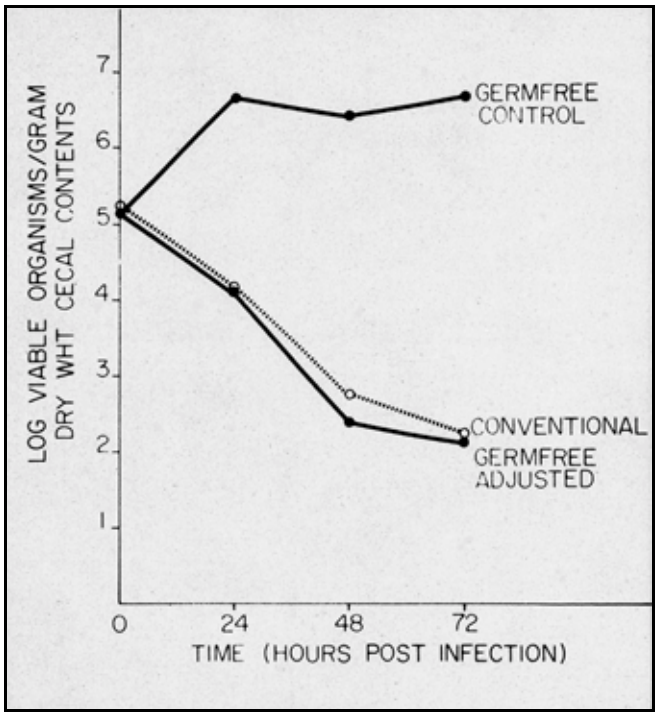

Figure 34: Multiplication of *Shigella flexneri* 2A in adjusted caecal contents from germfree and conventional mice.

1985). The caecum and small intestine of all streptomycin treated mice were colonised with *Ps. aeruginosa* and the organism translocated to either the mesentery, liver or spleen of the majority of these animals. None of the untreated mice were colonised Although the *S. typhimurium* challenge dose was much smaller than the *Ps. aeruginosa* dose, the caecum, small intestine and extra-intestinal organs of all streptomycin treated mice were colonised with the organism. *S. typhimurium* failed to colonise untreated mice. Administration of streptomycin, which lowered volatile fatty acid concentrations and increased pH of intestinal contents predisposed the animals to infection with non-indigenous organisms.

Volatile fatty acid production by the bacterial microflora is not the only factor regulating the bacterial populations of the intestine. There is evidence that the populations of *Escherichia coli* are not influenced by variations in intestinal volatile fatty acid concentrations. In general, *E. coli* tends to be more resistant to the affects of the acids than classic intestinal pathogens. When germfree mice were associated with different floras obtained from conventional mice and then implanted with an *E. coli* strain, there was no correlation between intestinal volatile fatty acid levels and *E. coli* population (*Freter* and *Abrams*, 1972). This indicates that the acids are not important in regulating *E. coli* populations in the intestine.

Experiments of *Freter* et al. (1973) provided data on possible *E. coli* population control mechanisms. Anaerobic continuous flow cultures of homogenates of intestinal contents from conven-

Table 14: Effects of streptomycin administration on various environmental conditions of caecal contents

Condition	Mean + SD of results from[A]:	
	Untreated mice	Treated mice
Eh (mV)	-128.90 ± 7.61	-118.57 ± 21.80
Protein[B]	6.17 ± 1.53	5.95 ± 1.79
Carbohydrate[B]	4.54 ± 3.22	6.90 ± 4.81
Dry wt/wet wt	0.24 ± 0.03	0.20 ± 0.06[C]
pH	6.42 ± 0.13	6.73 ± 0.28[C]
Aceticacid[D]	74.8 ± 9.0	53.1 ± 7.9[C]
Propionic acid[D]	19.6 ± 4.0	13.0 ± 4.4[C]
Butyric acid[D]	60.8 ± 9.0	20.7 ± 4.9[C]
Valeric acid[D]	2.5 ± 0.9	ND[C,E]

[A] 10 Experiments.
[B] Results given in milligrams per gram (wet weight) of caecal contents.
[C] Significantly different compared with untreated mice ($p<0.05$) by the two-tailed Student t-test.
[D] Results given in micro-equivalents per gram (wet weight) of caecal contents.
[E] ND: None detected.

tional mice mixed with *E. coli* in veal infusion broth, suppressed *E. coli* populations when compared with pure continuous flow cultures of *E. coli*. The diminished *E. coli* population levels were of magnitude observed *in vivo* in conventional mice suggesting that the control mechanisms in the flow culture and in the animal intestines were similar. Effluents from the continuous flow cultures inhibited *E. coli* multiplication, but the inhibition was reversed by addition of glucose. The results suggest that competition for nutrients, which are replaceable by glucose, is the activity overriding importance in the regulation of *E. coli* population levels in continuous flow cultures and possibly in the intestinal contents.

Bile acids, which are modified by indigenous flora components, may also function to exclude non-indigenous organisms from the intestinal tract. The primary bile acids, cholic acid and chenodeoxycholic acid, are synthesised by the liver and are conjugated to either taurine or glycine. Human bile also contains conjugates of a secondary bile acid, dehydrocholic acid, which is formed by the dehydroxylation of cholic acid.

In the intestine the conjugates are hydrolysed to release free bile acids by a variety of bacteria, particularly anaerobes. Only free acids are present in the faeces. The bacteria also convert the primary bile acids to secondary bile acids by oxidoreduction of hydroxyl groups and dehydroxylation reactions. *Floch* et al. (1972) demonstrated that a variety of both Gram-positive and Gram-negative bacteria are inhibited by free bile acids but are not affected by either human whole bile or by conjugated bile acids. Free bile acid may very well be responsible for the exclusion of non-indigenous organisms from the intestinal ecosystem.

Competition for colonisation sites on intestinal mucosa surfaces is another mechanism that has been proposed to explain the role of the indigenous bacterial microflora in the rejection of non-indigenous organisms from the intestine. This was suggested by discovery of the colonisation by normal flora bac-

teria of the mucus layer on the intestinal mucosal cells. The resulting mat of microflora presumably prevents contact and colonisation by non-indigenous bacteria, which need to adhere to the mucosa in order to survive in this open system. Specificity of attachment appears to be greater between indigenous microflora and host cells than between non-indigenous organisms and host cells. The non-indigenous organisms either fail to attach or are displaced by indigenous microflora once attached and are eliminated from the intestinal ecosystem.

The body of evidence suggests that multiple mechanisms are employed by the flora to exclude non-indigenous organisms from the intestinal tract. Recent data reported by *Hentges* et al. (1989) tend to confirm this hypothesis. Using Swiss white mice, they examined the influence of several oral antibiotics, administered at therapeutic levels, on resistance against colonisation by enterotoxigenic *E. coli*, *S. typhimurium* and *Sh. flexneri*.

In every case where an antibiotic produced an effect, resistance to colonisation with *S. typhimurium* and *Sh. flexneri* was enhanced. However, the situation with *E. coli* was the reverse. When an effect was observed the colonisation resistance for this pathogen was decreased. Therefore, identical factors appear to regulate colonisation of the intestine by *Sh. flexneri* and *S. typhimurium*. These factors differ from those that regulate the colonisation of the intestine by *E. coli*. This provides strong evidence that a universal mechanism governing colonisation against non-indigenous organisms in the intestinal tract does not exist but that several mechanisms are involved to actively exclude non-indigenous organisms from the ecosystem. In this way the indigenous intestinal flora maintains the ecological stability of the gut.

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5. Individual Variation in the Microbial Population of the Human Gut

Microbial colonisation of new-born infants begins immediately after birth; infants are colonised by flora from the body of the mother and other (human) contacts as has been reviewed earlier in this seminar. Initial colonisation is fortuitous, depending on the first suitable organism to arrive at a particular site as well as on factors such as route of delivery, the type of nourishment received (breast milk or formula) and the degree of exposure to 'hospital environment'. In most cases, after only a few weeks, the representation of microbial species within the neonatal flora is remarkably similar to the adult pattern of colonisation (Table 15). This Table shows the conventional summary of the composition of the digestive tract microflora. Even more detailed descriptions which go into genera and subgenera surpass perhaps the reality as such detailed list of flora components does not take in consideration that genera and even species and subspecies can be further subdivided in different serotypes. Particularly the latter may be of 'practical importance', since an important way in which the host organism 'decides' about the composition of its flora is not only by offering it a species - and possibly even strain - specific source of nutrients but by looking at it immunologically. Both selective mechanisms have been mentioned in this seminar several times.

Table 15: Microorganisms which may inhabit various sites of the digestive tract

Oropharynx	Nasopharynx	Upper intestines	Lower intestines
Viridans streptococci Staphylococci *Str. pyogenes* *Branhamella cattharhalis* *Neisseria* spp. Lactobacilli Corynebacteria *Haemophilus* spp. Obligate anaerobes (*Candida*, protozoa)	Staphylococci Corynebacteria *Haemophilus* spp.	Streptococci Lactobacilli (*Candida* spp.)	Staphylococci Streptococci (including enterococci) Lactobacilli Corynebacteria *Neisseria* spp. Obligate Gram-pos. and Gram-neg. anaerobes Aerobic Gram-negative enterobacilli *C. albicans* (Protozoa)

The fact that the composition of the gastrointestinal flora of the new-born is completely fortuitous as it depends on the flora of the mother and many other sources encountered early in life and the fact that since a flora has settled in the digestive tract it remains stable in composition over long periods, may indicate that indeed in the first weeks or months after birth, the immune system may determine which bacteria, capable of digesting their nutrients (mucus, cells) can be 'tolerated' and which should be 'rejected'. This then may explain why several investigators have reported that different humans often are colonised by a different intestinal flora (*Holdeman*, *Good* and *Moore*, 1976; *Mitsuoka* and *Ohno*, 1977; *Moore*, *Cato* and *Holdeman*, 1978).

Van de Merwe and co-authors (1983) have investigated to which extent family members (parents and their children) match when the composition of their faecal flora is carefully studied. They reported that in man not only the aerobic (for most part transient) but also the anaerobic part of the faecal flora differs between individuals. In ten young (several months to several years old) human twins (five monozygotic and five dizygotic twin pairs) *van de Merwe*, *Stegeman* and *Hazenberg* (1983) being faced with the difficult task to analyse the faecal flora of these children in great detail sought for a method by which they could handle a great number of samples with the limited technical assistance available. They found a rather elegant solution which enabled them to answer their question to which extent the human flora differs from one to another within one family and whether there is a genetic component involved in the selection made of the fortuitously encountered flora after birth. Fresh faeces were plated on selective media under carefully maintained strict anaerobic circumstances. After incubation, Gram-stains were made of the various different (looking) colonies. In this way the Gram-positive bacteria could be classified into six different morphology groups:

group 1, cocci growing in clusters or in chains;

group 2, ovoid larger cocci and short rods;

group 3, typical *Bifidobacterium*;
group 4, typical *Eubacterium*;
group 5, very short rods;
group 6, all other (unclassable) types of rods.

Gram-negative bacteria (rods) were put in a seventh group. Per faecal sample the seven morphological groups were expressed in percentages of the total number of bacteria in that particular sample. Individual faecal floras were represented by a single point in an Euclidian space with seven dimensions, one for each group of bacteria. Dissimilarity of faecal flora of twin siblings and between children of different siblings was expressed as a distance in the Euclidian space between the point representing each individual. These distances were calculated by taking the square root of the sum of square raised differences of corresponding parameters.

From the results of their study *van de Merwe* and co-workers concluded that the composition of the faecal flora is under influence of genetic determinants of the host. In their study the floras of monozygotic twin siblings, being individually constant, were less distant (different) than siblings of the dizygotic twins. As one may expect on the bases of observations reported by other groups, the distance in the Euclidian space between unrelated subjects appeared much greater than between siblings of dizygotic origin.

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6. Microbial Ecology of the Human Bile Duct

If in patients the bile duct gets occluded, either from outside by for example a pancreatic carcinoma, or from within by foreign material such as a T-tube used to drain the common bile duct for some time after surgery, stone formation may occur. A study by *Speer* and co-workers (unpublished data) suggests that bacteria are involved in this stone formation. This is not confined to the bile duct only but may also be the case in the ureters as will also be discussed in this paper.

The first clue that bacteria would be able to survive and proliferate in the common bile duct, was on recovered stones. Both the external surface and the interior part appeared to be formed by aggregated material. The stones were found to have alternating layers of blue and brown material in concentric rings (Figure 35).

The stones may grow so big that they eventually - in less than one to six months - may block the bile duct or the T-tube. The brown staining zones in the stones are composed of calcium bilirubinate. On light microscopy they show large numbers of bacteria (Figure 36).

On examination of these aggregates by electron microscopy, they show large numbers of bacteria in microcolonies surrounded by a polysaccharide layer (Figure 37); large areas of loose staining material could thus be formed by bacterial aggregates. If one examines the alternate layers where the brown material occurred in light microscopy, very flat plates can be seen which appear chemically deposited cholesterol (Figure 38). In addition to cholesterol, which is precipitated out, one may find non-edged crystals (Figure 39) that appear rich in calcium (Figure 40), perhaps calcium bilirubinate.

In the patients with a T-tube in the common bile duct bacteria adhere to the

plastic of the tube to make a fairly thick film (Figure 41). They deposit their products, the cholesterol and calcium bilirubinate into the polysaccharide film. The stones are then formed by alternating bacterial growth and chemical deposition of metabolic products.

These observations convincingly indicate that bacteria can live in bile and, in the presence of foreign material, even in fairly high numbers in the bile duct. In other words, bacteria may be able to set up microcolonies after entering a hostile environment, since bile is after all antagonistic to a number of bacterial species, and set up microcolonies. While doing this they may protect themselves from clearance mechanisms by firm adherence and by forming the polysaccharide film layer.

Another human system, which has the same earmarks, is formed by the ureters. Stone formation is here often caused by *Proteus* species which form 'struvite stones' by urease activity (*Nickel*, *Emtage* and *Costerton*, 1985). In this case, struvite and ammonium and/or calciummagnesium phosphates are found in very large amounts around the bacterial microcolonies. Deposition of chemical aggregates and bacterial growth occurs here also in fairly regular intervals responsible for the layering. This event can be reproduced *in vitro* by growing the organism on a polystyrene layer. The bacteria start growing to produce a film surrounding themselves by polysaccharides and they actually manufacture the struvite crystals progressively (Figure 42).

In summary: In some ecosystems which are normally not thought of as being heavily bacterially colonised, bacteria can survive as long as they can adapt a microcolonial mode of growth motive, covering themselves with exo-

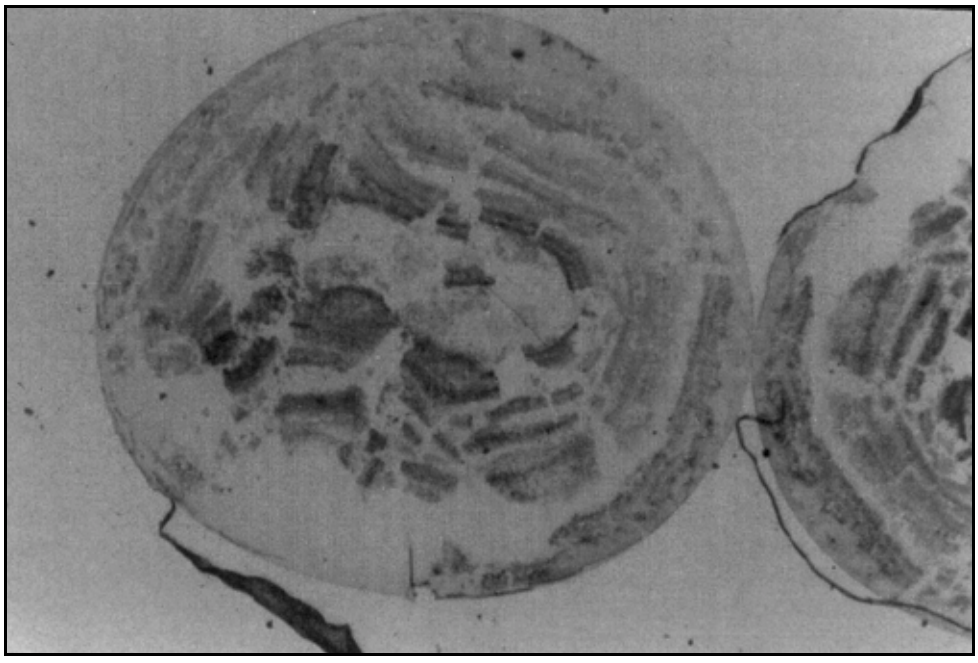

Figure 35: Light micrograph of a Haematoxilin Eosin preparation of a soft bile stone that had occluded the lumen of a biliary tube. The occluding deposit was composed of alternating concentric blue and light brown layers (x 3.8).

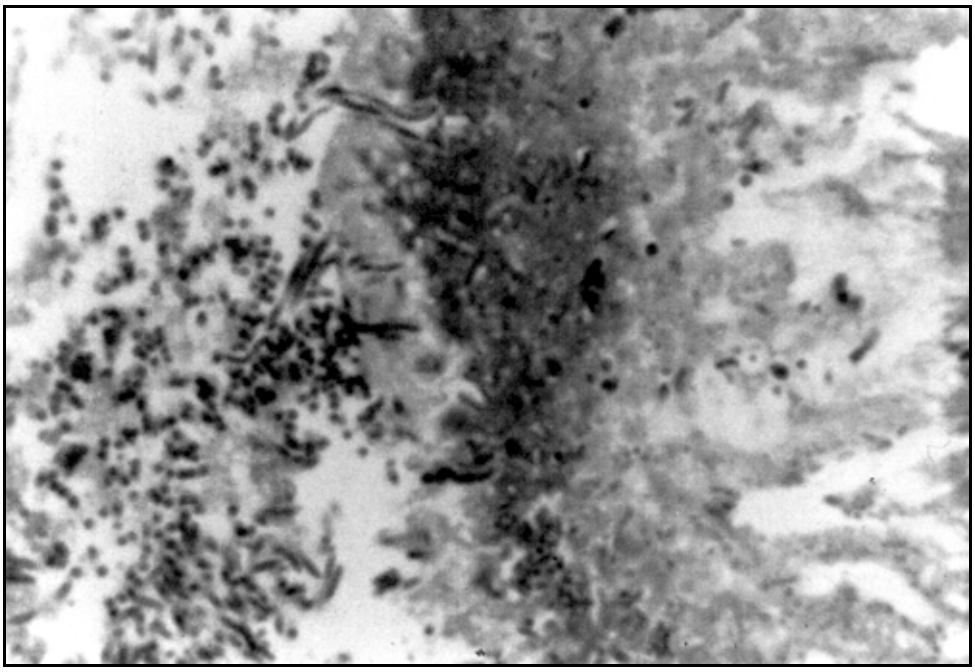

Figure 36: Higher magnification of the preparation seen in Figure 35 showing the very large number of bacterial cells that comprise the blue concentric layers of the deposit (x 580).

or extra-polysaccharide. This polysaccharide cover protects them from local antimicrobial factors. So far in many cases we may not have picked up this event by traditional microbiological methods, being unaware of the presence of bacteria, we may have disturbed these microcolonies during processing and exposed them to antibacterial substances such as bile. If however, the stones are carefully rinsed free of bile, the organisms mobilised by scraping off the surfaces and subsequently plated, large counts of as many as log 10 to 11 per cm^2 are obtained.

These data suggest that normally there may be a (scarce) bacterial flora in the bile duct which comes to prominence in the presence of foreign material or during obstruction of the normal bile flow.

J. William Costerton, Department of Biology, University of Calgary, Calgary, Canada T2N IN4.

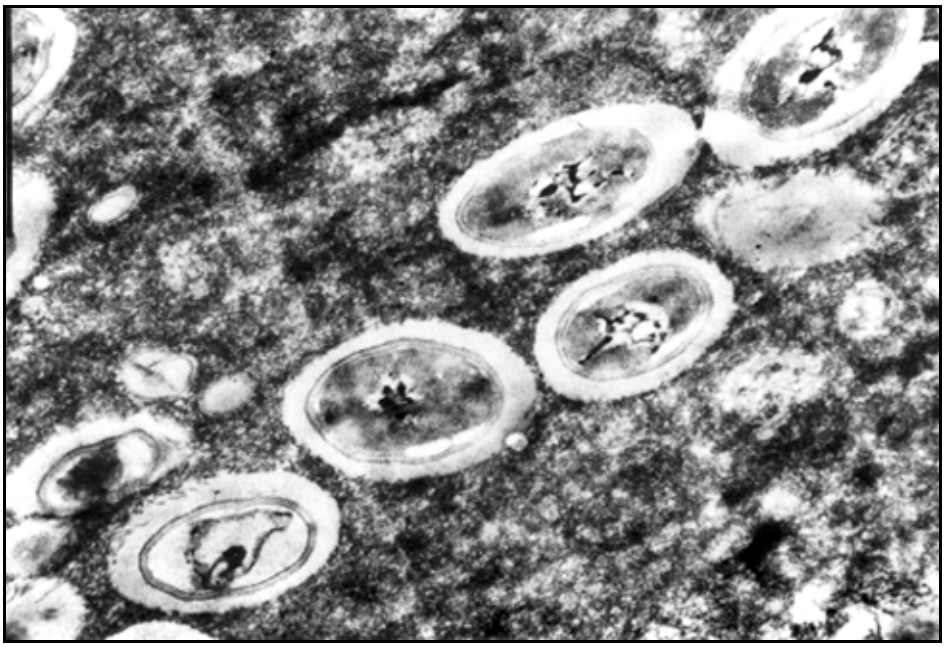

Figure 37: Transmission electron micrograph of a section of ruthenium red-stained and embedded biliary stone material showing a microcolony of bacterial cells embedded in this fibrous exopolysaccharide glycocalyx. These particular cells were rod-shaped and showed a rod-shaped cellular morphology, but adjoining microcolonies were composed of different morphotypes (x 42,000).

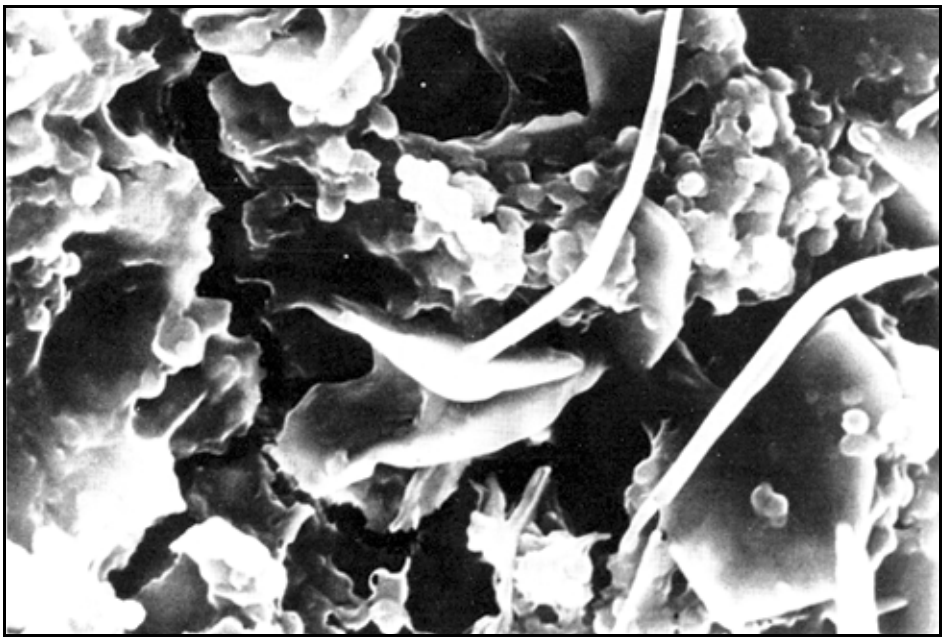

Figure 38: Scanning electron micrograph (SEM) of biliary stone material occluding a biliary tube showing coccoid bacterial cells in slime enclosed microcolonies amongst the characteristically plate-like flat crystals formed by cholesterol (x 38,500).

Figure 39: Less well defined irregularly shaped crystals from the same deposit showing (spot) where the beam was placed to produce the EDAX pattern seen in Figure 40 (x 38,500).

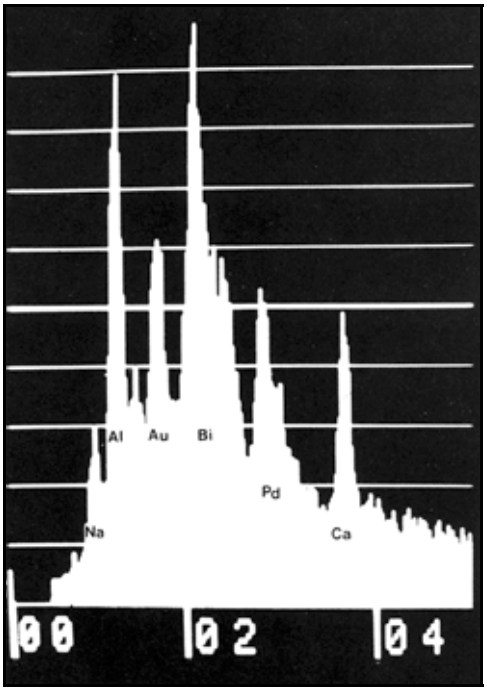

Figure 40: EDAX pattern of the crystal seen in Figure 39 showing the very high levels of calcium in these bulky crystals which are presumed to be calcium palmitate. The high levels of other atoms were contributed by the support structures (Al, Au) or by the processing (Na, Bi, Pd).

Figure 41: SEM of the surface of a biliary tube placed in a patient for <1 day showing the beginning of the colonisation of this smooth plastic surface by a wide variety of bacterial morphotypes (x 28,000).

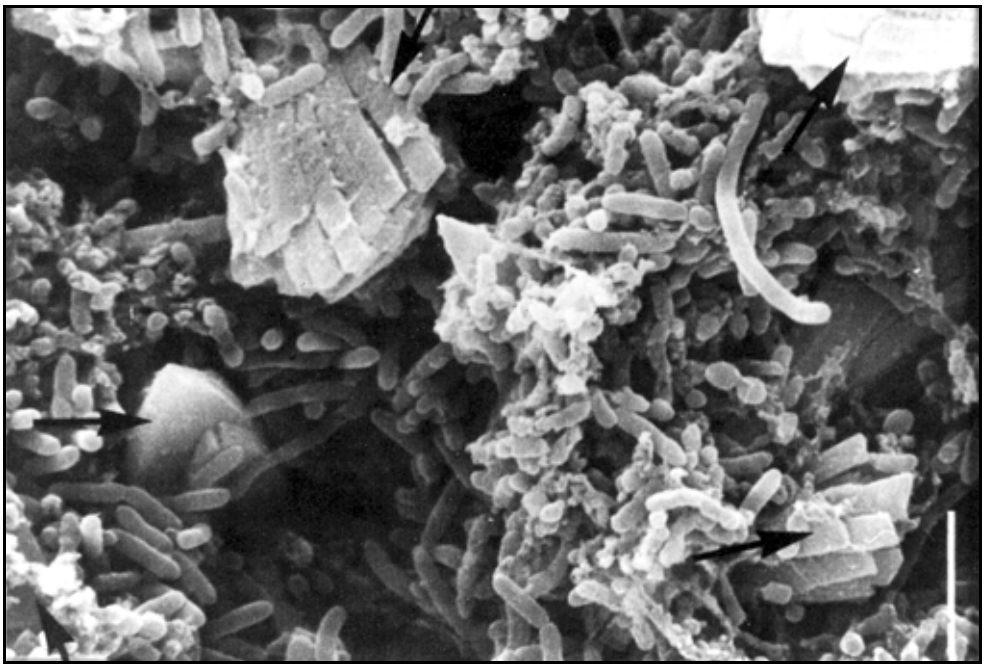

Figure 42: SEM of cells of *Proteus vulgaris* growing *in vitro*, in microcolonies, and producing crystals that were shown by EDAX to be struvite (Ammonium Magnesium Phosphate) as the initial step in urinary calculus formation (x 28,500).

7. Effects of Dietary, Genetic and Stress Factors on the Microbial Ecology of the Gut

A review of the literature reveals that the colonic microbial flora can be characterised in several different ways (*Lee*, 1985; *Holdeman, Good* and *Moore*, 1976; *Moore* et al., 1981); i.e. by:

a. the particular microbial species,
b. the metabolic characteristics of the microflora,
c. the quantity or mass of bacteria in a particular ecological niche and
d. genetic characteristics of the microorganisms which define particular metabolic or enzymatic capabilities.

In the fasting individual, only limited material enters the colon in the form of mucus, desquamated and obligatory products of cell membrane turnover. Among bacterial species which characterise the colonic microflora no qualitative changes have been observed during fasting, starvation or during ingestion of a chemically defined diet which is fully absorbed in the small intestine (*Hentges*, 1980; *McNabb* and *Tomasi*, 1981; *Simon* and *Gorbach*, 1987; *Tannock* and *Savage*, 1974; *Tomkins* et al., 1981). Expansion of the indigenous colonic flora occurs with introduction of growth substrates for this microflora. These are usually complex polysaccharides such as starch cellulose, pectins and other dietary constituents. Cellulose is only partly degraded in the human colon (*Wolin*, 1981). Such an expansion of the human colonic flora may be associated with changes in the population density of certain bacterial species in the caecum which favour lactic acid production and reduced acetic, butyric and propionic acid generation. A change in content of complex polysaccharides degraded in the caecum in some animal species, may alter the distribution of other bacterial species and their capability for survival in this ecological niche (*Mathiesen* et al., 1987) (Table 16). Changes in faecal microbial populations associated with chronic dietary intake of specific foodstuffs (*Tomkins* et al., 1981) have not been characterised consistently (*Moore* et al., 1981). However, review of the literature suggests that changes of bacterial species may occur in human groups on high fat - high meat protein intake associated with increased activity of ß-glucuronidase, azoreductase and bile acid excretion (*Hentges*, 1980; *Gorbach* et al., 1967; *Goldin* et al., 1980). It is now well recognised, that many bacterial metabolic

Table 16: Seasonal change in caecal microflora (reindeer)

Fall in bacterial populations in winter (17%)		
Species:	Summer:	Winter
Butyrivibrio	23 %	18 %
Streptococcus bovis	17 %	5 %
Bacteroides	10 %	26 %
Metabolic activity:	Summer:	Winter
Fibre	36 %	48 %
Cellulolysis	10 %	6 %
Xylanolysis	33 %	48 %
Starch	77 %	71%

Table 17: Estimated faecal populations of B-degrading bacteria

Subjects	No. of subjects	Estimated mean ± SD (Log^{10} count/gram)
B secretors	7	7.4 ± 1.4
A secretors	5	3.3 ± 2.6
O secretors	5	2.6 ± 2.6
Nonsecretors	5	2.1 ± 1.7
- (combined)	(15)	(2.7 ± 2.2)

characteristics, such as polysaccharide degrading activity, occurs across species. Greater differences occur in rank order of bacteria between individuals than between the same individual occur day to day; which may partially account for any change associated with dietary intervention. Stress factors (*Hentges*, *Good* and *Moore*, 1976; *Lee*, 1985; *Tannock* and *Savage*, 1974) such as alteration in food allocation, change in bedding and water supply to animals may change colonic flora radically. However, it is not known how such purtubations are expressed. Certainly, these stresses might alter immune expression (*McNabb* and *Tomasi*, 1981), release of catocholamine and neurotransmitters etc.

Blood group substances expressed by the host seem important for status of the hosts colonic flora. Expression of a higher density of bacteria with the specific blood group degrading activity indicate that genetic factors are important (*Hoskins* and *Boulding*, 1976b) (Table 17). However, there is little consistent data available which define, at this time, other genetic factors, which may contribute. The mucosal surface represents the major nutrient source for indigenous microbial organisms during the interdi-

Table 18: Faecal microflora population densities of PHA-fed (T) vs. non-PHA-fed (c) weanling rats over time (results are expressed as mean + SD log MPN organisms/g tissue)

		Total aerobes	Coliforms	Streptococci	*Lactobacillus*
I. (n = 4)					
Day 0	T	8.47±0.70 (NS)	8.37±0.82 (NS)	7.0±0.68 (NS)	2.97±3.54 (NS)
	C	8.70±0.40	8.40±0.31	7.64±0.38	2.49±2.91
Day 2	T	9.52±0.47*	8.39±0.72 (NS)	8.78±1.52*	6.51±1.70*
	C	8.76±0.60	8.62±0.64	7.17±0.64	3.54±0.22
Day 6	T	9.84±0.41*	9.39±0.67*	8.47±1.93*	8.10±0.17 (NS)
	C	7.80±0.78	7.37±0.82	6.89±0.37	7.68±0.29
Day 9	T	9.87±0.31*	9.80±0.18*	6.28±0.58 (NS)	7.49±0.60
	C	7.98±1.07	6.18±0.89	6.64±0.29	8.39±0.09
II. (n = 6; separate group)					
Day 11	T	NA	9.46±0.59*	NA	NA
	C		7.89±0.43		

MPN = Most probable number.
NA = Not assayed.
NS = Not significant.
* = Significant ($p<0.05$).

gestive phase. However, the exact nature of the control over these metabolic factors which include urea diffusion, mucus, desquamated cells and components of the mucosal cell membrane which turnover rapidly (half life about 3 hours) are poorly understood (*Lee*, 1985).

A new animal model in which augmented mucosal cell membrane turnover occurs, associated with feeding of phytohaemaglutinin (PHA) lectin derived from red kidney bean is of interest (*Banwell* et al., 1984, 1985). Such a single dietary stimulus which binds to the intestinal brush border membrane may have future importance in demonstrating how increased enterocyte brush border membrane shedding into the lumen may correlate with bacterial colonisation and mucosal adherence. Increased membrane turnover was greatly augmented in PHA fed rats and was associated with increased faecal protein and glycosphingolipid excretion. PHA induced changes in microbial ecology are associated with an increase of a specific ecological niche for the small intestinal indigenous microflora, demonstrated by *E. coli* and a *Streptococcus* spp. propagating 2-3 logs in PHA exposed animals without significant change in faecal anaerobic flora (*Banwell* et al., 1985) (Table 18).

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