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Colonization and Impact of Disease and Other Factors on Intestinal Microbiota

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Abstract The aim of this study was to review the process of microbial colonization and the environmental and host factors that influence colonization and microbial succession. The impact of some diseases on intestinal microbiota composition is also described. Microbial colonization of the gut by maternal vaginal and fecal bacteria begins during and after birth. During the first 2 years of life, specific microbes become established in a process designated microbial succession. Microbial succession in the gastrointestinal tract is influenced by numerous external and internal host-related factors, and by the second year of life, the intestinal microbiota composition is considered identical to that of adults. Nevertheless, intestinal microbiota in both infants and adults remain incompletely characterized and their diversity poorly defined. The main explanation is that many intestinal bacteria that live in an anaerobic environment are difficult or impossible to culture outside the intestine. However, recent advances in molecular biology techniques have initiated the description of new bacteria species. The composition of gut microbiota can be modulated by host, environmental, and bacterial factors, and strong evidence has emerged of substantial modifications during illness or exposure to threatening experiences. It has been postulated that improvements in hygienic measures have led to an increase in allergic diseases ("hygiene hypothesis"). Alterations in gut microbiota and their functions have been widely associated with many chronic and degenerative diseases, including inflammatory bowel disease, colon cancer, and rheumatoid arthritis.

Keywords Colonization · Inflammatory bowel disease · Intestine · Intestinal diseases · Microbiota

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Introduction

Fetuses are sterile in utero, and newborn infants are considered devoid of bacteria at birth [1]. However, low amounts of bacteria (5×10^3–5×10^4) are found before birth, acquired by rupture of fetal membranes [2]. Nevertheless, many researchers maintain that microbial colonization begins during and after the birth process, when the infant intestine is exposed to bacteria from the vagina and maternal intestine. At this stage, the dominant strains are facultative anaerobes such as enterobacteria and lactobacilli. Thereafter, other lactic acid bacteria become the predominant microorganisms in the gut microbiota, including bifidobacteria and coliforms [1, 3, 4]. Colonization is also determined by contact with the surrounding environment. After birth, environmental, oral, and cutaneous microbes from the mother are mechanically transferred to the newborn by various processes including suckling (breast milk contains up to 10^9 microbes/L in healthy mothers), kissing, and caressing. Moreover, neonates are continuously exposed to new microbes that enter the gastrointestinal tract with food.

Both adults and neonates are regularly exposed to food microorganisms but the effects differ. Microorganisms entering infants via milk are more likely to colonize than those entering healthy adults with stable bacterial communities. Thus, the factors regulating the fate of ingested microbes differ between infants and adults. Because the intestinal tract is a dynamic ecosystem influenced by host, intrinsic, and environmental factors, opportunities may arise that favor colonization by ingested microbes in infants. The present paper focuses on intestinal microbial colonization in early life, emphasizing factors regulating gut microbiota composition. It also considers the potential impact of disease, stress, hospitalization, and antibiotic therapy on intestinal microbial ecology in humans.

Microbial colonization

Colonization describes a bacterial population in the gastrointestinal tract that remains stable in size, with no need for periodic reintroduction of bacteria by oral doses or other means. This implies that colonizing bacteria multiply in a given intestinal niche at a rate that equals or exceeds their rate of washout or elimination from that site [1].

Normal colonization of the sterile newborn intestine is a complex process. Initial colonization is achieved by maternal vaginal and fecal bacteria. Bacteria usually start to appear in infant feces within a few hours after birth. The milieu in the infant intestine displays a positive redox potential at birth. The gastrointestinal tract is therefore first colonized by facultative anaerobes that lower the redox potential and thus permit growth of strict anaerobes, which normally appear in large numbers during the first week of life [5, 6]. Neonates are quickly colonized by facultative anaerobes (*Escherichia coli* and *Streptococcus*), reaching concentrations of 10^8 to 10^{10}/g of feces within 1–2 days; anaerobic microorganisms do not become established until the second month of life [7]. During the first week of life, the initial colonizers in both formula and breast-fed babies are enterobacteria and streptococci, which reach their highest numbers at the average age of 3.3 days [7]; by days 4–6, all babies born by vaginal delivery are colonized by anaerobic bacteria, whereas only 9% of those born by cesarean section have anaerobic bacteria [8]. By about day 10, all neonates display colonization by a heterogeneous bacterial microbiota; bifidobacteria predominate in breast-fed infants and a more complex microbiota is found in formula-fed children [1]. During the first month, bifidobacteria and *E coli* are the predominant bacteria, followed by *Lactobacillus*, *Bacteroides,* and Gram-positive bacteria, all in similar quantities. After about 1 year, *Lactobacillus, Bacteroides,* and clostridia numbers increase, while bifidobacteria and *E. coli* decrease; by this stage, the infant intestinal microbiota increasingly resembles that of the adult [9, 10], and the main bacterial groups isolated are *Staphylococcus aureus* (4%), *S. epidermidis* (20%), *Streptococcus fecalis* (30%), *Streptococcus faecium* [10], nonhemolytic streptococci [10], and Enterobacteriaceae (*E. coli* [20%], *Klebsiella aerogenes* [20%], *Proteus mirabilis* [2%], *Enterobacter cloacae* [1%], *Serratia* sp. [1%], and *Pseudomonas aeuroginosa* [0.5%]). Facultative anaerobe numbers are followed very closely by *Bifidobacterium* sp. Finally, in the second year, the intestinal microbiota is considered identical to that of adults, although facultative anaerobes are more abundant in children [9].

Specific microbes become established in particular hosts during different phases of development, in a process referred to as microbial succession. Microbial succession in the gastrointestinal tract is influenced by numerous external and internal host-related factors. Extrinsic factors include the microbial load of the immediate environment, food and feeding habits, antibiotic therapy, and composition of the maternal microbiota. Dietary and temperature stress also have an impact on microbial succession. Intrinsic or host-related factors influencing succession include host physiology, endogenous nutrients, and the microbiota.

Numerous factors govern microbiota stability and shifts (succession changes) in populations. These include intestinal pH, microbial interactions, environmental temperature, physiologic factors, peristalsis, bile acids, host secretions, immune responses, drug therapy, and bacterial mucosal receptors [1]. Both external and host factors control which ingested bacteria will be established in the intestine, and the order of succession of the colonizing strains is of major importance.

Diversity of gut microbiota: A challenge for microbiology research

The intestinal microbiota remains incompletely characterized and its diversity poorly defined [11]. The main explanation is that intestinal bacteria are adapted to an anaerobic environment, and many species that inhabit human gut are difficult or impossible to culture outside the intestine. Recent progress in molecular techniques has given rise to new tools for exploring and characterizing the human gut microbiota. Thus, studies based on 16S rRNA sequences have revealed the presence of a vast number of new species, the large majority of which cannot be cultivated [12–15], and 24%–49% of the human fecal microbiota remains unidentified [12, 16]. Although our knowledge of the composition of the intestinal microbiota has been improved by genetic techniques, only 1822 of the >20,000 rRNA gene sequences currently in GenBank have been documented as derived from the human gut, and 1689 represent uncultured bacteria [17]. Therefore,

further research is required to fully elucidate the gut commensal microbe composition.

Fluorescent in situ hybridization (FISH) studies of 16S rRNA showed a species diversity in adults of fewer than 20 dominant phylogenetic groups [12, 16, 18, 19]. In a study in five European countries, *Clostridium coccoides-Eubacterium rectale* probe (Erec 482) and *Clostridium leptum* probe (Clep 866) represented 28.0% and 25.2% of total bacteria, respectively, with large interindividual variations in bacterial composition. On the other hand, the distribution of major dominant groups in the microbiota appears to be comparable among countries studied [12].

Finally, fecal samples are often used to investigate intestinal microbes because of their easy collection. However, the degree to which the composition of fecal microbiota differs from that of mucosal microbiota has not been established. In a recent study of 13,355 prokaryotic ribosomal RNA gene sequences from multiple colonic mucosal sites and feces of healthy subjects, 62% of bacterial phylotypes were novel and 80% represented sequences from species that have not been cultivated [11]. Most of the inferred organisms were members of the *Firmicutes* and *Bacteroidete*s phyla [11, 20–22], with a wide interindividual variability of *Bacteroidetes* phylotypes [11, 20] and a low abundance of *Proteobacteria* sequences (including *Escherichia coli*) [11, 14, 20–22]. Phylotype richness estimations suggested that at least 500 phylotypes would be detected by means of continued sequencing [11]. Unfortunately, there have been no molecular studies of microbiota in children. Moreover, little is known about the composition of the microbiota at different sites of the gut in either infants or adults.

Factors regulating gut microbiota

Localization

Microorganism levels in the intestine are strongly dependent on the site of the sampling: the upper small intestine shows only low microorganism counts due to its proximity to the relatively sterile stomach, whereas the microbiota of the lower small intestine more closely resembles that of the colon. Bacterial counts also appear to vary among different sites in the large bowel. The highest bacterial counts are obtained from stool specimens [6].

Type of delivery

It is well established that the type of birth has a significant effect on the development of the intestinal microbiota. In vaginal delivery, colonization begins at parturition when the neonate swallows vaginal fluid [23]. Subsequently, due to the long birth process, there is a greater likelihood of isolating viable microbes from the infant's stomach and mouth that will later colonize the intestine [24]. In vaginally delivered neonates, bacteria begin to appear in the stool during day 1. These are initially mainly anaerobic but are followed within the first 5 days by *Bifidobacterium* sp. By day 10, most healthy full-term neonates display colonization by a heterogeneous microbiota.

In contrast, neonates delivered by cesarean section begin life in a bacteriologically clean state, and their first contact with bacteria is more fortuitous. Their microbiota is characterized by the lack of anaerobic bacteria, since they are generally colonized only by microaerophilic microorganisms, facultative anaerobes, and sporulated forms such as *Clostridium*. Although infants born by cesarean section are exposed to the maternal microbiota, initial exposure is more likely to involve environmental strains from equipment, air, and other infants, with nursing staff serving as vectors for transfer [6, 24]. In these cases, *Escherichia coli* and *Enterococcus* sp. are usually the first species to colonize the infant gut [6, 25, 26].

Diet

Diet may exert a major effect on the composition and activity of the gut microbiota. In the first stages of life, the type of diet (breast vs. formula feeding) markedly affects the colonization pattern [27, 28].

In breast-feeding babies, human milk promotes the growth of bifidobacteria and lactobacilli in the intestine, and frequent contact with nipple and milk ducts and the surrounding skin favors the growth of *Staphylococcus, Streptococcus, Corynebacterium, Lactobacillus, Micrococcus, Propionibacterium,* and *Bifidobacterium* species [29–33].

In contrast, the profile of formula-fed infants is much more complex and more similar to that of adults. *Bacteroides* is predominant among anaerobes and *Clostridium* species are abundant. The succession of organisms in the feces of formula-fed neonates is initially marked by higher levels of facultative anaerobes such as *Staphylococcus, Streptococcus,* and Enterobacteriaceae, while colonization by *Bifidobacterium* sp. generally begins several days later [3, 4, 6, 7, 34, 35].

At weaning, the microbiota radically changes and its composition starts to resemble that of adults. The introduction of solid food to breast-fed infants prompts a major disturbance in the microbial ecology of the large bowel, with a sharp rise in Enterobacteriaceae counts and colonization by *Bacteroides* sp. and clostridia [36]. This is not observed when formula-fed infants begin to take solids, when counts of facultative anaerobes remain high and colonization by anaerobes other than *Bifidobacteria* continues. It has also been reported that staphylococcal colonization rates remain

higher in breast-fed infants. This is probably due to prolonged contact with maternal skin during suckling [3, 6, 37].

Gestational age

The above-reported processes of colonization and bacterial succession at delivery refer to infants born at term, and the situation in preterm infants is different. Bacterial overgrowth by a limited number of species is more likely to occur in preterm infants, since they are colonized by few aerobic bacterial strains compared with full-term babies. This is probably because the preterm infant is cared for in a relatively aseptic environment and usually receives antibiotics shortly after birth, therefore displaying delayed colonization by a limited number of bacterial species. Finally, lactobacillus numbers are lower in preterm children, and this reduction has been correlated with previous antibiotic treatment and time spent in an incubator and with delayed colonization [38–42]. A few years ago, comparison between full-term and preterm infants showed a very similar number of coliforms and lactobacilli but a lower prevalence of colonization in the preterm group [43, 44].

Stages of life

During the various stages of a human life, the microbiota undergoes several modifications. The first step in the colonization process takes place before and/or during delivery and is characterized by a predominance of facultative anaerobes. Within a few days of birth, coliforms and streptococci become predominant. Obligate anaerobes appear some time later. Clostridia and lactobacilli may also be present in most hosts within a short period of time. These microorganisms are later associated with anaerobic bacteria such as *Bifidobacterium, Bacteroides, Eubacterium, Veillonella,* and *Clostridium*, depending on the origin of the milk (breast or formula).

Fecal bifidobacteria counts in 1- to 19-week-old formula-fed infants are reportedly comparable to those of age-matched breast-fed infants, even though counts may be higher in breast-fed infants up to 5–6 weeks old. *Bacteroides* counts are low in 1-week-old breast-fed and formula-fed infants but increase thereafter in the latter. Between 5 and 24 months of age, as the infant is progressively introduced to a variety of foods, differences between breast-fed and formula-fed infants are lost and fecal microbiota reach adult levels, although bacterial profiles suggest that almost all infants under 2 years old still differ from adults in having more bifidobacteria than *Bacteroides*. After 2 years of age, when the microbiota is practically identical to that of adults, *Bacteroides, Bifidobacterium, Eubacterium, Clostridium, Peptococcus, Peptostreptococcus*, and *Ruminococcus* are the predominant species. Finally, *Bifidobacterium* counts progressively decrease with aging [1, 7, 36, 37, 47–49].

There is some evidence of the possible additional acquisition of microbiota early in life, but once it has been established and immune tolerance is achieved, the intestinal microbiota remains relatively constant throughout life. The gut immune system learns to recognize and tolerate bacterial species that were acquired during early infancy. Consequently, a permanent change in gastrointestinal microbiota is very unlikely after this time [50].

Bacterial modulation (self-modulation)

Autochthonous bacteria, also known as indigenous bacteria, are ubiquitous microbes in the gastrointestinal ecosystem that occupy all available habitats and niches. To achieve this, gut bacteria produce substances that stimulate or inhibit the growth of other commensal or allochthonous species, i.e., microorganisms that are merely passing through the gut and are derived from food and water or from elsewhere in the host [1]. Pioneer bacteria can also modulate the expression of genes in host epithelial cells, thereby creating a favorable habitat for themselves, and can prevent growth of other bacteria introduced later into the ecosystem. Initial colonization is therefore very relevant to the final composition of the permanent microbiota in adults [51].

The preservation of gut microbiota in animals is necessary to prevent infections. It has been reported that germ-free animals are more susceptible to infection compared with animals colonized with microbiota, and that the oral administration of fecal suspensions to animals may prevent infection. All of this information has led to the concept that a healthy or balanced microbiota is necessary for maintaining the health of the host [52].

Impact of disease on intestinal microbiota composition

There is powerful evidence that alterations in the gut microbiota and its functions are contributing factors in many chronic and degenerative diseases. The following entities have been associated with changes in the gut microbiota.

Allergy

According to the hygiene hypothesis, environmental changes in the perinatal period have led to reduced microbial contact at an early age, resulting in a growing epidemic of atopic diseases, mainly in communities with high acquisitive power [53]. The immunological interpretation of this hypothesis suggests that if this cause-and-effect relationship exists, it may operate by increasing the predisposition to

atopy, perhaps by inhibiting the normal balancing of the immune response. An imbalance in T helper-type responses or in regulatory mechanisms as a result of the lack of microbial stimulation has been proposed as an underlying mechanism of the hygiene hypothesis. However, a specific infectious factor responsible for the hygiene hypothesis has yet to be established [54].

Recent advances in epidemiology and immunology demonstrate, however, that the hygiene hypothesis may need to be extended in three aspects. First, the importance of infections in causing immune deviation may be outweighed by other sources of microbial stimulation, perhaps most importantly by the indigenous intestinal microbiota. Second, immunomodulatory and suppressive immune responses complement the T_H1/T_H2 paradigm. Third, in addition to protection against atopy, protection against infectious, inflammatory, and autoimmune diseases may also depend on healthy host-microbe interactions implicated in the hygiene hypothesis [55].

Despite the above, allergic infants have been reported to show differences in gut microbiota and the main differences are as follows.

(a) The gut microbial pattern of infants with a high risk of allergy differs between infants who develop atopy at 12 months of age and those who have not been atopic. At 3 months of age, clostridia counts in stools are higher and bifidobacteria counts tend to be lower [56].
(b) Allergic children at 2 years are less colonized with lactobacilli, bifidobacteria, and *Bacteroides* and have high counts of aerobic microorganisms, particularly coliforms and *Staphylococcus aureus* [57].
(c) Levels of the adult type of *Bifidobacterium adolescentis* are higher in allergic than in healthy infants, who show greater numbers of *Bifidobacterium bifidum* [58].

Unfortunately, none of these studies used newly available techniques to identify uncultivable bacteria.

Inflammatory bowel disease (IBD)

IBDs, usually referred to as Crohn's disease (CD) and ulcerative colitis (UC), are chronic disorders with many similarities in pathogenic mechanism and clinical course that lack an etiological therapy. Both entities are mainly located in areas with high bacterial concentrations, such as the terminal ileum and cecum in CD and the rectum in UC [59]. Although a long search has failed to confirm a direct pathogenic role for a specific infectious agent, there is convincing evidence that commensal enteric bacteria and their products may provide a local environmental trigger that initiates and perpetuates IBD. Most of this evidence derives from animal studies, but several modifications of the intestinal microbiota have been reported in patients with IBD, including high concentrations of mucosal bacteria in both inflamed and noninflamed bowel tissue [60, 61], increased numbers of coliforms and bacteroids and a decrease in lactic acid bacteria [62]; increased *E. coli* [61, 63] and lactobacilli numbers in the colon [64], and a significant increase in *E coli* and *Bacteroides fragilis* in the ileum [65]. A higher bacterial load in the intestinal mucus of IBD patients has also been reported [66].

Finally, some modified *E. coli* strains have been reported in patients with UC and CD. Likewise, patients with IBD have larger amounts of bacteria attached to their epithelial surfaces compared with healthy individuals. Isolated bacteria belong to a wide range of genera; some are identified within the epithelial layer, while others are found at intracellular locations. Nevertheless, the role of these altered bacteria in IBD pathogenesis remains unclear [5, 60].

Obesity

One of the main metabolic functions of colonic microbiota is the digestion of metabolic substrates [10]. Major sources of nutrients are the upper intestinal tract and the colon itself. The available substrates include dietary fibers, starches, oligosaccharides, sugars, lipids, proteins, endogenous mucins, sloughed epithelial and enterocyte tissues, bacterial debris, bile acid, and cholesterol [7, 47]. The main products of bacterial metabolism are short-chain fatty acids (SCFAs). SCFAs produced by colonic microbiota include acetic, propionic, and butyric acids. Acetic and propionic acids are quickly absorbed and are a major source of energy for the host.

Taking into account that the typical European diet contains about 50 to 60 g of carbohydrates, the theoretical production of SCFA ranges from 0.5 to 0.6 mol of SCFAs with an energy value of 140 to 180 calories, and SCFA absorption represents nearly 10% of the daily energetic requirements [10, 67]. For this reason, a difference has been postulated between the microbiota of obese and that of nonobese individuals. Although this point has not been proven in humans, animals raised without exposure to microorganisms (gnotobiotic animals) were reported to have 40% less corporal fat compared with animals that acquired their microbiota from birth. The lower weight of gnotobiotic animals, despite their consumption of a larger amount of the same food [68, 69], can be explained by the ability of the gut microbiota to salvage energy from dietary polysaccharides.

The gut microbiota of leptin gene mutated mice (ob/ob) was recently reported to differ from that of their mothers or nonaffected brothers or sisters. The main differences were a reduction in *Bacteroidetes* and a proportional increase in *Formicutes*. Gut species differ between mice and humans but are similar at the division level (superkingdom or deep evolutionary lineage), therefore it is reasonable to postulate that a similar phenomenon may occur in humans [17].

Colon cancer

Dietary fat and a high consumption of red meat are associated with a high risk of colon cancer [70], whereas a high intake of fish, vegetables, fruit, some cereals, and calcium reduces this risk [71]. Although the evidence is not conclusive, the gut microbiota appears to be an environmental factor that may increase the risk of cancer. It has been suggested that the effect of diet on carcinogenic processes may be mediated by changes in gut microbiota, which in turn may produce procarcinogens, carcinogens and cocarcinogens [47].

Rheumatoid arthritis

Both genetic and environmental factors appear to be associated with the etiology of rheumatoid arthritis. Infections and microbes have been widely proposed as potential environmental factors [72]. Infections due to *Yersinia*, *Salmonella,* and *Shigella* have been the most frequently implicated as triggers of reactive arthritis [73], and the degradation products of these pathogens have been isolated in patients' synovial tissues [74, 75]. These findings have raised the possibility of a similar phenomenon implicating bacteria normally harbored by the gastrointestinal tract, leading to synovial inflammation in genetically susceptible subjects. Supporting evidence for this theory includes the following observations: degradation products of bacterial cell walls are normally found within circulating blood cells and may also end up in joint tissue; cell walls of several bacterial species representing normal human intestinal microbiota are arthritogenic in animal models; and patients with early rheumatoid arthritis were found to have a different intestinal microbiota from that of control patients [73].

The main differences in gut microbiota between subjects with and those without arthritis rheumatoid are in the amount of fecal anaerobic and facultative anaerobic microorganisms. The agent most frequently implicated in these differences is *Proteus mirabilis* [76, 77].

Other pathologic conditions

There is a small but growing body of evidence associating intestinal microbiota changes and related phenomena with several pathologic conditions.

Impact of hospitalization on gut microbiota

Hospitalization can prompt changes in the normal intestinal microbiota: it modifies bacterial resistance to antimicrobial agents, and changes in bacterial species have also been reported. It appears that the impact of hospitalization alone, with no antibiotic treatment, produces alterations in the normal microbiota [6].

Recent studies monitoring microbiota development in vaginally born infants and in infants born by cesarean section showed major differences between them in cultivable microbiota. In hospitalized neonates born by cesarean section, intestinal colonization by lactobacilli and bifidobacteria is delayed by 10 days and 1 month, respectively, in comparison with vaginal births. In these cases, the main species are *Klebsiella, Proteus, Pseudomona,* and *E. coli*. Similar delays are seen with the early use of antibiotics or sterile environments such as incubators [6, 78].

Effect of stress

It has been proposed that psychological stress has profound effects on intestinal microbiota in animals and humans [79, 80]. These changes have been attributed, among other factors, to a catecholamine bacterial exposure that produces a significant decrease in lactobacilli and bifidobacteria and an inverse effect on potentially pathogenic bacteria such as *E. coli*, *Yersinia enterocolitica, Bacteroides* sp., and *Pseudomonas aeruginosa* [81, 82]. It has also been reported that norepinephrine increases the growth and expression of virulence factors in some *E. coli* strains [80]. Recent experiments found that *E coli* produce a growth hormone-like peptide that induces its own growth and stimulates in vitro the growth of 12 other Gram-negative microorganisms [84].

Antibiotics

Antibiotics are very commonly used to treat a specific illness without considering their impact on gut microbiota. Most studies of normal microbiota and antibiotics have been done in volunteers or in patients undergoing decontamination procedures for intestinal surgery. These have reported a decrease in anaerobes, aerobes, and Gram-negative bacilli and an increase in yeast and *E. coli* [7]. One of the most dangerous complications associated with antibiotic use is overgrowth of *Clostridium difficile*. This bacterium produces diarrhea and pseudomembranous colitis, entities associated with outbreaks with a high rate of mortality in hospitalized patients [85–88].

To summarize, colonization of the infant gut is a complex process initiated by maternal bacteria; strict anaerobes appear during the first week of life followed by rapid colonization of the large bowel by facultative anaerobes. Intestinal colonization and succession are influenced by extrinsic factors, e.g., composition of maternal microbiota, food, and antibiotic therapy, and by host factors. Despite advances in our knowledge of intestinal ecology in recent years, the intestinal microbiota remains incompletely characterized because many species that inhabit the human gut cannot be cultivated. The introduction of new molecular and genetic techniques will yield information on the commensal microbial

composition in the gut of both infants and adults, allowing analysis of its influence on health status. There is strong evidence in support of the hygiene hypothesis, which claims that a reduced contact with environmental microorganisms in early life leads to a higher incidence of atopic diseases. In addition, a number of diseases (e.g., IBD, colon cancer, obesity, and rheumatoid arthritis) and conditions (e.g., stress and hospitalization) have a negative impact on intestinal ecology. The application of new genetic tools should improve our understanding of microbiota changes in these diseases and allow the development of alternatives to classical therapies.

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