Protective Nutrients for the Immature Gut

W. Allan Walker and Dingwei Dai*^

Mucosal Immunology Laboratory, Combined Program in Pediatric Gastroenterology and Nutrition, Massachusetts General Hospital and The Children's Hospital, Boston, Massachusetts, USA; *Shanghai Institute for Pediatric Research, Shanghai Second Medical University, China

Humans live in close association with vast numbers of microorganisms that are present on the skin, in the mouth, and in the gastrointestinal tract. Although fecal bacteria were first observed microscopically some 300 years ago by Van Leeuwenhoek, the degree of microbial colonization of the lower intestinal tract was not appreciated until relatively recently. That this is considerable is evidenced by the observation of Savage that there are approximately $10^{14}$ cells associated with the human body and that 90% of these are microorganisms, the vast majority of which reside in the colon (1). One gram of large intestinal contents contains about $10^{12}$ bacteria. Biologically important functions of the large gut include absorption and secretion of certain electrolytes and water, as well as the storage and excretion of waste materials. However, it is now recognized that the gut microflora is very important in the health of the human host. Through the process of fermentation, intestinal bacteria are able to produce a wide range of compounds that have both positive and negative effects on gut physiology, as well as other systemic influences (2–4). For example, the metabolism of complex carbohydrates to short-chain fatty acids (SCFA) may result in an increased energy yield from the system, whereas proteolytic species can produce toxic compounds. There is therefore considerable interest in the manipulation of the composition of the gut flora toward the most salutary relationship—that is, an increase in numbers of health-promoting genera, such as bifidobacteria and lactobacilli. In this review, we describe the role of nutrients in the protection of the human immature gut and their effects on the modulation of human colonic flora and its activities.

THE GUT MICROFLORA AND ITS HEALTH EFFECTS

The human colon is an extremely complex ecosystem in which individual bacteria exist in a multiplicity of different microhabitats and metabolic niches. The microbiota consist of several hundred different bacterial species. Information regarding the composition of the gut microbiota has largely arisen as a result of studies on feces. Several studies have shown that Gram-negative anaerobes of the genus Bacteroides are the single most numerous group in the large gut, accounting for up to 3% of the
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FIG. 1. Generalized scheme of the human gut microbiota composition. The different bacterial groups are divided on the basis of whether they exert properties that are potentially damaging or health promoting for the host. The central vertical line gives approximate numbers of organisms in feces. (Reproduced with permission from ref. 6.)

total fecal flora. Other numerically predominant groups are Gram-positive rods (bifidobacteria, eubacteria, clostridia, lactobacilli) and Gram-positive cocci (ruminococci, peptococci, peptostreptococci). Chief among these are the bifidobacteria, which may constitute as much as 25% of total fecal counts. Several other groups exist in lower proportions, including enterococci, coliforms, methanogens, and dissimilatory sulfate-reducing bacteria (5,6) (Fig. 1).

In general, intestinal bacteria may be divided into species that exert either harmful or beneficial effects on the host (see Fig. 1). Pathogenic effects include diarrhea, inflammation, necrosis and ulceration, liver damage, carcinogenesis, and intestinal putrefaction. Health-promoting effects may be caused by the inhibition of growth of harmful bacteria, stimulation of immune functions, decrease of gas production, improved digestion and absorption of essential nutrients, and synthesis of vitamins B and K.

FEEDING AND GUT MICROFLORA

At birth, colonization of the previously germ-free human gut begins. Normally, the first microbes to be established are derived from the mother during delivery and subsequently from other external environments (e.g., neonatal intensive-care unit
There have been many reports on the establishment of intestinal microflora (7–12). Numerous factors affect the nature of intestinal microflora, especially the local environment and diet. It has been recognized that profound differences exist with respect to the composition of the gut microbiota in response to the infant’s feeding (Fig. 2). The newborn intestine is first colonized with enterobacteria, and their number reaches $10^9$ per gram of feces. Yoshioka et al. (7) reported that by day 6 bifidobacteria were the predominant organisms in the stool of breastfed infants, exceeding enterobacteria by a ratio of 1,000:1, whereas enterobacteria were the predominant organisms in formula-fed infants, exceeding bifidobacteria by approximately 10:1. At 1 month of age, bifidobacteria were the most prevalent organisms in both groups. But the number of these organisms in the stool of formula-fed infants

![Diagram](image)

**FIG. 2.** The succession of bacterial populations in the large bowel of breastfed infants (filled columns) and formula-fed infants (hatched column).

*When fewer than seven babies were examined the results have been adjusted to a fraction of seven.

†Counts of facultative anaerobic bacteria $\geq 10^9$g feces are raised in comparison with counts in normal adults.

(Reproduced with permission from ref. 10.)
was approximately one-tenth that of breastfed infants. A diet of breast milk creates an environment favoring the development of a simple flora of bifidobacteria and few other anaerobic and small numbers of facultatively anaerobic bacteria. In contrast, formula-fed infants have a more complex microbiota, which contains bifidobacteria, bacteroides, clostridia, and streptococci (8,9).

The introduction of solid food to breastfed infants causes a major disturbance in the microbial ecology of the large bowel as numbers of enterobacteria and enterococci rise sharply and colonization by *Bacteroides* spp., clostridia, and anaerobic streptococci occurs. This was not observed when formula-fed infants began to take solids; instead, counts of facultative anaerobes remained high while colonization by anaerobes other than bifidobacteria continued. At 12 months, the anaerobic bacterial populations of the large bowel of both groups of infants are beginning to resemble those of adults in number and composition, and there is a corresponding decrease in the number of facultative anaerobes (10).

The studies discussed earlier were on normal, healthy newborns. Bell *et al.* (11) found that the incidence of pure cultures of aerobic bacteria was higher in the stool of the premature neonate and critically ill infants than in normal full-term infants.

The reasons for the differences in the fecal flora of breastfed and formula-fed infants are not yet fully known but appear to be related to the following:

1. **The type of protein**—whey-based formula produces a flora more like that of the breastfed baby (9);
2. **The availability of iron**, which is determined by the amount of iron in infant formula and the presence of lactoferrin in human milk. Bifidobacteria and lactobacilli do not need iron, whereas *Bacteroides* species and enterobacteria require iron for growth. Lactoferrin supplementation may suppress the growth of facultative organisms in the infant gut (12);
3. **The presence of oligosaccharides**—some N-acetylglucosamine-containing oligosaccharides in breast milk are shown to be “growth factors” for bifidobacteria (13);
4. **The pH**—human milk is thought to be necessary to maintain an acidic pH in the gut owing to its poor buffering capacity compared with cow’s milk and formula milks, with their high calcium and phosphate content. Bullen and Willis (14) noted that the pH of stool in breastfed infants was 5.1 at age 7 days, whereas it was as high as 6.5 in formula-fed infants. The low pH level promotes the growth of bifidobacteria and lactobacilli but inhibits other bacteria. In addition, secretory IgA (SIgA) may act to suppress the growth of coliforms in the infant gut.

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Because of immaturities of intestinal host defenses, the preterm neonate is more susceptible to pathological colonization leading to intestinal infection. The incomplete development of host defense at the mucosal level could explain this increased susceptibility (15–17). Nutrition may play an important role in the development of the mucosal barrier. Bacteria colonize the gut by adhering to a receptor of glycoprotein
or glycolipid in the microvillus membrane. Pathological bacteria are defined in part by their expression of adhesins on their surface and the capacity of these adhesins to bind to the microvillus glycoconjugates in a lectinlike fashion (18). Thus inhibiting bacterial colonization may reduce the risk for bacterial infection.

Feeding preterm infants may include supplements of age-appropriate maternal milk (milk secreted after birth in mothers who deliver prematurely). Human preterm milk may provide near optimal nutrition for the preterm infant, and its role in meeting preterm infants’ host defense needs may be equally important. Human preterm milk appears to be qualitatively different from term milk and may, as such, be uniquely suited to the host defense needs of the preterm infant (16). There are various growth factors, hormones, and nutrients in human milk (16,19) that are thought to promote gastrointestinal maturation and strengthen mucosal barriers (Table 1).

Apart from these trophic factors, there are many immunological components and nonimmunological factors in human milk, which are thought to be anti-infective agents for the newborn (13,16,20). Compared with milk from other species, human milk is unique with regard to its content of complex oligosaccharides (13,20). For many years, these components have been thought to play a role only in the development of a normal gut flora in breastfed infants. Now there is striking evidence that free oligosaccharides and glycoconjugates are potent inhibitors of bacterial adhesion to the microvillus membrane, an initial stage of the infective process (13,20). In vitro assays have shown the ability of these molecules to competitively inhibit microbial adhesion and enterotoxin binding by acting as receptor analogs (20). Gangliosides have been shown to act as receptor analogs for the heat-labile toxins from Escherichia coli and Vibrio cholerae (21). Other oligosaccharides in human milk have also been shown to inhibit in vitro attachment of the classic and El Tor strain of V. cholerae (22), Streptococcus pneumoniae, and Haemophilus influenzae (23), and inhibit localized adherence of enteropathogenic E. coli to HEp-2 cells (24). Glycoproteins and glycolipids also interfere with the binding of enterotoxigenic E. coli to epithelial cells (25). Many protective oligosaccharide and glycoconjugate components in human milk are now known. We list the prominent ones in Table 2.

It is known that glycosylation in the microvillus membrane is under developmen-

| TABLE 1. Nutrients and hormones that may promote gastrointestinal maturation |
|-----------------------------|-----------------------------|-----------------------------|
| Nutrients                 | Hormone and growth factors | Others                      |
| Iron                      | Growth hormone             | Nucleotides                 |
| Zinc                      | Glucocorticoids            | Polyamines                  |
| Vitamin A                 | Insulin                    |                             |
| Vitamin B₁₂               | Thyroxine                  |                             |
| Folic acid                | Bombesin                   |                             |
| Glutamine                 | Somatomedin                |                             |
| Arginine                  | Intestinal peptide YY      |                             |
| Taurine                   | Epidermal growth factors   |                             |
| Lactose                   | Nerve growth factors       |                             |
| Amino sugars              | Insulin-like growth factors|                             |
|                           | Transforming growth factors|                             |
TABLE 2. Oligosaccharides and glycoconjugates in human milk that inhibit enteropathogen adhesion or its toxin binding

<table>
<thead>
<tr>
<th>Receptors</th>
<th>Pathogens</th>
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<tr>
<td>Fucosylated pentasaccharide</td>
<td>Enteropathogenic <em>E. coli</em></td>
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<tr>
<td>Fucosylated oligosaccharide</td>
<td><em>E. coli</em> (the heat stable enterotoxin)</td>
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<tr>
<td>Gal(1-4)GlcNAc(1-3)Glc</td>
<td><em>Campylobacter jejuni</em></td>
</tr>
<tr>
<td>Ganglioside GM₁</td>
<td><em>Streptococcus pneumoniae</em></td>
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<tr>
<td>Glycolipid Gb₃</td>
<td><em>Vibrio cholerae</em> (toxin)</td>
</tr>
<tr>
<td>Mannosylated glycopeptide</td>
<td><em>E. coli</em> (the heat labile enterotoxin)</td>
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<tr>
<td></td>
<td><em>C. jejuni</em> (toxin)</td>
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<td></td>
<td><em>Shigella</em> (toxin)</td>
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<tr>
<td></td>
<td>Enteroheamorrhagic <em>E. coli</em> (verotoxin)</td>
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<td></td>
<td>Enteroheamorrhagic <em>E. coli</em></td>
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tal regulation (15,26). In the adult animal, mature glycosylation results in mature carbohydrate side chains on microvillus membrane glycoproteins and glycolipids. In contrast, the newborn intestine has immature glycosylation, which results in differences in the availability of glycoconjugates and may account for enhanced pathogen toxin binding (15). We have previously reported in an animal model that sialyltransferase activity is increased in newborns and that fucosyltransferase and galactosyltransferase activities are decreased (27,28). We have also shown that these and other glycosyltransferases may be developmentally regulated by pretreatment with cortisone (27,28). Previous studies have suggested that the human neonatal intestine may show pathological colonization of bacteria because of immaturity in glycosylation of the mucosal surface molecules (glycoconjugates) (29), and preliminary studies have suggested that cortisone can modify these immature glycoconjugates to cause a more mature pattern of bacterial colonization (15,26,29). Many factors other than cortisone affect the developmental regulation of glycosylation in the intestine, including nutritional factors such as vitamin A deficiency (30) and nutrient composition differences in the diet (31). However, further studies are needed to clarify the nutritional regulatory mechanism of glycosylation and to define the nutrient requirements for this process.

PROBIOTICS, PREBIOTICS, AND THE GUT MICROFLORA

It is becoming increasingly accepted that the intestinal microbiota may play an important role in the maintenance of host health. Keeping the equilibrium of microflora seems to be a contributory factor. *Bifidobacterium* is the numerically predominant bacterial genus in the feces of breastfed infants, and this may contribute to the protection which breastfeeding provides against intestinal infections. Because of their potentially beneficial properties (Table 3), there have been attempts to increase the numbers of these bacteria in the human intestine. Diet can influence microbial colonization in two ways: (i) administration of live microorganisms by mouth (probiotics); or (ii) the oral intake of bacterial stimulants that are directed toward specific
components of the endogenous flora (prebiotics). The two approaches are more formally defined as follows:

- **Probiotics** are live microbial food supplements that beneficially affect the host animal by improving its intestinal microbial balance (32).
- **Prebiotics** are nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth or activity of one or a limited number of bacteria in the colon that can improve host health (5).

Both probiotic and prebiotic supplements are targeted toward beneficial microorganisms and therefore tend to operate through that part of the gut flora containing lactic acid bacteria and bifidobacteria. Lactobacilli (e.g., *Lactobacillus acidophilus*, *L. casei*, *L. delbruekii*) and bifidobacteria (e.g., *Bifidobacterium adolescentis*, *B. bifidum*, *B. longum*, *B. infantis*) are commonly used as probiotics. Feeding yogurt containing putative probiotic organisms may introduce more bifidobacteria or lactobacteria into the intestine. Bifidobacteria given in this way can pass through the terminal ileum and may be detected in the feces at $10^9$ organisms per gram (33). However, they rapidly disappear from the feces when oral supplementation is discontinued. Similar results have been obtained with *L. casei* strain GG (34). These studies indicate that long-term colonization with probiotics did not occur. These invading species need to compete for nutrients and colonization sites with a previously established microflora comprising several hundred other bacterial strains and species that already occupy the available physical, physiological, and metabolic niches.

Those prebiotics that have hitherto been described are specifically used to stimulate the growth and activities of bifidobacteria. Among the natural nondigestible oligosaccharides that fulfill these criteria as colonic food, fructo-oligosaccharides are the only products presently recognized and used as food ingredients that meet all the criteria allowing classification as prebiotics (5). In a recent *in vitro* study using both pure strains of colonic bacteria and mixed human fecal cultures, Wang and Gibson (35) showed that in comparison with other simple or complex carbohydrates, fructo-oligosaccharides selectively stimulate bifidobacterial growth, while restricting the growth of potential pathogens such as *E. coli* and clostridia to some extent. This effect was subsequently confirmed *in vivo*, when human volunteers were given a strictly controlled diet supplemented with oligofructose or insulin at a level of 15 g/d (36). Moreover, in defined co-culture experiments, various species of bifidobacteria inhibited the growth of *E. coli* and *Clostridium perfringens* owing to secretion of an anti-

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**TABLE 3. The beneficial effects of bifidobacteria on human health**

<table>
<thead>
<tr>
<th>Effect, related to bifidobacteria</th>
<th>Benefits</th>
</tr>
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<tbody>
<tr>
<td>Inhibits the growth of potential pathogens</td>
<td><em>Inhibits the growth of</em></td>
</tr>
<tr>
<td>Lowers blood ammonia levels</td>
<td>Promotes immunological attack against malignant cells</td>
</tr>
<tr>
<td>Produces vitamins B and folic acid</td>
<td>Improves host resistance to pathogens</td>
</tr>
<tr>
<td>Promotes immunological attack against malignant cells</td>
<td>Reduces blood cholesterol levels</td>
</tr>
<tr>
<td>Improves host resistance to pathogens</td>
<td>Restores the normal microflora during antibiotic therapy</td>
</tr>
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</table>
microbial substance that was independent of changes in the culture pH. Plating experiments also showed that this antimicrobial substance variably suppressed several other groups of pathogenic organisms, including species belonging to the genera Salmonella, Listeria, Campylobacter, and Shigella, as well as V. cholerae (37).

BACTERIAL FERMENTATION IN THE HUMAN COLON

Many different substrates reach the large intestine and are utilized by bacteria. The principal nutrient substrates for bacterial growth are resistant starches, plant cell wall polysaccharides, and host mucopolysaccharides, together with various proteins and peptides (2,4). Most of the simple sugars and oligosaccharides ingested and digested by humans are absorbed in the small intestine. However, some low-molecular-weight carbohydrates such as lactose, raffinose, and stachyose, together with fructo-oligosaccharides (such as oligofructose or inulin), sugar alcohols, sorbitol, and xylitol, pass into the colon and are rapidly fermented (38).

The most numerous, as well as the most versatile, polysaccharide utilizers in the colon belong to the genus Bacteroides. Other bacteria able to grow on carbohydrates are saccharolytic species belonging to the genera Bifidobacterium, Ruminococcus, Eubacterium, Lactobacillus, and Clostridium. Because of the extremely complex nature of the gut ecosystem, many groups of bacteria are unable to degrade polymerized carbohydrates directly. These species grow by crossfeeding on fragments produced by primary polysaccharide degraders. Saccharolytic bacteria are highly adapted for growth on complex carbohydrates by means of their ability to produce a variety of polyhydrolases and glycosidases. Although some bacteria in the colon can synthesize many different types of saccharolytic enzymes, carbohydrate metabolism is likely to be dependent on the cooperation of different enzymes and various bacterial species taking part in the process.

The principal end products of carbohydrate fermentation are short-chain fatty acids (SCFA), which include acetate, propionate, and butyrate in the molar ratio of 60:20:18, respectively (2,4). Several gases (hydrogen, carbon dioxide, and methane) and bacterial cell mass (bacterial mass) are also produced in this process (2,39). Protein fermentation also leads to SCFA, H2, CO2, and bacterial mass and to the generation of branched-chain fatty acids (BCFA) such as isobutyrate, isovalerate, and α-methylbutyrate, ammonia, amines, and phenols, together with various other organic acids. In some circumstances other fermentation intermediates accumulate, such as ethanol, lactate, succinate, and pyruvate, and these may, in addition, be further fermented to SCFA. Carbohydrate metabolism is quantitatively more important than amino acid fermentation in the colon, particularly in the proximal bowel, where substrate availability is greatest.

Every bacterial species has its own characteristic profile of SCFA products, and these are often used in species identification. Bacteroides produce mainly acetic, propionic, and succinic acids. Bifidobacteria and eubacteria produce mainly acetic and lactic acids. It is not known which bacteria are responsible for each SCFA in the final colonic fermentation mixture, and in particular it is not known which species contribute to butyrate production. Rasmussen et al. (40) have described differences in fe-
cal SCFA of neonates and adults that were expressed in a high relative contribution of acetic acid in the fecal SCFA of infants. A higher contribution of acetic acid to the SCFA spectra has also been observed in breastfed as compared with bottle-fed infants (40–42).

The intestinal microflora functions through fermentation. Many physicochemical factors can influence the pattern and extent of fermentation of particular substrates. These include nutrient substrate availability, the physicochemical environment of the colon, various host conditions, metabolic interaction among bacteria, and individual dietary preferences (2,3).

THE PHYSIOLOGICAL ROLE OF SCFA

The primary function of human colon microbiota is to salvage energy from carbohydrates not digested and absorbed in the upper gut. This is achieved through fermentation and absorption of its major products, SCFA, which represent around 40% to 50% of the available energy of the carbohydrates (4). The principal SCFA—acetate, propionate, and butyrate—are metabolized by the colonic epithelium (butyrate), liver (propionate), and muscle (acetate). In recent years the physiological significance of SCFA has been receiving considerable attention. These observations are summarized later.

Energy Supply

SCFA contribute to total energy requirements. Theoretically, it is estimated that around 300 to 800 mmol SCFA are formed by intestinal bacteria in the human colon each day (43). This amount of SCFA would provide 400 to 1,000 kJ (90 to 240 kcal), representing 5% to 10% of the host’s total daily energy requirements. The SCFA load could be much greater in those individuals consuming very-high-fiber diets or in those with malabsorption. Large quantities of malabsorbed carbohydrate may be fermented to SCFA, and therefore in patients with malabsorption the colon could have a significant role in meeting total energy needs.

Effect on Colonic Epithelial Cell Transport

SCFA are absorbed rapidly, primarily by passive diffusion of the nonionized acid. SCFA absorption promotes the absorption of sodium, potassium, and water, and an increase in luminal bicarbonate concentration. This process appears to occur by the following mechanism, described by Binder et al. (44). After absorption, the SCFA are ionized at the intracellular neutral pH to hydrogen (H⁺) and fatty acids (FA⁻). The H⁺ is exchanged for sodium (Na⁺) by an antiport transport mechanism, and the FA⁻ (particularly butyrate) is exchanged for chloride (Cl⁻) by a similar mechanism. In addition, FA⁻ are metabolized to bicarbonate (HCO₃⁻) in the cell, which is also exchanged for Cl⁻. Thus the net effect is that SCFA stimulate Na⁺ and Cl⁻ absorption and bicarbonate secretion. SCFA do not therefore appear to contribute to the
osmotic load and may actually constitute an important protection against diarrhea through the removal of sodium and water from the colon.

**Substrate Supply for Enterocytes**

The human colonic epithelium derives 60% to 70% of its energy from bacterial fermentation (45). SCFA are partly metabolized to CO₂ and ketone bodies that act as precursors for lipid biosynthesis in the mucosa. Roediger (45) found that more than 70% of oxygen consumption by colonocytes grown in vitro was caused by butyrate metabolism. SCFA also stimulate mucosal proliferation both in the large bowel and, when instilled into the isolated cecum, in the small bowel. The mechanisms of their trophic effects on the jejunal epithelium are currently being actively investigated (46).

**Modulation of Nucleic Acid**

The role of butyrate in modulation of nucleic acid is of particular interest, especially its effects on the regulation of gene expression and cell growth. Butyrate can reversibly alter the in vitro properties of human colorectal cancer cell lines by prolonging doubling time and slowing growth rates. Low concentrations of SCFA reduce DNA synthesis and suppress proliferation in a variety of cell types (2). Smith's work has indicated that butyrate inhibits the enzyme histone deacetylase in the cell nucleus, thereby allowing the hyperacetylation of histone proteins (47). This has the effect of opening up the DNA structure, thus facilitating better access by DNA repair enzymes. Recently, Ohno et al. (48) showed that butyrate increased the secretion of macrophage inflammatory protein-2 (MIP-2) in stimulated rat small intestinal epithelial cells (IEC-6) by increasing histone acetylation. Shah et al. (49) found that n-butyrate reduced the expression of β-galactoside α2,6-sialyltransferase mRNA in Hep G2 cells by a post-transcriptional mechanism. In addition, butyrate is well established as a growth inhibitor and inducer of differentiation in many cell lines.

**Antibacterial Effect**

Production of SCFA and the anaerobic flora that fermentation supports prevents the establishment of pathogenic bacteria such as *Salmonella* species. Recently, Jacewicz and his colleagues (50) reported that butyrate enhanced the expression of Gb3, the Shiga toxin receptor, and its synthetic enzyme (UDP-galactose: lactosyl ceramide galactosyltransferase) in human cultural intestinal cells (CaCo-2A cells). Moreover, butyrate suppresses the expression of α2,6-sialyltransferase but promotes the expression of β1,4-galactosyltransferase and N-acetylglucosyltransferase in cultured T84 colonic cells (51). Since some pathological bacteria and toxins use glycoconjugates on the microvillus membrane of the intestine as acceptors before invasion and cell destruction, it may be hypothesized that SCFA protect the intestine by regulating glycosylation.
Effects on Carbohydrate and Lipid Metabolism

Animal experiments have shown that acetate infusions reduce plasma glucose concentrations. However, acetate given either orally or intravenously has little effect on glucose metabolism and does not stimulate insulin release in man. Propionate-supplemented diets have been shown to lower blood cholesterol in rats and pigs (52,53), but in man the effects are less apparent. More research is needed to determine the exact effects of SCFA on glucose and cholesterol homeostasis in humans.

In the light of the growing interest in the physiological actions of SCFA in the human host, it may become worthwhile to manipulate SCFA in the colon for specific clinical situations such as the treatment and prevention of intestinal disease. To achieve the ideal SCFA profile for each situation, it is necessary to do further studies on the bacteria responsible for producing individual SCFA and their preferred nutrient substrates.

GUT MICROFLORA AND NECROTIZING ENTEROCOLITIS

Necrotizing enterocolitis (NEC) is one of the leading causes of morbidity and mortality in newborn infants, with some reports estimating an incidence of more than 10% among very low-birthweight infants weighing less than 1,500 g and with an associated mortality as high as 35% among affected infants (54,55). Although the etiology of NEC is still unclear, impaired intestinal barrier function and the resultant translocation of bacteria and their products seem to be the most consistent risk factors (56,57).

The human host has developed multiple defense mechanisms that harmonize to prevent intestinal bacteria and endotoxins from reaching systemic organs and tissues (17). These defenses include mechanical barriers, the stabilizing influence of a normal intestinal microflora, and the immune system. Many, if not all, of the defenses that prevent bacterial translocation are impaired in patients at risk of developing NEC. For example, the combination of an immature gastrointestinal mucosal barrier and an underdeveloped gastrointestinal immune system would certainly predispose the premature or low-birthweight infant to bacterial translocation.

Bacterial overgrowth in the intestine is one of the major factors that promotes bacterial translocation. In an animal model, even with a normal intestinal barrier function, bacterial translocation will occur if certain enteric bacteria reach or exceed intestinal population levels of $10^9-10^{10}$ bacteria per gram of cecum content or stool (57). Based on studies in germ-free mice colonized with single strains of bacteria, it appears that not all bacteria are able to translocate equally well (58). Although indigenous Gram-negative enteric bacilli translocate in large numbers to mesenteric lymph nodes, Gram-positive bacteria translocate at intermediate levels and obligate anaerobes at only very low levels. These results suggest that enteric bacilli such as *E. coli*, *proteus*, *pseudomonas*, and *enterobacter* are associated with a higher incidence of bacteremia in high-risk patients because these bacteria translocate more efficiently from the gastrointestinal tract than other bacteria, especially obligate anaerobes (57). Bell *et al.* (11) found that the incidence of pure cultures of aerobic bacteria was
higher in the stools of infants nursed in neonatal intensive-care units than in normal full-term infants. Therefore it appears that NICU infants are at increased risk for bacterial translocation owing to the high levels of Gram-negative aerobic bacteria and low levels of anaerobic bacteria in their gut microflora. The term colonization resistance is used to describe the phenomenon whereby certain members of the normal gut microflora (e.g., strict anaerobes, lactobacilli) protect the host against intestinal colonization and subsequent infection with potential bacterial pathogens.

Many diverse bacteria can elicit an intestinal infection in the newborn period (Table 4). The presence of a mechanism that suppresses the growth of these organisms in the intestine is obviously favorable in preventing disease in young infants. It has been repeatedly documented that the occurrence of disease caused by enteropathogenic E. coli, salmonellae, or shigellae is significantly more likely in formula-fed than in breastfed infants (59,60).

The incidence of NEC was noted to vary in relation to variation in the intestinal microflora cultured from infants in neonatal intensive-care units. Increased colonization with E. coli and K. pneumoniae was associated with an increased incidence of NEC, which suggests that these organisms are related to the pathogenesis of NEC (61–63).

Panigrahi et al. (64) showed that NEC-associated bacteria (E. coli) have a greater propensity than non-NEC-associated bacteria of the same species to prevent adherence of Gram-positive bacteria to the enterocytes and to cause pathological changes typical of NEC in an animal model, and the injury could be prevented by co-infection with Gram-positive isolates from the homologous infant. The same investigators' recent results have shown that the same E. coli isolates can cross Caco-2 cell monolayers in the absence of ultrastructural change or damage. The transcytosis of E. coli was reduced three- to fivefold in the presence of Enterococcus faecium, previously shown to prevent NEC-like injury in the animal model. There was a mild increase in the rate of E. coli transcytosis when studies were conducted with younger, undifferentiated cells; these immature cells had no brush border, but retained well-defined tight junctions. A further reduction or complete blockage of E. coli transcytosis was observed when E. faecium was used as the co-infection in studies with these undifferentiated cells (65). These data suggest that bacterial translocation and the microflora in the neonatal gut play a pivotal role in the development of NEC.

Despite the lack of direct evidence for bacterial overgrowth resulting in NEC, it

<table>
<thead>
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<th>TABLE 4. Bacterial enteropathogens in newborn period</th>
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<tbody>
<tr>
<td>Escherichia coli</td>
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<tr>
<td>Staphylococcus epidermidis</td>
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<tr>
<td>Clostridium difficile</td>
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<tr>
<td>Klebsiella pneumoniae</td>
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<tr>
<td>Salmonella</td>
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<tr>
<td>Shigella</td>
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<tr>
<td>Campylobacter</td>
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<td>Yersinia enterocolitica</td>
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was noted that oral antibiotics (66), formula acidification (67), and IgG/IgA administration (68) could decrease bacterial translocation and reduce the incidence of NEC.

Several factors that are involved in NEC are beyond the control of the neonatologist and pediatrician, the most important of which would be the prevention of premature birth. Nevertheless, there is clinical and laboratory evidence to suggest that certain therapeutic approaches directed at improving intestinal mucosal immunity, hastening mucosal maturation, and promoting the development of a normal gut flora will decrease the incidence of NEC.

SUMMARY AND CONCLUSIONS

The normal human microflora is a complex ecosystem in part dependent on enteric nutrients to establish colonization. The gut microbiota is important to the host in respect of many metabolic functions and in resistance to bacterial infection. Normal intestinal microflora are relatively constant during a lifetime, but certain factors may affect this equilibrium. Diet and environmental conditions can influence this ecosystem. A breastfed infant has a preferred intestine microbiota in which bifidobacteria predominate over potentially harmful bacteria. Oligosaccharides and glycoconjugates, natural components in human milk, may prevent intestinal attachment of enteropathogens by acting as receptor homologs. Probiotics and prebiotics modulate the composition of human intestine microbiota to the benefit of the host. Bifidobacteria and lactobacilli are commonly used as probiotics. Non-digestible oligosaccharides in general, and fructo-oligosaccharides in particular, are used as prebiotics. The beneficial effects may result in the suppression of harmful microorganisms or the stimulation of bifidobacterial growth. In the future, control and manipulation of the intestinal microflora may be an approach to both therapeutic and preventive medicine.

REFERENCES


DISCUSSION

Prof. Haschke: What do you think about the safety of probiotics in premature infants? Adding probiotics means adding living bacteria to a formula. The preterm gut might react differently from the term gut. If there was inflammation, bacteria might cross the gut and enter the bloodstream. Are there any animal models that could simulate this?

Dr. Walker: That is a very pertinent question. We don't want to run before we can walk in these studies. We're trying to do basic science experiments in our laboratory—to simulate as far as we can both in vivo and in vitro conditions in which bacteria interact with the intestine. If these studies prove to be as helpful as I think they might, then we have to cautiously do some clinical trials. What happens in a transplanted human intestine is not necessarily what is happening in the infant itself. Eventually, we are going to have to try some probiotics on the living infant, even if they do cross the epithelium. This is a multistep process, and I don't mean to infer from my comments that we should start feeding probiotics to infants at risk of necrotizing enterocolitis immediately. In the long run, though, this approach may turn out to be a much better approach than using antibiotics, because antibiotics eventually result in overgrowth of resistant organisms and may cause more harm than good.

Prof. Haschke: But what will your approach be before going into clinical trials?

Dr. Walker: First we need to do more studies in our model systems. Then we need to be able to show that probiotics are beneficial and that they can displace pathogenic organisms. And then I would use probiotic bacteria already available in yogurt and other products, to see if a beneficial effect can be shown.

Dr. Guesry: You presented very nice in vitro data on lactoferrin and nucleotide, but nobody has ever been able to show any significant reduction in morbidity with either lactoferrin or nucleotides. How do you explain that?

Dr. Walker: It's possible that in infants lactoferrin is not metabolized in vivo in the intestine to produce the conjugates that interfere with bacterial colonization. It's also possible that lactoferrin as an intact molecule does not get across the epithelium to interact with its receptor on TH1 helper cells. You're absolutely right. We need to pursue this in a manner that is beneficial to the patient. We may have to use different dosages or different modifications of intraluminal events.

Dr. Schanler: You mentioned the effect of corticosteroids on bacterial colonization. I know that the data on steroids in NEC are equivocal, but were you talking about an antenatal steroid effect, or about the pharmacological effect of steroids? And how does that affect bacterial colonization?

Dr. Walker: What I alluded to is that using our pretreatment model system—that is, treating before the animal delivers or immediately after—we can modify the nature of bacterial colonization and translocation and alter glycosyltransferases that we believe affect that colonization process. It's a big step from those observations to what has actually been done. I quoted the Bauer study, which suggested that the use of prenatal steroids in mothers at risk for delivering prematurely reduced the incidence of NEC [1], but there are also studies contradicting that finding [2]. I was just pointing out that there is an experimental basis for accepting the initial observations that prenatal steroids might help prevent NEC.
Dr. Schanler: Another comment. We talk about single nutrient additives—single proteins like lactoferrin by itself or one oligosaccharide—but don’t you think that the most protective effect would be the whole mixture? I don’t understand why we should expect one protein to be the magic protein. That’s one of the reasons why human milk is unique—it is so complex!

Dr. Walker: You’re absolutely right. I don’t believe that NEC is caused by a single mechanism or a single organism, so we need multiple protective mechanisms. Lactoferrin has not been shown to be effective as a single molecule, but collectively, oligosaccharides, lactoferrin, and nucleotides added to premature formulas may be effective in helping to protect the intestine from infection and inflammation.

Dr. Berseth: Can you suggest how your research at the cellular level will be translated into clinical reality?

Dr. Walker: We’re trying to answer questions at the cellular level, and this does not necessarily translate to the living infant. The points that have been made about clinical trials are very important. We need to continue our studies in human fetal model systems to the point where we think we should take the next step, which would be clinical trials. Your own studies, where epidermal growth factor and its influence on the intestine, illustrate yet another component of this complex process. For example, growth factors affect the intestinal cell differently at different stages of development, so how do we choose the right time for their introduction? We need to have more information about these very complex problems, and it is necessary for someone to coordinate the basic science observations. We’ve made a step closer to the human by going away from cancer cells and animal models to human in vitro models. Now we need to work with our clinical investigator colleagues to come up with the best recommendations for clinical studies. Invariably, what we see in vitro or even “simulated” in vivo is not necessarily what is happening in the human infant in vivo.

Dr. Baibarina: Which is more important, secretory IgA or specific immunization?

Dr. Walker: They are not mutually exclusive. I did not talk about IgA because its protective properties are thought to be immunologic rather than nutritional. IgA is a unique immunoglobulin for the mucosal surface and is produced in response to antigens or microorganisms that cross the epidermal surface. The IgA content of breast milk under prolactin stimulation is a direct and specific response to organisms that might cause harm to the infant. In general, immunoglobulin specifically responds to an epitope on an antigen or a microorganism, and it prevents binding of the antigen or the microorganism to the epithelial surface. Thus it’s a specific process. Other substances such as oligosaccharides may have a more generalized effect on bacterial colonization.

Prof. Wu: One of your slides stated that NEC only occurred in premature infants. In our hospital, many cases occur in term infants. So the statement is not true, in China at least.

Dr. Walker: I meant to imply that being premature is a major risk factor. It is my understanding that most reported cases of NEC, about 90%, occur in premature infants. I’m aware that full-term infants can develop it, and that may or may not be a different process. I was just attempting to give some clinical relevance to what I talked about experimentally.

Dr. Putet: What changes in gut colonization occur when pasteurized human milk is used, or when milk is supplemented with protein or lactose? And does hydrolyzed protein affect gut colonization?

Dr. Walker: When you pasteurize and freeze milk, you affect many of its protective properties. You end up with maybe 75% of the immunoglobulin, all the cells killed, and many cytokines modified. In other words, if freezing may strikingly affect the protective properties of the milk. My personal bias would be that the mother should express her milk for her own infant. However, when you give humanized supplemental formula you probably encourage colonization with bifidus lactobacilli. So there may be some beneficial effects, but not as much as
when the infant is given mother’s own expressed milk. I don’t know what hydrolysis of pro-
tein does to colonization in the premature infant, though there are studies suggesting that the
bacterial flora is altered by the presence of hydrolyzed nutrients in the gut or by using par-
tenteral rather than enteral feeding [3]. I don’t know if anyone has specifically studied immune
responsiveness with hydrolysates compared with nonhydrolysates.

Prof. Ziegler: I have a question about butyrate. You showed data suggesting that butyrate
increases the release of IL-8. Butyrate is of course regularly produced from lactose as a
byproduct of fermentation in the colon, especially in the breastfed infant. I know of other
data—for instance, in the piglet—showing that butyrate can serve as a major energy substrate
and even has trophic effects [4]. So where is the balance here in terms of positive and negative
effects?

Dr. Walker: That’s an important point. The studies we’ve been doing have been on small
intestinal cells. You’re absolutely correct in suggesting that butyrate, as a fermentation pro-
duct, acts as an energy source in the colon; it is particularly important under conditions such as
short bowel syndrome. In the small intestine, butyrate affects histone acetylation and upregu-
lates the production of cytokines a hundredfold. This does not mean that it is not functioning
differently in the colon, or in the small intestine, when used as an energy source.

Prof. Cooper: When there are outbreaks of necrotizing enterocolitis I imagine these must
be related to specific bacterial colonization at those times. Do you have any idea of what spe-
cific organisms may be involved?

Dr. Walker: We mainly see NEC shortly after feeding has been introduced. I tried to show
that by the nature of the feeding you modify the nature of the organism, and that the immature
intestine tends to not handle pathological organisms very well; in particular, the release of tox-
ins may cause an inappropriate response. I believe the combination of inappropriate coloniza-
tion and an abnormal inflammatory response to the interaction of bacteria with epithelial cells
is the major contributor to the pathogenesis of NEC. I don’t think there is any specific organ-
ism, because epidemiologic studies have shown many different types of organisms involved.
The problem lies in the conditions of colonization and the reaction of the immature intestine.

Prof. Cooper: There seems to be a difference sometimes in the epidemic form of NEC,
which seems to follow a specific pattern, and the background cases that one sees intermittently.

Dr. Walker: Some of that has to do with the nature of how infants with NEC are handled.
They are put into a hospital environment where specific organisms exist from the hospital per-
sonnel and they are given antibiotics, because they’re thought to be septic, so you are modify-
ing some of the other gut organisms. I was suggesting that if we could use another nonpatho-
genic organism to interfere with the colonization, we might prevent some of the other
processes that occur.

Prof. Lucas: You’ve touched on the idea of using breast milk biological proteins, such as
lactoferrin, in formula. How important do you think it might be that these are actually synthe-
sized in the breast, in terms of surface components or packaging that might subsequently in-
fluence their handling or activity in the gut?

Dr. Walker: I don’t know the answer to that. It could very well be that the nature of glyco-
sylation or the glycoprotein components might be important in what is going on in the intes-
tine. That’s another area of research.

Prof. Haschke: I should like to discuss nucleotides. In your opinion, which nucleotides are ac-
tive and at what concentrations? At present, we add them in the amounts considered to be present
in breast milk, but do we need more? And if so, is there any possibility that they might be toxic?

Dr. Walker: We’ve studied this in the experimental setting. We’ve been able to show that
for general responses, such as proliferation and differentiation, using the concentrations and
combinations of nucleotides that exist in breast milk seems to be effective. The fortuitous response shown with organ culture was when we were trying to see if single nucleotides had an effect, and we showed that adenosine monophosphate affected apoptosis. Now AMP is a very important component with a host of cell functions, so further work is needed to clarify this. Pickering’s study [5] used nucleotides added to formula at the concentrations that occur early on in breastfeeding (nucleotide levels in breast milk decline with time). This was a human study done under controlled conditions that made some important observations.

I don’t believe there is toxicity effect. However, we have preliminary data to show that nucleotides may enhance some of the inflammatory cytokine responses, which might represent a potential negative effect.

Prof. Berger: There is preliminary evidence that free radicals may play a role in necrotizing enterocolitis. We know that bacteria, depending on whether they’re anaerobic or aerobic, have an antioxidant enzyme capacity. Is there a possibility of synergism in antioxidant protection? In other words, is it possible that the bacterial flora help in the catabolism of various reactive oxidant species? For example, I do know that clostridium species have xanthine oxidase, which could therefore increase the input of free radicals. If there were some synergism in the antioxidant capacity of the bacteria, there could be a relationship to the type of flora in NEC.

Dr. Walker: We’re just at the beginning of our understanding of this. Like most paradigms, the more you get into it, the more complex it becomes. The immaturity of the intestine is sometimes protective for the neonate, and when you mention clostridia, it reminds me that the receptor for clostridium A toxin is underexpressed in the neonate. That’s why neonatologists can grow clostridia in the stools of neonates, but they don’t get pseudomembranous colitis. So Nature is not totally against the neonate. But your point is very well taken and we need to look more carefully at that.

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