Intestinal Microbiota in Neonates and Preterm Infants: A Review

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Abstract: The fetal gastrointestinal tract is sterile until birth when microbes colonize the gastrointestinal tract, and a dense, complex microbiota develops. This enormous cell mass performs a variety of activities that affect both the intestinal and systemic physiology. The microbiota provides nutritional, metabolic, immunological, and protective functions. The neonatal gastrointestinal tract is an organ at risk. Increasing awareness that the human flora is a major factor in both health and disease has led to different strategies to manipulate the flora. Manipulation with prebiotics and probiotics has shown promising results although a better understanding of the gut bacterial colonization process is required before attempts to change the flora should be made. In this review, we summarize the data regarding developmental microbial ecology in the neonatal gastrointestinal tract, and the modulation of such microbiota. The discussion focuses on the control and manipulation of bacterial colonization in the neonatal gut for the prevention and treatment of bacterial intestinal disease in both in human infants and on animal models. Since the best available methodologies should be utilized in studies of nutritional sciences, a recapitulation of the latest techniques for the study of the gastrointestinal flora is presented. Future progress is likely to arise from the use of genomic techniques to track of diet-induced changes in microbiota.

Keywords: Neonatal gastrointestinal tract, microbiota, colonization.

The GI tract of a normal fetus is sterile. During the birth process and shortly thereafter, microbes from the mother and the surrounding environment colonize the gastrointestinal tract of the infant until a dense, complex microbiota develops. The establishment of the gut microbial population is not strictly a succession in the ecological sense. Rather, this colonization is a complex process influenced by microbial and host interactions as well as by internal and external factors. The climax intestinal flora is attained in successive stages [1]. The enteric flora contributes to health by facilitating carbohydrate assimilation and interaction with the developing immune system and also contributes to disease [2]. The environmental conditions under which babies are born and nurtured may affect their exposure to microbes and may subsequently influence the composition of their gut microbiota. The environment of the intestine is derived from 3 main factors: dietary intake, bacterial ecology, and factors such as peristalsis and glandular secretions that are intrinsic to the intestine. The aim of this non exhaustive review is to identify data that contributes directly to the understanding of the establishment and development of gut microbiota.

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I. PHYSIOLOGICAL COLONIZATION

The human fetus receives nutrients, growth factors, and immunoglobulins *via* active or passive placental transport. Swallowing of amniotic fluid nourishes the fetal intestine and prepares this organ for birth. Preterm delivery interrupts the transfer of these factors that are critical to prepare and protect the newborn infant from bacteria that will colonize the intestinal tract postnatally.

I.1. ***The process of colonization*** is greatly influenced by the successive shift from formula feeding to weaning. Culture studies have indicated that, in general, infants are initially colonized by *enterobacteria* and gram-positive cocci, which are thought to create a reduced environment that is favourable for the establishment of *Bacteroides, Bifidobacterium,* and *Clostridium* by day seven [3]. A full-term breast-fed infant has an intestine microbiota in which *Bifidobacteria* and *Lactobacillus* predominate over potentially harmful bacteria, whereas in formula-fed infants, coliforms, enterococci, and *Bacteroides* predominate [4]. In full-term infants, a diet of breast milk induces the development of a flora rich in *Bifidobacterium* sp. Other obligate anaerobes such as *Clostridium* sp. and *Bacteroides* sp. are isolated less frequently, and enterobacteria and enterococci are also rare [1]. *Clostridia* have consistently been found at lower levels in breast-fed babies; thus the presence of this group of bacteria may indicate that the babies have been fed formula. The intestinal microflora in breast-fed infants can be followed by different biochemical

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parameters [5]. Acetic acid is found at higher concentrations in breast-fed infants than in formula-fed infants. Degradation of mucin begins later in breast-fed infants than in formula-fed infants. The conversion of cholesterol to coprostanol is also delayed by breastfeeding.

As an example of phylogenic analysis, *Park et al.* employed a molecular approach to study the feces of one infant on the first, third, and sixth days after birth and showed that microbiotic diversity changes very rapidly in the days following birth. In addition, the acquisition of unculturable bacteria expanded rapidly after the third day [6]. Of the 325 isolated clones, 220 were characterized as known species, while the other 105 clones were characterized as unknown species. On the first day of the life, *Enterobacter, Lactococcus lactis, Leuconostoc citreum,* and *Streptococcus mitis* were present in the infant's feces with the largest taxonomic group in number of clones isolated being *Lactococcus lactis*. On the third day of life, *Enterobacter, Enterococcus faecalis, Escherichia coli, Streptococcus mitis,* and *Streptococcus salivarius* were present. On the sixth day, *Citrobacter, Clostridium difficile, Enterobacter* sp., *Enterobacter cloacae,* and *Escherichia coli* were present. At this point the largest taxonomic group was *E. coli.* Geographical differences in the composition of the intestinal microflora in infants have also been reported. For example, enterobacteria, enterococci, bifidobacteria, lactobacilli, and bacteroides show a different distribution in developed and developing countries [5].

I.2. ***Peristalsis*** is developmentally regulated and controls microbiota changes along the length of the intestine. In term infants, as in adults, migrating motor complexes pass as waves along the gastrointestinal tract. Feeding results in further complexes superseding the background wave pattern. In preterm neonates, however, migrating complexes are not present until around 34 weeks gestation [7]. In these infants, the mechanisms necessary for maintaining a stable temporo-spatial relation in the intestine are not fully developed. In the fetus the environment of the intestine is mostly controlled by the amniotic fluid, and thus, the role of peristalsis in regulating lumenal homeostasis is correspondingly less important. In preterm infants, however, the intestinal environment is affected by the outside world. Thus, the possibility of build-up of substances within the intestine exists because the propulsive action of the intestine is not yet fully developed [8]. Characteristics of upper esophageal sphincter and primary peristalsis are present as early as 33 weeks postmenstrual age, undergo further maturation during the postnatal period, and are significantly different from those in adults [9, 10]. Fetal swallowing contributes greatly to amniotic fluid homeostasis and fetal somatic development. Fetal gastric emptying cycles normalized during the early third trimester. The near-term evidence of delayed emptying may contribute to newborn infant feeding satiation [11].

The environment of the epithelial surface. In preterm neonates, the degree of mixing of lumenal contents may be small due to immature peristaltic activity. This may result in an unstirred layer of greater thickness than is present in neonates born at term. . Changes in the gastrointestinal tract longitudinally are determined to a large extent by peristalsis. Molecules passing from the contents of the intestine are propelled by peristalsis of the intestine to the epithelial cell apex. These molecules encounter the unstirred mucus layer and the deep mucus layer, both of which are present in the neonatal intestine [8]. The effect of each layer on the absorption of the molecules depends on the chemical nature of the molecules. The unstirred layer is a significant barrier to lipid-soluble molecules, whereas the acidic microclimate has a large effect on weak electrolyte uptake. The unstirred layer may not be a distinct layer on the mucosal surface but may serve as a barrier in which molecules diffuse at a rate different from that predicted by the diffusion coefficient of water. Increased agitation of the content in this layer enhances the diffusion barrier. In preterm neonates, the degree of mixing of lumenal contents may be small because of immature peristaltic activity and therefore might result in an unstirred layer of greater thickness than is present in term neonates. In premature neonates, pancreatic and biliary functions are not as well developed as in adults. As a result, the unstirred layer is a significant barrier to lipid absorption.

I.3. ***Mucus secretion*** is well developed in neonates although its composition changes during development [8]. While the role of mucus secretion is thought to be important, quantification in neonates is difficult. Therefore, studies have been conducted using rats models [12]. The deep mucus layer is significantly more acidic than the lumen, and changes at the surface of the epithelium are less variable than those in the bulk phase in different parts of the gastrointestinal tract [13, 14]. The acid microclimate has a direct effect on transport of dipeptides, which, unlike amino acids, are transported into the cell in association with hydrogen ions. Human neonates produce a microclimate sufficient for these absorptive functions, but little is known about the microclimate in preterm neonates [8].

I.4. ***Intestinal permeability.*** In preterm infants (26-36 weeks gestation), intestinal permeability is greater during the first two days of life than during days five through eight. Permeability is greater in preterm infants than in terms infants only when measured within two days of birth. This results suggests rapid postnatal adaptation of the small intestine in preterm infants [15]. The barrier function of the intestinal epithelium transiently decreases during the first week after birth in preterm neonates that are enterally fed. Both a diminished barrier function and a low absorptive capacity during the early postnatal period, particularly in neonates born at less than 28 weeks gestation, may underline the high vulnerability of these patients to intestinal complications. Given the finding that epithelial integrity was restored on initiation of enteral feeding, early administration of enteral nutrition may offer an effective strategy to support intestinal adaptation to extrauterine life in preterm neonates [16].

Marker of intestinal permeability. Immaturity of the intestinal epithelial barrier function and absorptive capacity may play a role in the pathophysiology of intestinal complications in preterm neonates during the early postnatal period. Thus, identification of non-invasive markers would be of utmost clinical relevance. Lactulose and mannitol have been used to test the passive intestinal permeability as neither of these molecules is metabolized and both are wholly and solely excreted by the kidney. Urinary recovery

is a measure of the intestinal uptake. Lactulose is though to pass across the gut wall by a paracellular pathway whereas mannitol passes across the gut wall by a transcellular pathway. The studies by Weather *et al.* indicated that the immaturity of the gut in preterm infants was responsible as opposed to a process for adaptation to enteral nutrition [17]. In addition, Mills used 2,3 Butanediol, which was detected in urine samples of premature infants by capillary gas chromatography. The presence of this biochemical marker indicated bacterial fermentation of pyruvate in the gut by abnormal gut colonization with acetoin-producing micro-organisms, an abundant supply of nutrient lactose in the colon, and an increase in intestinal permeability [18]. The lactulose-to-rhamnose ratio was determined as a marker of intestinal permeability. The urinary excretion percentages of D-xylose and 3-0-methyl-D-glucose were determined as markers of passive and active carrier-mediated mono-saccharide absorption, respectively [16].

I.5. ***Gradient from stomach to colon.*** The establishment and succession of bacterial communities in preterm infants produces an increasing gradient from the stomach to the colon and provides spatial distribution within each gut compartment. Basically, the intestine is comprised of four microhabitats: the intestinal lumen, the unstirred mucus layer, the deep mucus layer, and the surface of mucosal epithelial cells [19]. Blakey *et al.* studied the developing microflora in the throat, stomach, and feces of 28 preterm babies during their first three weeks of life using classical culture methods [19]. The flora at all levels of the gastrointestinal tract differed from that of healthy breast-fed and artificially-fed full-term babies. Colonization of the throat and stomach was delayed beyond the first four days of life in 74% of the preterm babies studied. Flora of the stomach was sparse and resembled fecal flora. The fecal flora was established more rapidly although only in 70% of the babies during the first 4 days of life. Initially *Bacteroides* sp. were predominant, but *Escherichia coli* and other aerobic gram-negative bacilli gradually increased in frequency. Lactic acid-producing bacteria usually appeared late in the third week of life.

I.6. ***Translocation*** describes the transmucosal passage of viable and non-viable microbes and their by-products (endotoxins) across the intact intestinal barrier [20]. Predisposing factors in the pathogenesis of systemic infections, such as prematurity, promote impaired mucosal barrier function and consequently foster gut permeability [20]. Under these conditions, indigenous bacteria, viruses, and toxins, which are normally confined within the gastrointestinal tract, may reach systemic organs and tissues. Human infants who received nutrition solely by the parenteral route have an increased risk of gram-negative bacterial translocation from the gastrointestinal tract into the systemic circulation and other organs [21]. Moy gives confirmation of translocated gastrointestinal bacteria in a neonatal model by demonstrating that transformed *E. coli* K1 fed to healthy rabbit pups spontaneously translocated from the intestinal lumen and subsequently disseminated to the mesenteric lymph nodes, spleen, and liver [22]. Bacterial translocation is one important cause of nosocomial infections following major abdominal surgery. Seehofer showed that synchronous liver resection and colon anastomosis led to increased bacterial translocation compared to the single operations in a rat model. Oral administration of probiotics was shown to minimize this translocation. From these studies, the authors proposed that bacterial overgrowth in the cecum and impaired hepatic regeneration, but not histo-logical changes or alterations of paracellular permeability, are the potential pathogenic mechanisms for translocation following the surgeries [23].

A high proportion of bacterial translocation in neonates results not only from immaturity of host defense functions, but also from the dominant colonization of aerobic bacteria in the intestine. Bacteria colonization develops differently in breast-fed, formula-fed, premature, and full-term infants. In a model of neonate rats, Yajima showed that the frequency of isolation of bacteria from mesenteric lymph nodes and other peripheral sites did not mirror the composition of the intestinal flora. Among the translocated bacteria, *Staphylo-coccus* may be especially hard to recognize and difficult for the host defense system to destroy. Furthermore, breast-feeding inhibited systemic bacterial translocation in the suckling period of the rat [24]. Additionally, Katalaya demonstrated that 1) the adherence of bacteria to the intestinal mucosal surface is an important factor in bacterial translocation, 2) the intestinal mucus modulates bacterial adherence, and 3) increased levels of mucosa-associated bacteria are associated with a loss of intestinal barrier function to bacteria [25].

The mechanisms by which probiotic agents, such as enteral *Lactobacillus* enhance the intestinal defenses against potential luminal pathogens has been examined in a neonatal animal model. Enterally-administered *Lacto* GG decreases the frequency of *E. coli* K1A translocation in a neonatal rabbit model [26].

I.7. ***The intestinal mucosal immune system*** is fully developed at birth for full-term infants. The immune system is composed of the innate, the specific immunity, and with regards to newborns, the immunity passively acquired from the mother by means of IgG antibodies and human milk. The innate immunity involves humoral elements such as complement system proteins, acute phase proteins, cytokines and cellular elements such as monocytes, macrophages, granulocytes, dentritic cells and natural killer lymphocytes. The innate immunity has a limited capacity to distinguish between microorganisms, and often has a similar response to different microorganisms. The components of specific immunity are the lymphocytes and their products (e.g. antibodies). It responds specifically to each microorganism and has a memory. Stages of fetal immune system development are summarized in Table **1** [27]. All newborns have an increased risk of microbial infections as compared with older children and young adults [28]. Extremely premature newborns (< 28 weeks gestation) have a 5-to 10-fold higher incidence of microbial infection than even term newborn [29]. Whether near-term newborns have an intermediate risk of acquiring sepsis immediately after birth or within the first few months of life is unknown. There is an intriguing possibility that the well known immaturity of the fecal immune system has a biological protective purpose. It helps to prevent "premature rejection" by the host – the mother. This immaturity may therefore represent an adaptive

Table 1. Stages of Fetal Immune Development [27]

Fetus age (weeks)	Innate Immunity	Humoral Immunity	Celular Immunity	Passive Immunity
5-6	Macrophages in the liver and blood		T-cell precursor in the liver	
9-10	Start of the complement synthesis	B precursor in the liver	T-cell precursors in the thymus	
12-14	Macrophages in lymphonodes and APC MHC class II	Pre-B cells with IgD, IgG and IgA	T-cells CD4+ and CD8+ in the liver and spleen	Start of mother's IgG transfer
16-17	Mature macrophages in the liver and circulating neutrophils	Large number of B-cells in the spleen, blood and bone marrow	T-cells in the blood and lymphoid tissues/ rearrangement of receptors	
20-30		B-cells secrete antibodies	Gradual increase of T-lymphocytes secreting lymphokines	Gradual increase of IgG transportation

APC : antigen presenting cells; MHC: major histocompatibility antigens

response to preventing premature birth. Yet, the ontogeny and sequences of maturation of the immune system in the late preterm infant has not been well studied [28].

The protective function of the gut requires the microbial stimulation of initial bacterial colonization. Breast milk contains prebiotic oligosaccharides, including inulin-type fructans, which are not digested in the small intestine but enter the colon as intact large carbohydrates that are then fermented by the resident bacteria to produce short-chain fatty acids. The nature of this fermentation and the resulting pH of the intestinal contents dictate proliferation of specific resident bacteria. For example, infants fed breast milk containing prebiotics support increased proliferation of *Bifidobacteria* and *Lactobacilli* (probiotic), whereas formula-fed infants produce more *Enterococci* and *Enterobacteria*. Probiotics, stimulated by prebiotic fermentation, are important to the development and sustainment of intestinal defenses. Probiotics, for instance, can stimulate the synthesis and secretion of polymeric IgA, the antibody that coats and protects mucosal surfaces against harmful bacterial invasion. In addition, appropriate colonization with probiotics helps to produce a balanced T helper cell response (Th1=Th2=Th3/Tr1) and to prevent a T cell imbalance (Th1>Th2 or Th2>Th1) that may contribute to clinical disease. A Th2 imbalance contributes to atopic dermatitis while a Th1 imbalance contributes to Crohn's disease and *Helicobacter pylori*-induced gastritis). Furthermore, toll-like receptors on gut lymphoid and epithelial cells recognize bacterial molecular patterns (e.g. endotoxin, lipopolysaccharide, flagellin, etc.) and modulate the intestinal innate immunity and an appropriate adaptative immune response. Both animal and clinical studies have shown that inulin-type fructans will stimulate an increase in probiotics (commensale bacteria), which have been shown to modulate the development and persistence of an appropriate mucosal immune response. These results are compelling, however, Forchielli and Walker recommend additional studies to show that prebiotics directly or indirectly stimulate intestinal host defenses. Thus, prebiotics could potentially be used as a dietary supplement to stimulate a balanced and effective mucosal immune system in newborns and infants [30].

II. CONTEXT INFLUENCES AND PATHOLOGY

II.1 ***Intestinal microbiota and allergy development.*** The infant's immature intestinal immune system develops as it comes into contact with dietary and microbial antigens in the gut. The evolving indigenous intestinal microbiota have a significant impact on the developing immune system [31, 32]. Disturbance in the mucosal immune system are reflected in the composition of the gut microbiota and vice versa [33]. Distinctive alterations in the composition of the gut microbiota appear to precede the manifestation of atopic disease, which suggests a role for the interaction between the intestinal immune system and specific strains of the microbiota in the pathogenesis of allergic disorders [34]. Further more, dietary lipids as immunomodulators may prevent allergic sensitization by down-regulating inflammatory response whilst protecting the epithelial barrier, and probiotic bacteria have been shown to reinforce the different lines of gut defence (immune exclusion, immune elimination and immune regulation). On this basis Isolauri *et al.* proposed a new strategy against allergic disease based on the administration of tolerogenic gut-processed peptide fragments of a specific protein, in addition to the use of specific dietary compounds such as fatty acids and antioxidants, and introducing a microbial stimulus for the immature immune system by means of cultures of beneficial live microorganisms characteristic of the healthy infant gut microbiota [33].

II.2. ***Breast milk*** is associated with a lower risk of necrotizing enterocolitis (NEC) and slower growth in the early postnatal period [35]. Exclusive breastfeeding protects against asthma [36]. Lem showed in a mouse model of maternal transmission of asthma susceptibility that breast milk is sufficient to mediate allergen-independent maternal transmission of asthma risk to offspring [37]. In breast-fed infants, *Bifidobacterium* predominates with *Lactobacillus* and *Streptococcus* as minor components while in formula-fed infants, gram-negative organisms such as *E. coli* and *Klebsiella* are more likely to colonize the gut [38].

II.3. ***Antibiotic therapy*** affects the gut colonization in three important ways: 1) antimicrobial agents can have specific effects on individual components of the microbiota

rather than a general non-specific suppression of all microbes, 2) the resultant microbial profile influences the populations that emerge after treatment has stopped, and 3) the effect of antibiotic therapy can persist beyond treatment [39].

The postnatal maturation of the gut, which is partially modulated by bacterial colonization, results up in the establishment of an efficient barrier to luminal antigens and bacteria. The use of broad-spectrum antibiotics in pediatrics alters the gut bacterial colonization and, consequently, may impair the maturation of the gut barrier function. Animal model studies have shown that Clamoxyl treatment altered the normal colonization pattern of the gut microbiota and the normal maturation profile of 10-30% of the genes in the different intestinal segments [40].

***Influence of the antibiotic therapy on intestinal microbiota*.** Therapy with broad spectrum antibiotics is frequently observed in pediatric practices, children within their first year of life being particulary affected. One major consequence of such early antibiotherapy is the alteration of the normal colonization process by the gut microbiota. Neonatal antibiotic treatment has been shown to reduce the biodiversity of the fecal microbiota, to delay the colonisation by beneficial species such as bifidobacteria or lactobacillus and to induce colonization by antibiotic-resistant opportunistic strains [41]. Penders *et al.* demonstrate on fecal samples from 1032 infants that oral use of antibiotics during the first month of life resulted in decrease number of bifidobacteria and bacteroïdes fragilis group species and may have a major effect on the composition of the gut microbiota, particularly on obligate anaerobies [42]. An experimental study in the rat clearly demonstrate that amoxicilline deeply affects the maturation of 10-30% of the genes involved in the intestinal barrier function at the suckling-weaning interface, a period during which the gut is challenged by a lot of novel food born antigens [40]. Changed patterns of early-life gut colonisation is reported to be associated with allergic sensibilisation, metabolic priming and development of regulatory lymphocyte population [43, 44].

There is rapidly increasing evidence from experimental studies that the initial colonization of the intestine is a moment of pivotal importance in long-term health. The potential for long term persistence of early colonising bacteria suggests that much more thought should be given to the late consequences of perinatal antibiotherapy [44].

II.4. ***Type of birth*.** The type of delivery of the neonate has a significant effect on the development of the intestinal microbiota [45]. The primary gut flora in infants born by cesarean delivery may be disrupted for up to 6 months after birth [46]. A longer vaginal delivery increases the likelihood that viable microbes can be isolated from the stomach and mouth of the infant [47, 48]. Although infants delivered by cesarean section are also be exposed to their mother's microbiota, their initial exposure is most likely to environmental isolates from equipment, air, and other infants, with the nursing staff serving as a vector for transfer [49]. In industrial countries, obstetric and hygienic procedures aimed at reducing the spread of pathogenic bacteria in maternity and neonatal facilities may result in delayed development of the gut microbiota or even to the absence of certain groups of intestinal bacteria during succession.

II.5. ***Premature neonatal gut.*** The pattern of bacterial colonization in the premature neonatal gut is different from that in the healthy, full-term infant gut. Infants requiring intensive care acquire intestinal organisms slowly. The establishment of bifidobacterial flora is retarded, and delayed bacterial colonization with a limited number of bacterial species tends to be virulent. Schwiertz showed an increase in similarity of the bacterial communities in hospitalised preterm infants in contrast to breast-fed, full-term infants. A strikingly high similarity was observed between bacterial communities from different preterm infants regardless of birth weight, feeding regime, and antibiotic therapy. This work underscored the fact that the initial colonization of the newborn GI tract is highly dependent on the environment and that cross-transmission of bacteria is a serious problem in the hospital [2].

II.6. ***Comparison between lower genital tract of pregnant woman, neonate, infant, and adult feces.*** A comparison of viable counts of common groups of bacteria found in the lower genital tract of pregnant women, in feces of neonates (< 1 wk of age), and in fecal samples from infants (> 1 month of age) and adults was described by Mackie [39]. Conventional culture methods were used to collect data (Table **2**).

II.7. ***The problem of low birth weight infants.*** Low birth weight contributes substantially to infant mortality and to childhood disabilities. The principal determinant of low birth weight in the United States is preterm delivery. Poverty is strongly and consistently associated with low birth weight [50]. Sakata *et al.* have employed classical culture techniques to study the development of the fecal flora in very low birth weight infants (VLBW, 810-1350 g) and full-term newborns. The intestine of the VLBW infants was first colonized by *Enterobacteria* and *Streotococci* similar to full-term infants, however, both microorganisms predominanted for a longer period of time and the establishment of *Bifidobacterial* flora was delayed in the VLBW infants. Emergence of *Bacteroides*, *Clostridium,* and *Lactobacillus* was also delayed. The observed decreased milk intake of the VLBW infants could also contribute to this delay [51].

One study on duodenal microflora in VLBW neonates showed a high incidence of duodenal gram-negative colonization with *Enterobacteriaceae* counts of up to 10^8 CFU/g [52]. This gram-negative predominance may be due to the immaturity of the gastrointestinal tract as it occurred beyond four days of age in infants that had been fed enterally, increased with age, and was associated with a longer stay in pediatric units (Fig. **1**) [52].

The state of health at a given gestational age probably depends on the balance between the many developing structures and the achievement of their functions. The diversity of possible influences explains the difficulties in clarifying the etiology of disease states such as NEC [53]. The use of animal models and relatively non-invasive clinical methods will dramatically improve our knowledge of the development of bacto-intestinal function, and thus, the

Table 2. Viable Counts of Common Bacteria Species from Pregnant Women, Neonates, Infants, and Adults [39]

Bacterial Group	Lower Genital Tract of Pregnant Women[2]	Feces[3]		
		Neonate	Infant	Adult
Aerobes or facultative anaerobes				
Bacilli	Moderate	Low	Low	Low
Corynebacteria	Moderate	Low	Low	Low
Enterobacteria	Low	Moderate	Moderate	Moderate
Enterococci	Low	Moderate	Moderate	Moderate
Lactobacilli	Moderate	Low	Moderate	Moderate
Micrococci	Low	Low	Low	Low
Propionibacteria	Moderate	Moderate	Moderate	Moderate
Staphylococci	Low	Low	Low	Low
Streptococci	Moderate	Moderate	Moderate	Moderate
Range (log_{10}CFU)[1]	7.7-8.6	8.2-9.1	8.0-8.7	6.9-8.8
Anaerobes				
Range (log_{10}CFU)	8.3-8.8	7.8-9.3	9.8-11.3	10.5-11.5
Bacteroides	Moderate	High	High	High
Bifidobacteria	Moderate	High	High	High
Clostridia	Low	Low	Moderate	Moderate
Eubacteria	Moderate	Moderate	High	High
Fusobacteria	Moderate	Moderate	High	High
Peptostreptococci	Moderate	Moderate	High	High
Ruminococci	Moderate	Low	Moderate	Moderate
Veillonella	Moderate	Moderate	Moderate	Moderate

[1] Viable counts summarized as high [≥log_{10}9 colony forming units (CFU), moderate (log_{10}6–8 CFU), and low (≤log_{10}5 CFU)].
[2] Numbers reported as log_{10} CFU/mL or g secretion.
[3] Numbers reported as log_{10} CFU/g feces (wet wt).

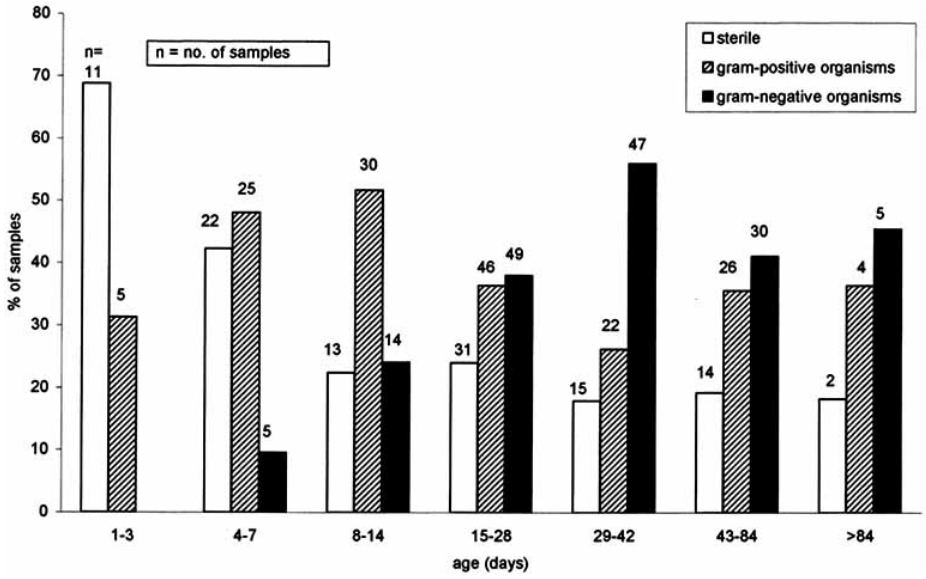

Fig. (1). Duodenal aspirate culture by organism type in relation to age.

medical care of preterm infants, especially nutritional support, will continue to improve.

II.8. ***Necrotizing enterocolitis*** (NEC) is a devastating condition with high morbidity and mortality that specifically affects preterm and VLBW infants. NEC may be the consequence of synergy among three of the major risk factors: prematurity, enteral feeding, and bacterial colonization. Together these factors result in an exaggerated inflammatory

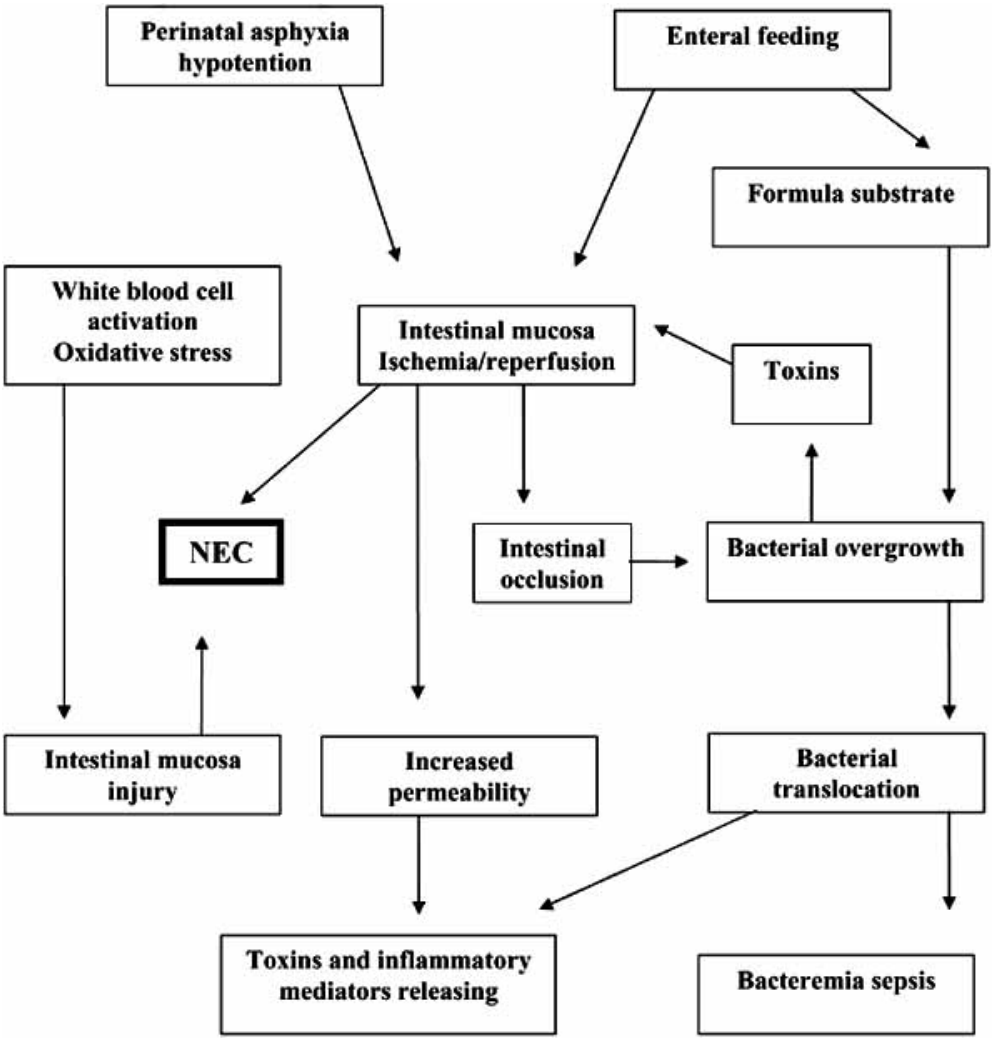

Fig. (2). Summary of the pathogenesis of necrotizing enterocolitis.

response that often leads to ischemic bowel necrosis. Human milk may reduce the incidence of NEC by decreasing pathogenic bacterial colonization and promoting growth of non-pathogenic flora as well as by maturation of the intestinal barrier and amelioration of the proinflammatory response [54]. The pathogenesis of NEC is unclear with many possible factors including stress that provokes a mesenteric ischemia with digestive stasis and exogenous bacterial pathogens [55]. Since the bacteria multiply as a result of both digestive stasis and immature immune function of the intestinal barrier, a weakened intestinal wall could easily be overwhelmed. Food ingestion also aggravates the problem. Both vascular and infectious factors often co-exist in premature infants, whereas in full term newborns, infection is the main factor (epidemic trend). The pathogenic process are summarized in Fig. (**2**) [56].

The critical clinical features of NEC include severe generalized infection and digestive symptoms. Abdominal radiography shows a distension of the digestive loops. Without appropriate treatment, three characteristic symptoms, vomiting, abdominal distension, and bloody diarrhea, emerge with an overall deterioration and can ultimately result in septic shock and then death.

Early and aggressive treatment must be initiated for any infant suspected of having NEC. Umbilical catheters should be removed whenever possible, oral feeding should be stopped, and nasogastric tube drainage should be instituted. Fluid and electrolyte deficits require rigorous attention [56]. Parenteral antibiotic therapy should commence after appropriate cultures (blood, cerebrospinal, urine, and stool) are obtained. The initial therapy should include an extended-spectrum cephalosporin and vancomycin. Inclusion of clindamycin in the management of NEC has been questioned [57].

The original observation that bacterial proliferation was a factor for NEC prompted suppression of the gut flora by administration of topical antibiotics in order to prevent the condition. Evidence suggests that oral antibiotics reduce the incidence of NEC in low birth weight infants, however, concerns about possible adverse outcomes, specifically the development of resistant bacteria, persist [58]. Feeding these infants with breast milk has been suggested to reduce the colonization by pathogenic organisms and induce colonization by commensal organisms by modulating inflammatory reactions and decreasing intestinal injury [54]. Unfortunately, no controlled studies have demonstrated

prevention of NEC following the feeding of colostrums or breast milk to human neonates.

III. ANIMAL MODELS

Only most important animal models are quoted here:

III.1. ***High incidence of bacterial translocation*** in neonates results not only from the immaturity of host defense functions, but also from the dominant colonization of aerobic bacteria in the intestine. Yajima *et al.* examined the incidence of bacterial translocation and identified the translocated bacterial species in neonatal rats. These findings were related to the intestinal microflora and to the type of feeding. The frequency with which species of bacteria were cultured from the mesenteric lymph nodes and other peripheral sites did not mirror the composition of the intestinal flora [24].

III.2. ***The potential protection,*** of human gut microflora against *E. coli* heat-labile enterotoxin (LT)-mediated abrogation of oral tolerance to unrelated co-ingested proteins has been studied in adult gnotobiotic mice [59]. Both specific IgG subclasses and IgE hyporesponsiveness was induced in LT+ovalbumin-fed gnotobiotic mice indicating that the human gut microflora can protect against the LT-mediated abrogation of oral tolerance. This protective effect, however, only occurs when the gut microflora is associated from birth. Colonization of germ-free mice with a single bacterial strain (*E. coli)* did not induce protection. These results support the hypothesis that the natural establishment of the gut microflora in neonates crucially influences resistance to LT-mediated abrogation of oral tolerance by reinforcing suppression of both Th1- and Th2- controlled responses. Further more, the results suggest that sequential bacterial colonization of the gut may be involved in this phenomenon.

III.3. ***An infant human flora-associated (IHFA) rat model*** has been developed [60-62]. This model system permits investigations on the interaction between diet, flora, and mucosa in newborn animals. Studies with these animals have documented the awful consequences for newborns following perturbations of the mother's flora (e.g. anti-biotherapy). In addition, these results have demonstrated the necessity of the presence for normal maternal flora for a normal installation of the digestive microflora of the newborn [63].

III.4. ***The use of "pup in a cup" technique*** circumvents the difficulty of controlling diet composition and caloric intake [64] and provides a means to study the effects of altered nutrition during the suckling period in rats [65].The model has been adapted by Beierle *et al.* for use in mouse pups thereby allowing the use of transgenic animals. This technique permits nutritional manipulation in neonatal mice, a mammalian model for which the genome has been sequenced and transgenic mutants are available [66].

III.5. ***Animal models and pre/probiotics.*** Studies on the effects of probiotic (*Lactobacillus reuteri* and zinc) supplementation in infant rhesus monkeys indicated that the supplementation is safe, improves iron status, and decreases diarrhea severity [67]. Using a model of newborn rat pups, Sherman showed that prophylactic therapy with recombinant human lactoferrin (rhLF) and the probiotic *Lactobacillus GG* act to enhance defenses against invasive *E. coli* in the nascent small intestine. RhLF is a natural glycoprotein that serves as a defense against infections. This protein is secreted, notably, into colostrums, milk, and tears [68].

The potential health-improving effects of both a prebiotic (fructo-oligosaccharides 5.7% (w/w)), and a probiotic (viable *Bifidobacterium lactis* and *Streptococcus thermophilus*) infant formula have been evaluated in a rat model by Montesi *et al.* [69]. Analysis of the composition of cecal microbiota by both classical plate count of the main bacteria groups and by PCR amplification of a V3 fragment of 16S rRNA genes and subsequent denaturing gradient gel electrophoresis (DGGE) revealed that both diets induced a significant reduction of *Clostridia* and *Bacteroides* spp. compared to control diets. A diet including prebiotics also reduced the number of coliforms and increased the presence of *Bifidobacteria*. DGGE analysis showed a significant increase of 16S RNA gene fragments in rats fed with either probiotics or prebiotics indicating a more diverse speciation. Detection of *Bifidobacterium* sp. with genus-specific primers was limited to prebiotic-fed rats, wherease the use of Lactobacillus group-specific primers produced similar results in rats fed with each of the diets. These molecular results were in agreement with the plate count results.

IV. MICROBIOTA ANALYSIS METHODS

IV.1. ***Culture techniques.*** Traditionally, GI microbiota have been studied via cultivation-based techniques, which are labor intensive and require previous knowledge of individual nutritional and growth requirements. Thus, culture-based approaches provide an incomplete picture of microbial diversity in the gastrointestinal tract.

IV.2. ***Molecular techniques.*** Recently, molecular ecology techniques that are based on the 16S ribosomal RNA gene (rDNA) have become increasingly popular and useful. These methods have proved to be reliable for the detection and identification of bacteria species [70, 71]. The rDNA analysis is based on physical and chemical proprieties of DNA molecules. Utilization of these molecular techniques bypass the cultivation step and enable characterization and quantification of the microbiota, while providing a classi-fication scheme to predict phylogenetic relationships. A summary of current molecular ecological approaches for studying the gastrointestinal microbiota is provided by Zoetendal [72].

In short, clone libraries are sequenced to identify the composition of the microbiota often to the species level. Microbial community structure and evolution can be analyzed *via* fingerprinting techniques, while dot blot hybridization or fluorescent *in situ* hybridization can be used to measure the abundance of particular taxa. Emerging approaches, such as those based on functional genes and their expression and the combined use of stable isotopes and biomarkers, are being developed and optimized as a means to study the metabolic activity of groups or individual organisms *in situ*.

Denaturing gel electrophoresis fingerprinting techniques have great potential for microbial ecology as these methods allow the application of statistical analysis [73]. An example

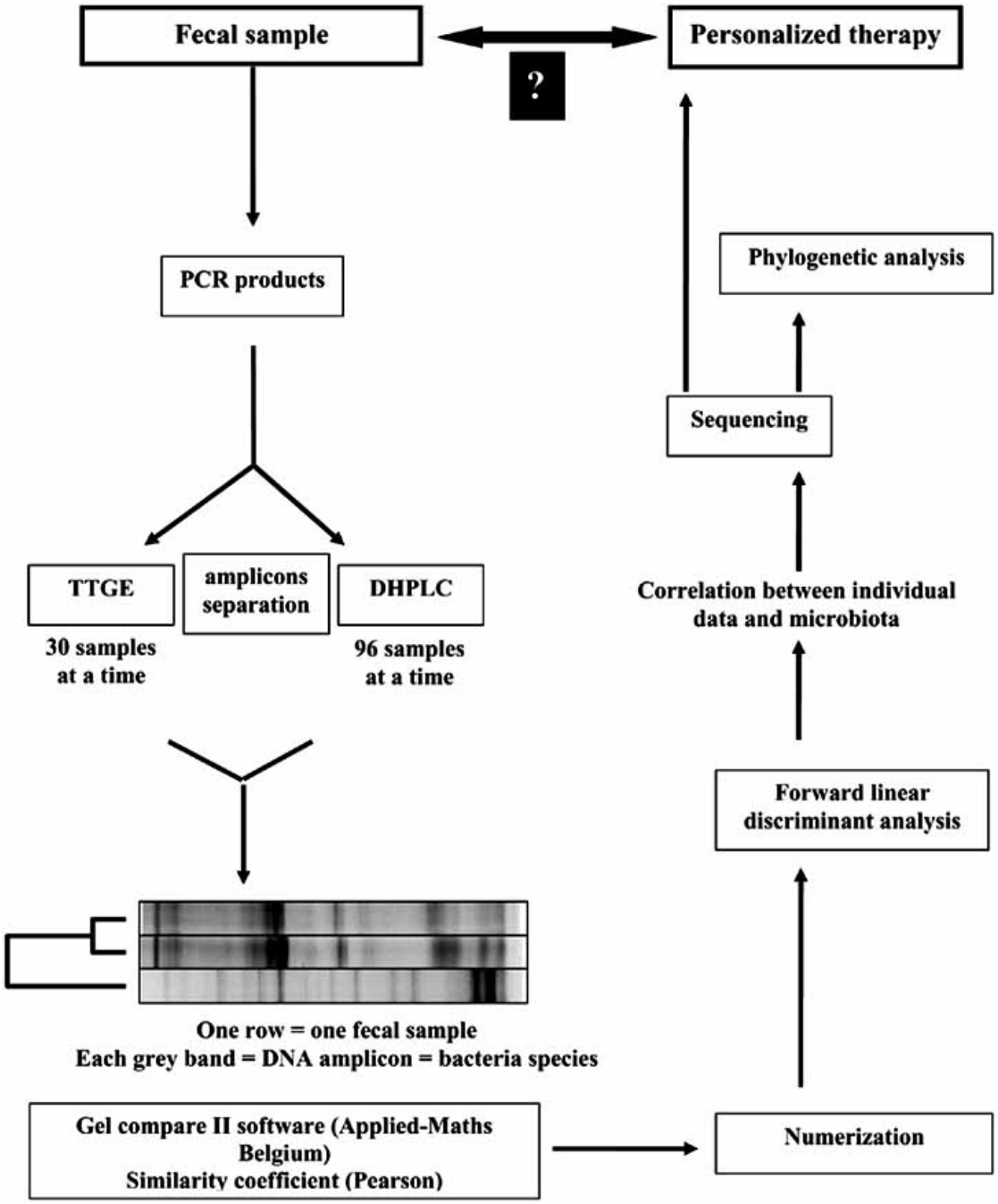

Fig. (3). Scheme for molecular analysis of fecal samples. The question of personalized therapy is highlighted in red.

fingerprinting technique is given by Temporal Temperature Gradient gel Electrophoresis (TTGE) [74]. This culture-independent molecular method has proven most appropriate in dynamic studies of dominant species diversity within complex ecosystem like the colon [75]. In our laboratory, we utilize molecular analysis of fecal samples to search for correlations between individual data (age, sex, clinical data, therapy, etc) and dominant microbiota (Fig. **3**) (de La Cochetière *et al.* ASM 2006). Our results support the concept of permissive microbiota (de La Cochetière *et al.* EECMID 2006) and raise the question of personalized therapy.

IV.3. ***Metagenomics.*** Much of the extant microbial genetic diversity, referred to as the metagenome, remains unexploited. Metagenomics utilizes a whole-genome shotgun sequencing approach to study the genomes of all microbes, regardless of their ability to be cultured. This method, which is both cost-effective and culture-independent, is used to identify microbes and analyze microbial genomes. By treating the microbial community as a single dynamic entity, metagenomics explores the genome content of the whole community and provides analysis of changes in content and expression as a function of location, time, and various states of perturbation, e.g., progression towards and regression

from disease following treatment [76]. From this type of analysis, a better understanding of the biology of the organisms found in the gastrointestinal tract and of to the process of adaptation to co-exist and interact with their host will undoubtedly arise. This new type of data will further our understanding of the impact the microbiota has on preterm human physiology. The completed genomes of *Bifidobacterium longum* NCC2705, *Lactobacillus acidophilus* NCFM, *Bacteroides thetaiotaomicron* VPI-5482, *Lactobacillus johnsonii* NCC533, and *Lactobacillus plantarum* WCFS1 have already been sequenced [77].

V. NUTRITION AND MICROBIOTA

The nutritional management of preterm infants may have a major impact on growth and development. Various feeding strategies have been used including the use of expressed maternal milk, donor human milk, breast milk fortifiers, adapted formula milks, and total parenteral nutrition. Feeding is one of the variables important for the acquisition of intestinal flora. In breast-fed infants, *Bifidobacterium* is the primary organism, and with *Lactobacillus* and *Steptococcus* are minor components. In formula-fed infants, the flora is very different with similar amounts of *Bacteroides* and *Bifidobacterium* and some *Staphylococcus*, *E. coli*, and *Clostridia* as minor components [38].

Preterm infants, especially those who have been growth restricted *in utero*, have fewer nutrient reserves at birth than term infants. Additionally, preterm infants are subject to physiological and metabolic stresses that can affect their nutritional needs, such as respiratory distress or infection. Recommendations based on data from intrauterine growth and nutrient balance studies assume that the optimal rate of postnatal growth for preterm infants would be similar to that of normal fetuses of the same postconception age. In practice, however, these target levels of nutrient input are not always achieved, and this failure may result in important nutritional deficits [78].

V.1. ***Nutrition requirements*** for preterm infants have been recommended by the international consensus group:

- Energy 110-120 kcal/kg/day
- Protein 3-3.8 g/kg/day
- Fat 4.5-6.8 g/kg/day
- Calcium 120-230 mg/kg/day
- Phosphorus 60-140 mg/kg/day

Manipulation of the neonatal gut with human milk is a useful strategy to prevent and treat intestinal diseases [4]. Colonization of the gut ecosystem in infancy by specific bacterial species may be important in the initial regulation of the developing immune system [79]. Previous review has focused on the specific effect of nutrients on the development of the immune system in early life [80]. Before employing a strategy to modulate the flora in infants, several questions should be considered: Should a breast-fed type flora with limited ability to ferment complex carbohydrates be retained? Can supplementation with probiotics and prebiotics achieve a flora with adult characteristics but with more lactic acid bacteria in weaned infants? Are there any health risks associated with such manipulation of the flora? [39, 81].

V.2. ***Short-chain fatty acids in the intestine*** (acetate, propionate, and butyrate) are the products of bacterial metabolism of carbohydrates in the gastrointestinal tract. In the preterm neonate, carbohydrate digestion is not fully developed. Carbohydrates that are readily absorbed in more mature infants provide a source of short-chain fatty acids in preterm infants. Short-chain fatty acids are not detectable at birth, but the concentrations increase thereafter. Boehm et al studied the possible effects of a prebiotic mixture of short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides [82]. The results from over 400 preterm and term infants clearly demonstrated that the prebiotic mixture specifically stimulated the growth of *Bifidobacteria* and *Lactobacilli* and reduced the growth of pathogenic bacteria. Furthermore, prebiotic treatment improved the response to vaccination and reduced allergic reactions in an animal models [83]. Knol *et al.* focused on the effect of *Bifidobacteria* dominance on the presence of clinically relevant pathogens such as *Pseudomonas aeruginosa*, *Enterobacter*, *Klebsiella*, *Proteus*, *Streptococcus* group B, *Clostridium difficile*, *Bacillus subtilis* and *Acinetobacter*. The stimulation of *Bifidobacteria* by prebiotic oligosaccharides reduced the presence of clinically relevant pathogens in the fecal flora. This finding indicated that prebiotic substances may indeed have the capacity to protect against enteral infections [84]. Lidestri et al evaluated the possible effect of dietary oligosaccharides on calcium homeostasis in preterm infant [85]. Marini showed that prolonged administration of probiotics in preterm infants induced an increase in probiotic-specific IgA and IgM antibodies. This increase in antibodies may explain the virtual disappearance of viable germs in stools despite continuous administration of probiotics. Probiotic supplementation did provide some positive influences such as a decreased ratio of aerobic/ anaerobic bacteria and an increase ratio of gram-positive/ gram-negative bacteria [86]. In breast-fed infants, the predominant short-chain fatty acid is acetic acid. Furthermore casein inhibited the bacterial flora responsible for high acetate production [87].

V.3. ***Glutamine supplement in premature infants.*** Traditionally, glutamine has not been used as a nutritional supplement because with healthy individuals with a normal diet under nonstressed conditions do not require glutamine supplementation. Several studies in animals suggested the possibility that glutamine supplementation might prove beneficial in critically ill humans including VLBW neonates [88]. A strong theoretical rationale for providing premature infants with supplemental glutamine at levels typically received in utero has been proposed [89-92]. The decreased bacterial translocation through mucosal surfaces, mechanisms of actions begin to be understood [93-98]. Tubman, however, argues that the available data from good quality, randomized controlled trials suggested that glutamine supplementation does not confer clinically significant benefits for preterms infants [99].

V.4. ***Probiotics*** are commonly defined as "viable bacteria that exhibit beneficial effects for health based on improvement of the balance of intestinal bacterial flora" [100].

These bacteria seem to function in immune regulation as well as via other mechanisms. Most of their effects have been studied on animal models [101, 102].

Specific anaerobes, classified as lactic acid bacteria (*Lactobacillus, Bifidobacterium*), may play a protective role in bacterial translocation via immunologic mechanisms. This role is promoted by fermentation that metabolizes varying quantities of lactic, acetic, and formic acids, vitamin synthesis, and production of antimicrobial bacteriocins and fatty acids [103]. Duffy summed up the potential benefits of anaerobic bacterial growth: 1) strengthening of the gut mucosal barrier function, 2) balance of microbial ecology, 3) adherence to intestinal mucosa and impeding invasive pathogens, 4) metabolism of dietary proteins and enzymes by the intestinal microflora, and 5) resilience of the epithelium to gut mucosal permeability [20].

Selected strains of *Lactobacillus* (*L. acidophilus* B62F04 and *L. casei* GG) exhibit adhesive properties to human intestinal cells. The mechanism of adhesion appears to involve a proteinaceous component that is species-specific for adherence in *Bifidobacterium* and *Lactobacillus*. Purified substances extracted from *B. infantis* cells could be administered to infants in an attempt to reduce the prevalence of atopic diseases [79].

V.4.1. *Genus Bifidobacterium*. Members of the genus *Bifidobacterium* are of particular interest because they are the numerically predominant bacteria during the first month of life in infants regardless of diet. Bifidobacteria form 60 to 91% of the total bacterial community in the feces of breast-fed babies and 28 to 75% of that in formula-fed infants [38]. Thus, these bacteria may play an important role in the ontogeny of the immune system associated with the gut mucosa. Young proposed that T-cell responses differ depending on the microbial signal delivered to the dendritic cells, the cytokine milieu, the antigenic dose and the dendritic-cell subset that has been activated (DC1 or DC2) [79]. *B. bifidum*, *B. longum*, and *B. pseudocatenulatum* activate dendritic cells to produce IL-10 and permit Th2 expansion and associated immunoglobuline E (IgE) responses to antigens. Th2 cells preferentially produce IL-4, which signals an antibody isotype switch to IgE production. IgE that is bound to mast cells is cross-linked by an allergen exposure. The mast cells then degranulate resulting in an inflammatory response. When this process occurs in the gut mucosa, gut permeability increases and systemic exposure to these antigens increases causing the development of atopic dermatitis. *B. infantis* fails to induce production of IL-10 by dendritic cells. Although the observed upregulation of CD18 expression indicates that dendritic cells were activated, the extent of activation is not sufficient to drive a Th1 or Th2 responses [79].

V.4.2. *Genus Lactobacillus*. Genomic analyses of lactic acid bacteria (LAB) have revealed a number of interesting features that are important for the roles of these microorganisms in health. Adherence/attachement factors, mucus-binding proteins, cell surface exopolysaccharide clusters, mannose-specific adhesion proteins, prophage-encoded proteins suspected of lysogenic conversion function, bacteriocins, two component regulatory systems and signalling pathways, stress and acid tolerance factors, and bile salt hydrolases have been identified in these LAB [77, 104]. For *Lactobacillus*, the protective layer function does exist not only on the GI tract mucosa, but also on all exterior body surfaces including the eye, the nose, the mouth, the respiratory tract, the vagina, and the skin.

V.5. *Prebiotics* describe the non-digestible food fiber components that contribute to host health by activating proliferation and function of beneficial intestinal bacteria [100].

Epidermal growth factor (EGF), which is present in breast milk, has both trophic and maturational effects on intestinal mucosa. Thus, EGF may provide protection for neonates from gut origin infection by decreasing the incidence of spontaneous bacterial translocation in the newborn [105].

V.6. *Synbiotic* are a combination of both probiotics and prebiotics nutritional supplements. Although the word synbiotics was originally coined to describe the combined action of pre- and probiotics, the term is now increasingly used in a wider sense to describe all of the substances released by microbial fermentation in the lower gut. Most of these substances appear to influence the immune system, increase resistance to disease, and, most importantly, prevent complications following surgery such as infections and thrombosis. Human breast milk is considered to be the best symbiotic product.

Gut microbiotic flora is significantly reduced in the sick, especially in connection with severe disease, care in the ICU, and little food intake or parenteral nutrition. Preterm neonates exhibit similar characteristics. Administration of both pre- and probiotics can modify appetite, sleep, mood, and circadian rhythm. This function is likely through the production of metabolites by microbial fermentation in the gut. Synbiotics are thus expected to improve the health of preterm neonates.

VI. CONCLUSIONS AND PERSPECTIVES

The intestinal ecosystem is characterized by dynamic and reciprocal interactions between the host and its microbiota. Although the importance of the gut microbiota for human health has been increasingly recognized, the early bacterial colonization in the neonatal gut is not yet completely understood. The mechanisms underlying these interactions are complex and influenced by many factors. The relative importance of these factors is difficult to organize into a hierarchy. A better knowledge of the microbiota and the impact of antibiotics will provide an essential step towards understanding the development of this important bacterial community.

Recent research in the area of probiotics, prebiotic oligosaccharides, and synbiotic combinations is leading to a more targeted development of functional food ingredients. Improved molecular techniques for analysis of the gut microflora and its development, increased understanding of metabolisms, and interaction between host and environment, and new manufacturing biotechnologies are facilitating the production of such food supplements. Thus, our increased understanding is fostering our ability to modulate the gastrointestinal microbiota for therapeutic outcomes.

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