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* actinomycetem- comitans
		A. *viscosus*	
Streptococcus sp.	*Neisseria* sp.	A. *naeslundii*	
S. *mutans* group*		A. *odontolyticus*	*Bacteroides* sp.
S. *mutans*		A. *israelii*	B. *forsythus*
S. *sobrinus*			B. *gingivalis*
		Arachnia	B. *intermedius*
S. *milleri*			
		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.

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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 synthe-

sised 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 gastro-intestinal 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.
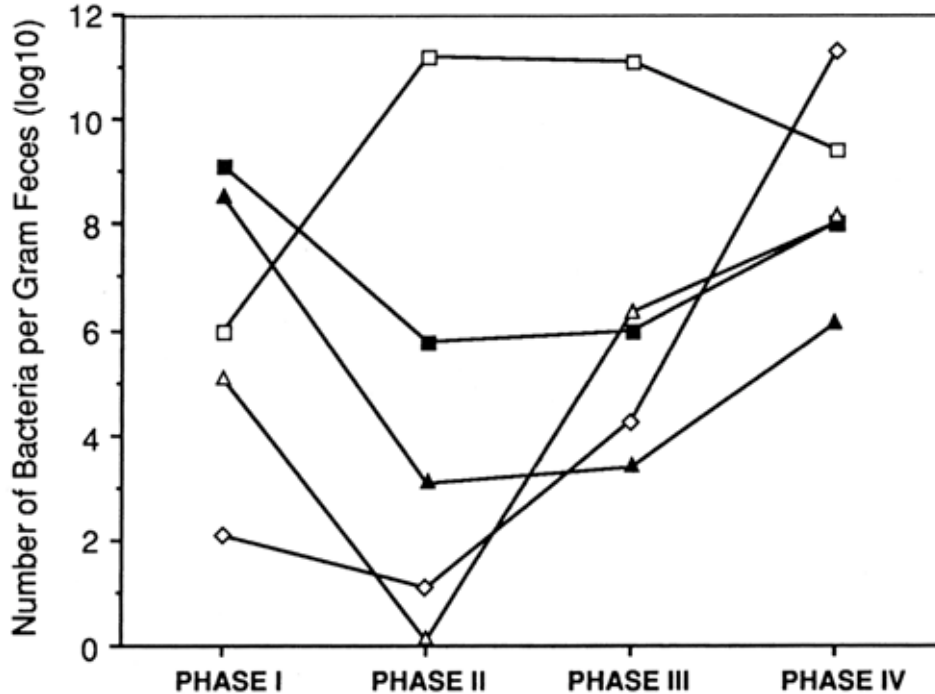

Figure 22: Sequential development of the normal intestinal flora in breast-fed infants.
■: *Enterobacteriaceae*; □: *Bifidobacterium*; ▲: *Streptococcus*; △: *Clostridium*; ↕: other anaerobes

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

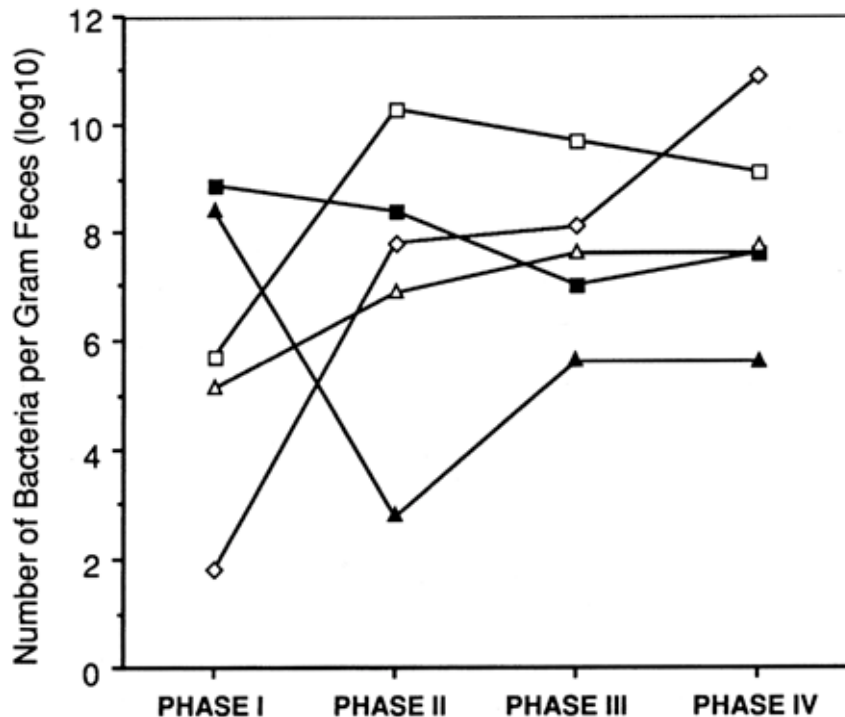Regardless of the animal species, Enterobacteriaceae and enteric strepto

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-
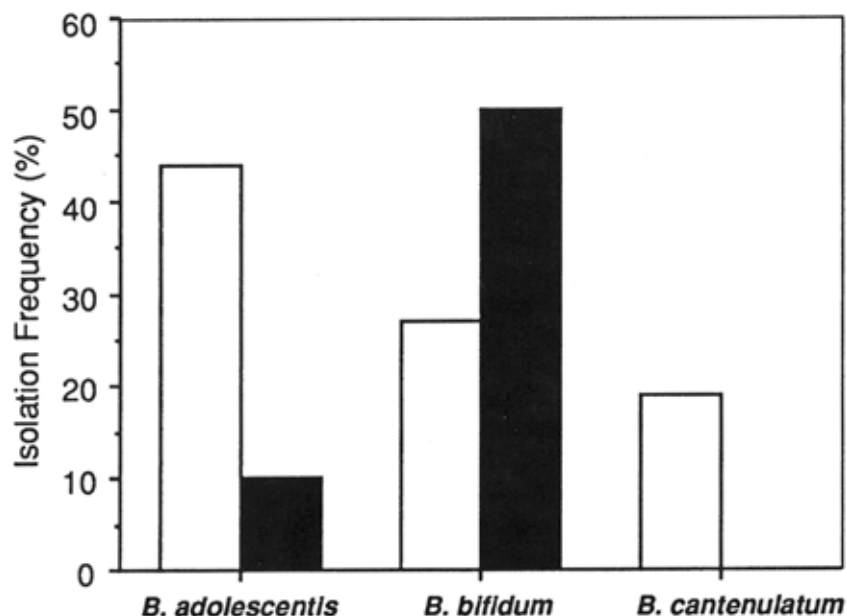

Figure 24: Isolation frequency of Bifidobacterium spp. from faecal samples of breast-fed (□) and formula-fed (■) infants (Modified from: *Mevissen-Verhage* et al., 1987).

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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 signifi-cantly 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-
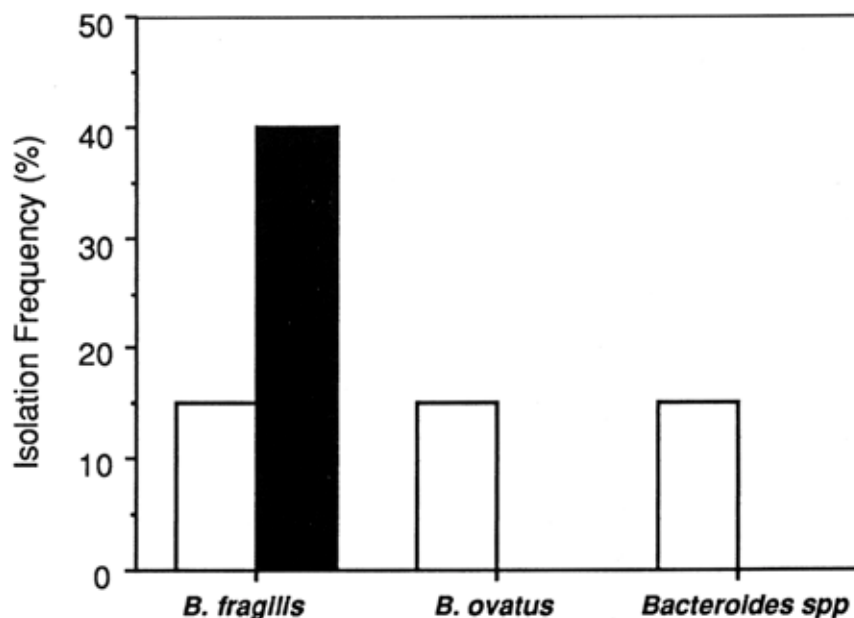

Figure 25: Isolation frequency of Bacteroides spp. from faecal samples of breast-fed (□) and formula-fed (■) infants (Modified from: *Mevissen-Verhage* et al., 1987).

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 physico-chemical 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

Oxidation-Reduction Potential

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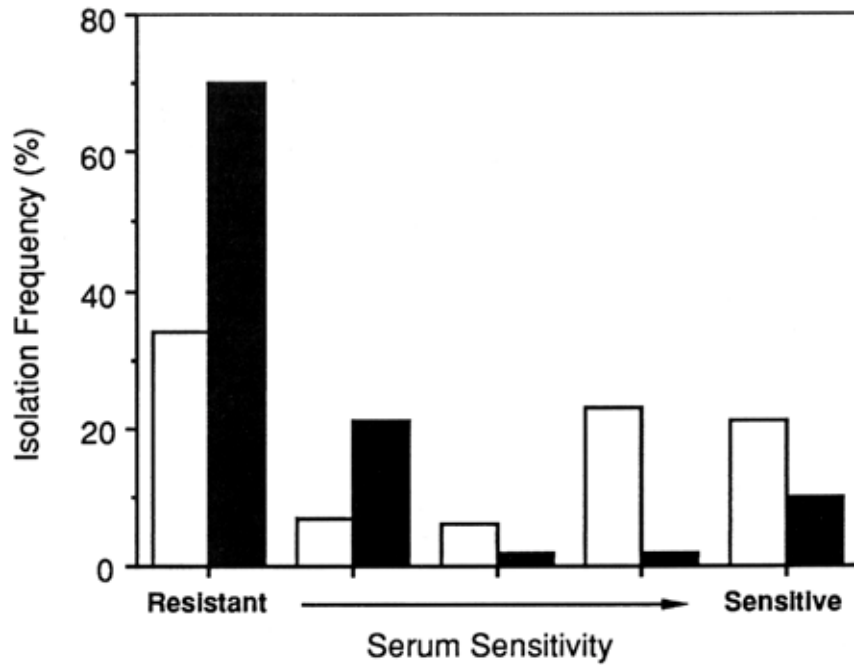
Figure 26: Bactericidal effect of human serum on strains of Escherichia coli isolated from the faeces of breast-fed (□) and formula-fed (■) infants (Modified from: *Braun*, 1981).
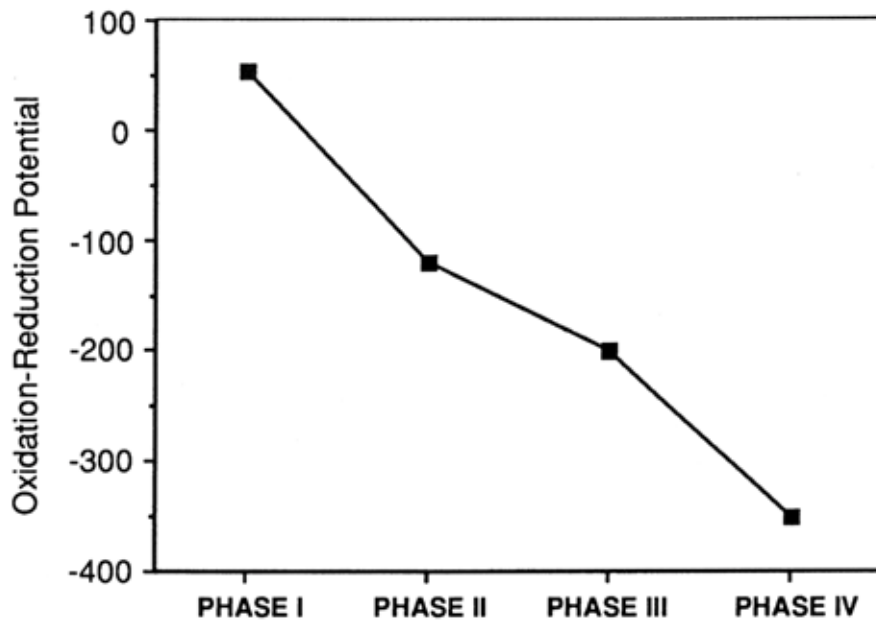

Figure 27: Oxidation-reduction potential in relation to the sequential development of the intestinal flora. (Modified from: *Grütte*, *Horn* and *Haenel*, 1965)

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).
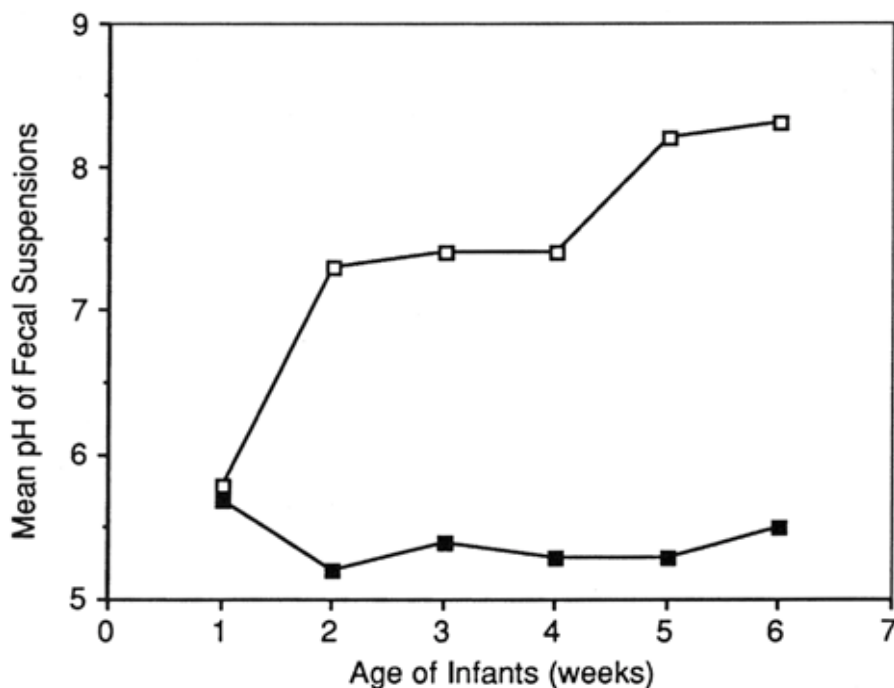

Figure 29: Mean pH of faecal specimens from breast-fed (■) and formula-fed (□) infants (Modified from: *Bullen*, *Tearle* and *Stewart*, 1977).

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 devel-

opment 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 Infants

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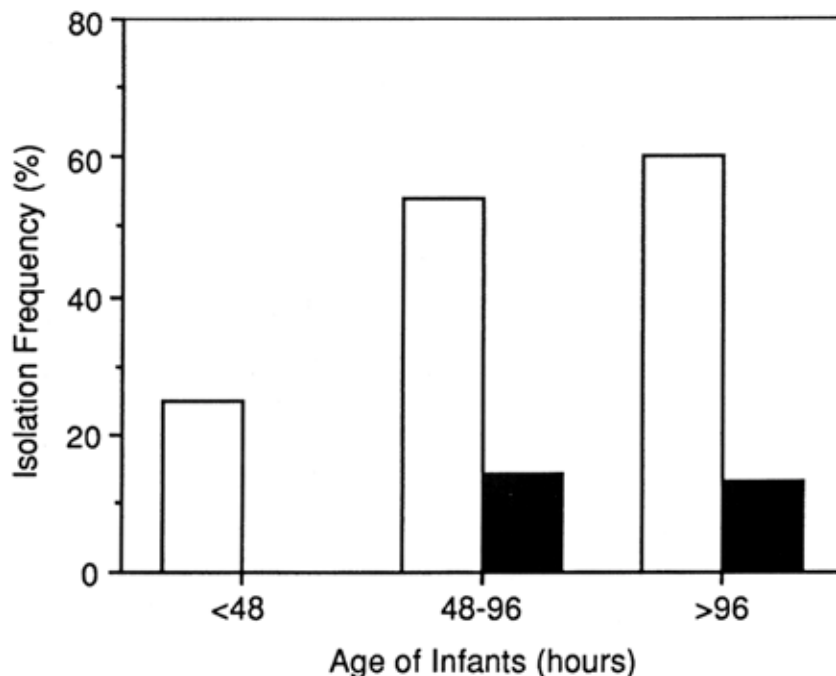

Figure 30: Isolation frequency of *Bacteroides fragilis* from infants born vaginally (□) and by Caesarean section (■) (Modified from: *Long* and *Swensson*, 1977).

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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-
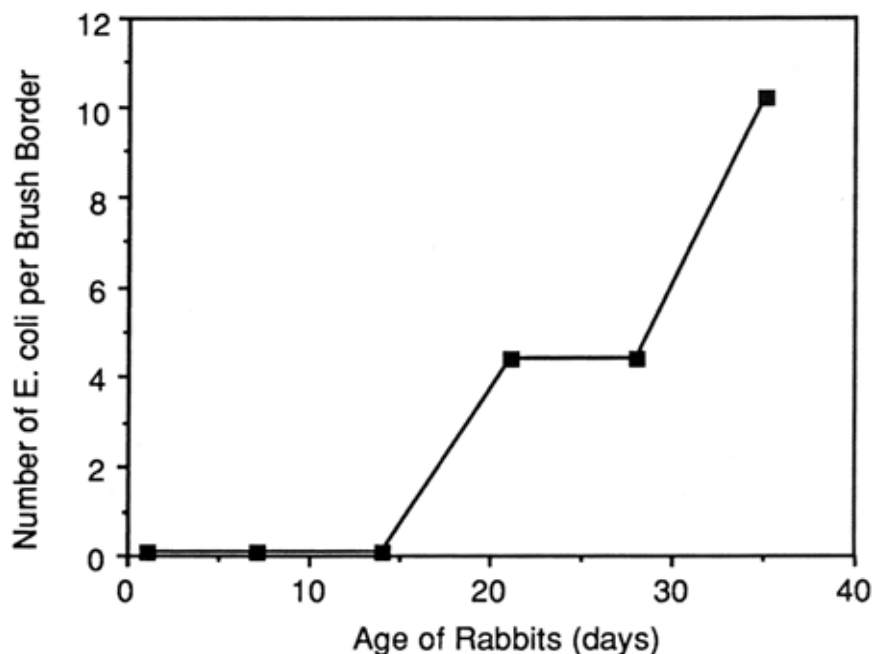

Figure 31: Variation in the adherence of *Escherichia coli* to rabbit intestinal brush borders with respect to age (Modified from: *Cheney* and *Boedeker*, 1984).

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.

Clostridium difficile colonisation of infants

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-
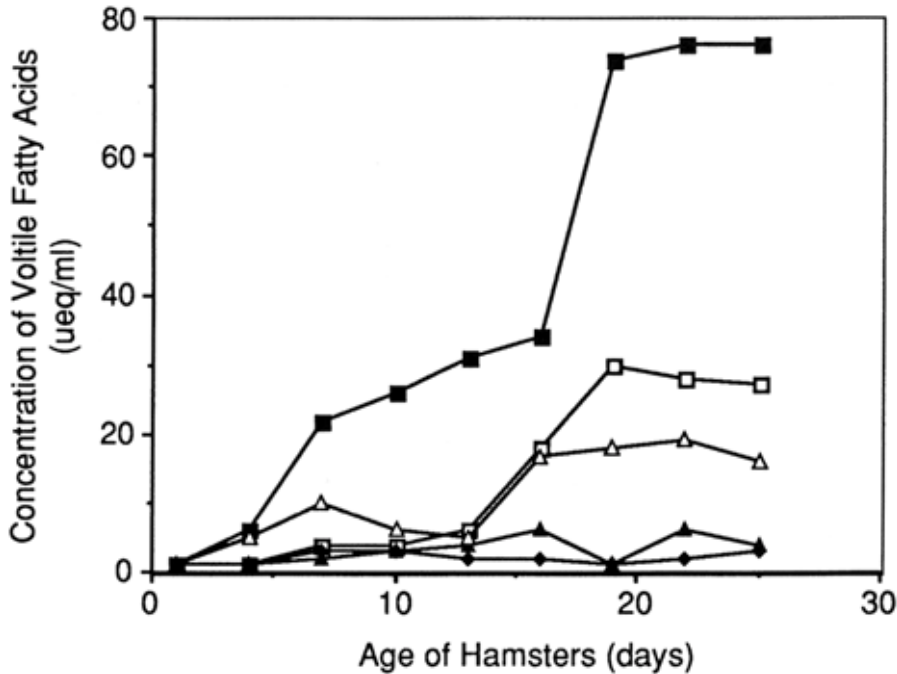

Figure 32: Concentration of volatile fatty acids in the caeca of infant hamsters. ■: Acetic acid; □: Butyric acid; △: Propionic acid; ▲: Valeric acid; ◆: Isovaleric acid (Modified from: *Rolfe*, 1984).

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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
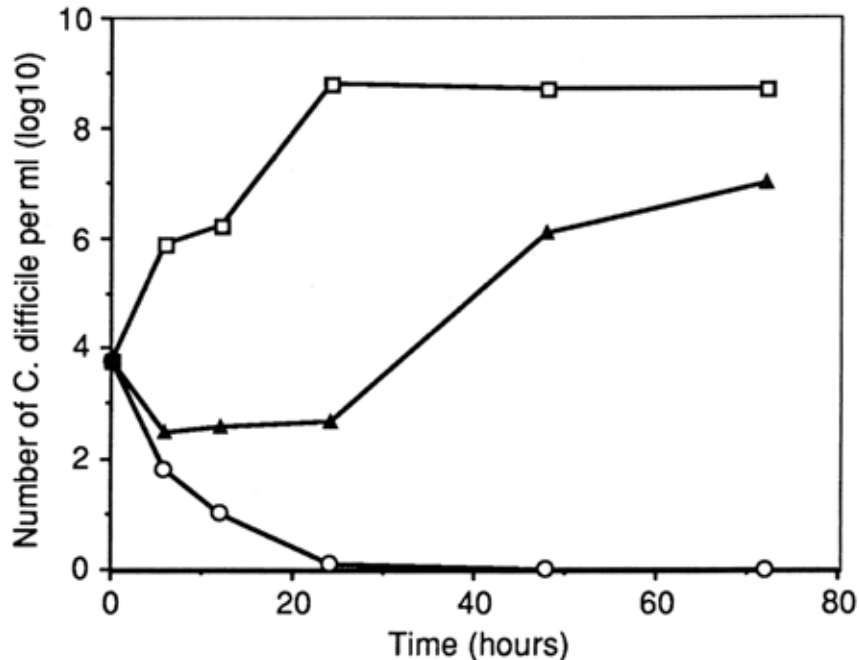

Figure 33: *In vitro* growth of *Clostridium difficile* in the presence of volatile fatty acids. □: Volatile fatty acids in hamsters I to 10 days of age; ▲: Volatile fatty acids in hamsters 10 days of age; ○: Volatile fatty acids in hamsters 16 days of age and older (Modified from: *Rolfe*, 1984).

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 con-

tents 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

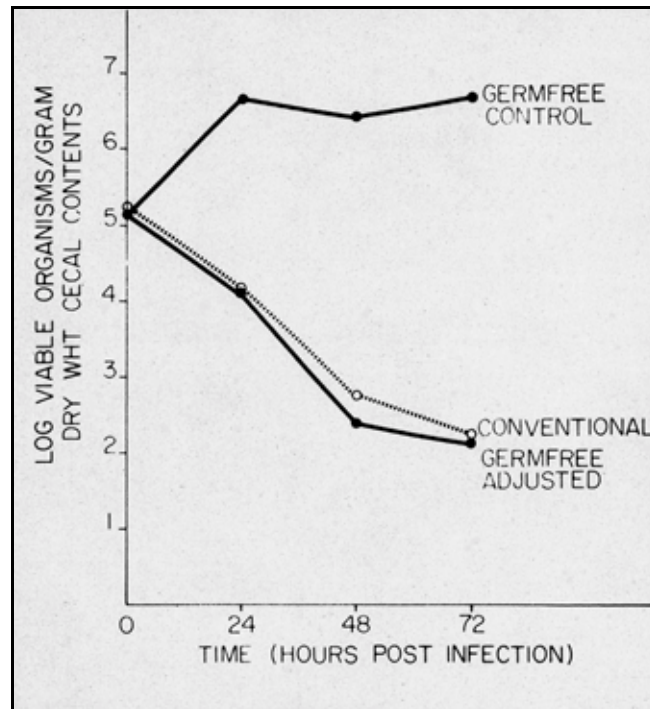|	Germfree	Conventional
Eh	- 49 mV	- 234 mV
pH	6.80	6.08
Total volatile fatty acids	0.04 N	0.15 N
Concentration of Acetic acid	0.04 N	0.10 N
Concentration of Propionic acid	0	0.005 N
Concentration of Butyric acid	0	0.045 N

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-
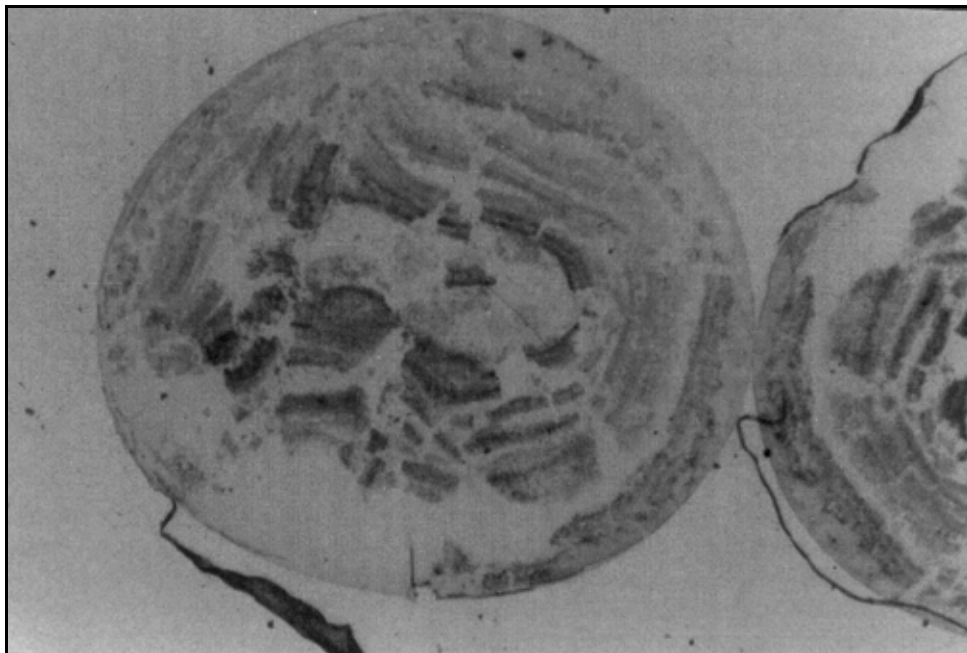

Figure 35: Light micrograph of a Haematoxilin Eosin preparation of a soft bile stone that had occluded the lumen of a biliary tube. The occluding deposit was composed of alternating concentric blue and light brown layers (x 3.8).
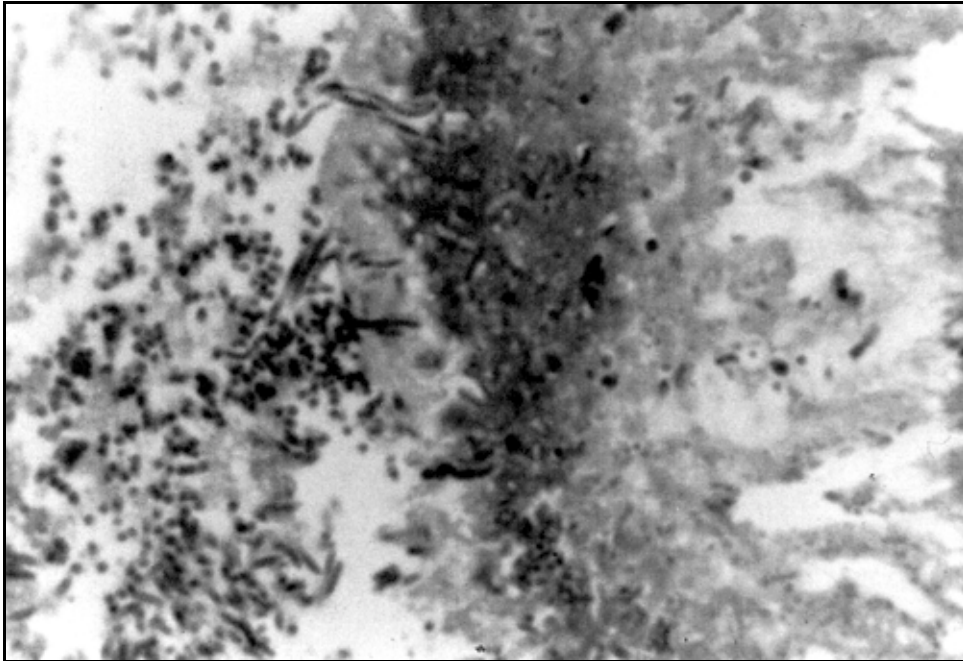
Figure 36: Higher magnification of the preparation seen in Figure 35 showing the very large number of bacterial cells that comprise the blue concentric layers of the deposit (x 580).

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.

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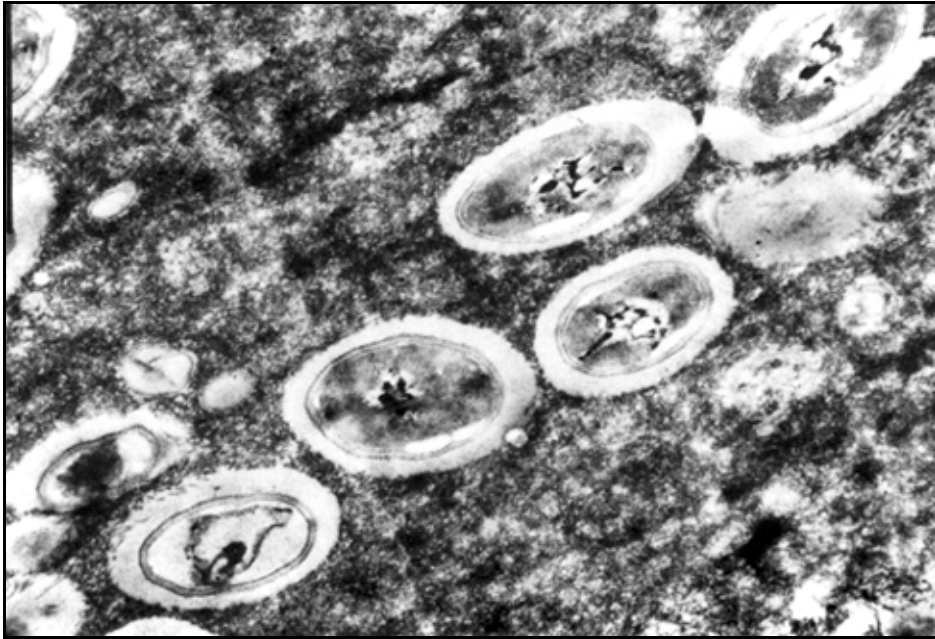
Figure 37: Transmission electron micrograph of a section of ruthenium red-stained and embedded biliary stone material showing a microcolony of bacterial cells embedded in this fibrous exopolysaccharide glycocalyx. These particular cells were rod-shaped and showed a rod-shaped cellular morphology, but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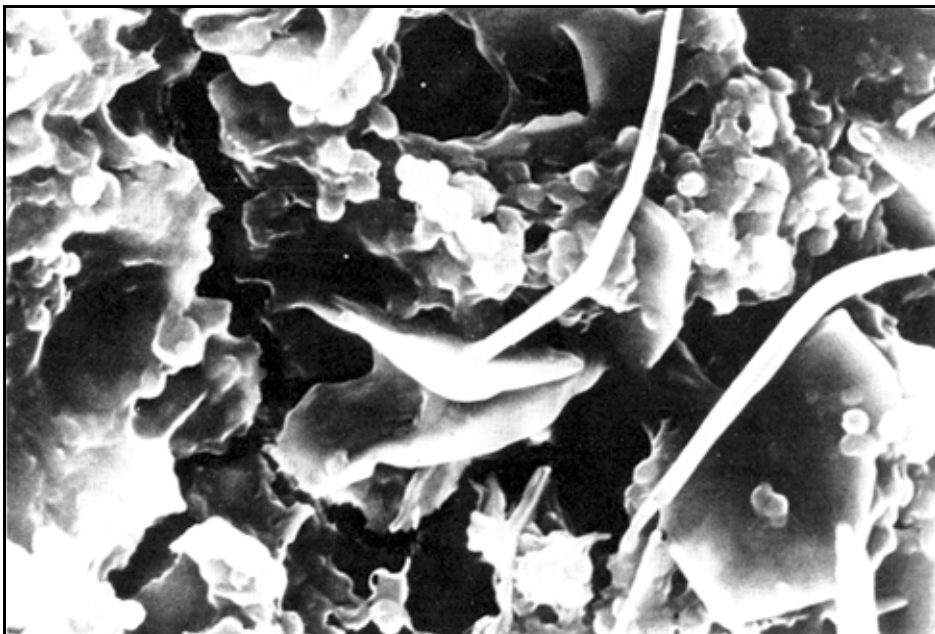

Figure 38: Scanning electron micrograph (SEM) of biliary stone material occluding a biliary tube showing coccoid bacterial cells in slime enclosed microcolonies amongst the characteristically plate-like flat crystals formed by cholesterol (x 38,500).
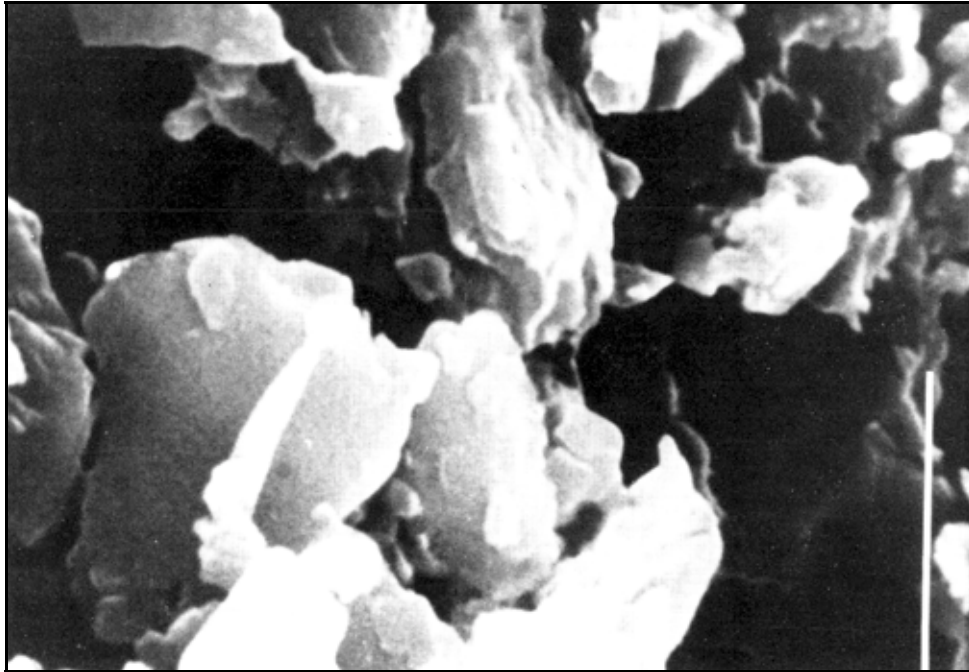
Figure 39: Less well defined irregularly shaped crystals from the same deposit showing (spot) where the beam was placed to produce the EDAX pattern seen in Figure 40 (x 38,500).
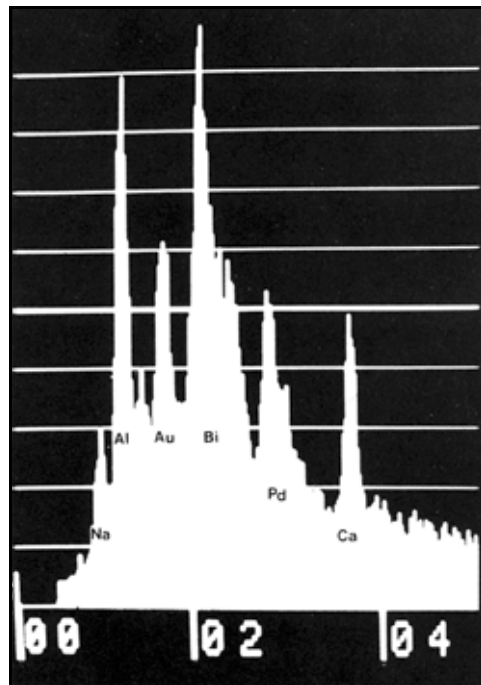

Figure 40: EDAX pattern of the crystal seen in Figure 39 showing the very high levels of calcium in these bulky crystals which are presumed to be calcium palmitate. The high levels of other atoms were contributed by the support structures (Al, Au) or by the processing (Na, Bi, Pd).

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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).
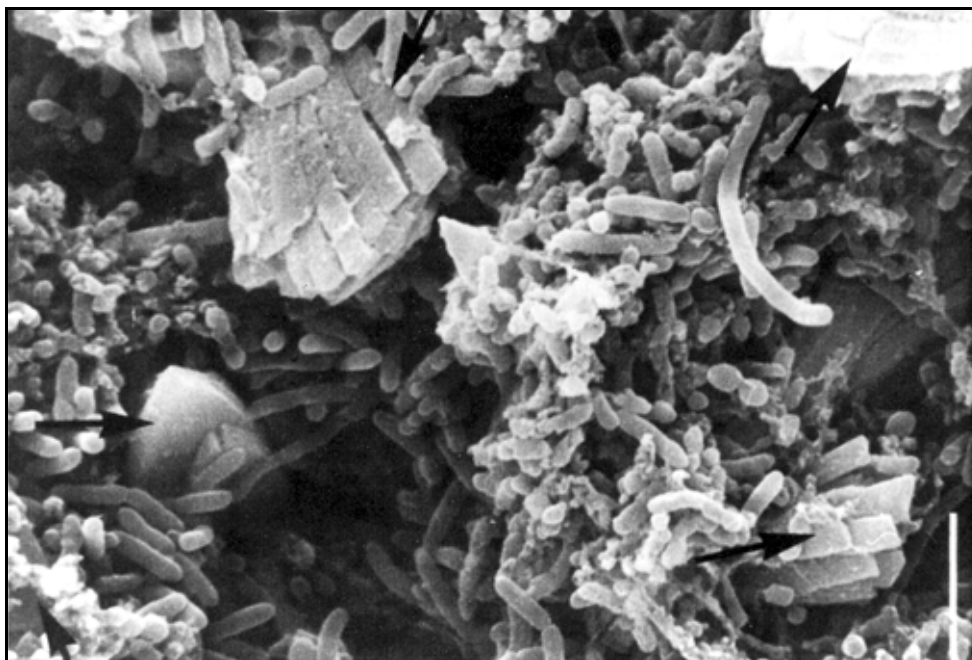

Figure 42: SEM of cells of *Proteus vulgaris* growing *in vitro*, in microcolonies, and producing crystals that were shown by EDAX to be struvite (Ammonium Magnesium Phosphate) as the initial step in urinary calculus formation (x 28,500).

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 micro-organisms 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 polysaccha-rides 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 ex-

pression (*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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