

Intestinal Microbiota in the Control of Pathogens

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The intestinal mucosa contains innumerable species of commensal microorganisms, at equilibrium end up preventing the persistence of or even eliminating pathogenic microorganisms. The manner by which the intestinal microbiota eliminates pathogenic microorganisms has been extensively studied by diverse researchers throughout the world. This is mainly due to the fact that the indiscriminate use of antibiotics has given rise to a global problem of great importance to public health. Consequently, diverse products that constitute alternatives to the use of antibiotics, such as probiotics, which act against pathogens. Currently, probiotics are being utilized commercially on a large scale in the production of animal products, and to a lesser degree in such human foods. We know of the existence of many other bacteria present in normal intestinal microbiota that are not known. Exact knowledge on how these microorganisms interact is the objective of researchers throughout the world, specifically by understanding the true function of all intestinal microbiota, thereby gaining the ability to combat intestinal pathogens in a safe and healthy manner in relation to public health.

Keywords microbiota; probiotic; microorganisms; public health; molecular biology.

1. Introduction

Intestinal mucosa, the greatest contact surface that we have with the external environment, represents an extensive gateway for diverse pathogenic microorganisms. This mucosa contains innumerable species of commensal microorganisms that are formed until a few weeks after birth and, at equilibrium, exercise diverse activities in the intestinal lumen and mucosa, and somehow end up preventing the persistence of or even eliminating other pathogenic microorganisms. The manner by which the intestinal microbiota eliminates pathogenic microorganisms has been extensively studied by diverse researchers throughout the world. This is mainly due to the fact that the indiscriminate use of antibiotics has given rise to a global problem of great importance to public health.

Currently, we know that this problem of bacterial resistance via misuse of antibiotics involves not only the use of underdoses, but also overdoses that end up selecting the bacteria (commensal or pathogenic) already resistant to the antibiotic utilized, in the intestine, thus becoming a source for the dissemination of genes conferring resistance to other bacteria that come into contact with these resistant microorganisms. Bacterial resistance to antimicrobials can also occur on account of dissemination of resistant bacteria originating from animals or from antimicrobial residues present in products of animal origin. Due to this threat, the European Union currently bans the use of antimicrobials in animal production, which has led other countries interested in the global agribusiness market to update their practices and adapt to international norms. Consequently, diverse products that constitute alternatives to the use of antibiotics, such as probiotics, which are microorganisms from healthy intestinal microbiota, have been identified and tested for their ability to inhibit pathogenic bacteria. Such probiotics act against pathogens, stimulating the immune system in a general manner (by augmenting immunoglobulins, defense cells, interleucins,...), diminishing intestinal pH by producing organic acids, bactericidal substances such as peroxides, the acids themselves, bacteriocins, or competing for adhesion sites in enterocytes or for nutrients. Currently, probiotics are being utilized commercially on a large scale in the production of animal products, and to a lesser degree in such human foods as yogurts, fermented milk, etc. Research studies have shown that the entire inhibition mechanism of probiotic bacteria is highly complex and that there are still many factors that influence bacterial inhibition by other bacteria. One example of this is the fact that many bacteria coexist without being inhibited by others. Given current technological advancements, we know of the existence of many other bacteria present in normal intestinal microbiota that are known despite current lack of precise knowledge about the requirements for creating a culture medium for their isolation. But the use of molecular techniques such as *Temperature Gradient Gel Electrophoresis*, which permits visualization of many of these bacterial groups that cannot be cultured, that represent about 80% of the bacteria present in the intestine, we can elucidate the interactivity among all these intestinal microorganisms, some of which exist only due to the presence of others.

Exact knowledge on how these microorganisms interact is the objective of researchers throughout the world, specifically by understanding the true function of all intestinal microbiota, thereby gaining the ability to combat intestinal pathogens in a safe and healthy manner in relation to public health. Although much is known about this subject, there are innumerable gaps in the quest to ascertain the complete action mechanism of such intestinal microbiota. Although the future prospects of these bacteria for inhibiting intestinal pathogens are promising, we remain far from achieving a complete understanding.

2. Intestinal Mucosa

The largest surface of contact with the external environment is the intestinal mucosa, comprising an area up to 100 times larger than that of the skin. Therefore, it is a surface exposed to exogenous antigens of greater importance to the organism, representing, therefore, the principle route of entry for the majority of pathogens [1; 2].

Intestinal Mucosa: There are many species of adherent or free microorganisms that form the normal microbiota. It is believed that these microorganisms, present in a density of approximately 10^{14} microorganisms, represent from 500 to 1000 different species, with their number being 10 times greater than the number of cells that form our organs and tissues [3; 4]. The gastrointestinal tract is a complex ecosystem that can be both a reservoir of beneficial and pathogenic bacteria [5]. However, once established, normal intestinal microbiota serves as a protective barrier for the host against colonization by pathogenic agents [6].

3. Intestinal Microbiota

Prominent among the great diversity of microorganisms found in the intestinal mucosa are such genera as *Bifidobacterium*, *Lactobacillus*, *Eubacterium*, *Clostridium*, *Peptococcus*, *Ruminococcus* [7], among others, that to be predominant need to adhere to mucosa receptors denominated adhesion sites of the intestinal mucosa [8]. The bacteria with this capacity permanently remain in the intestinal mucosa without requiring periodic reintroduction are known as autochthone biota. Another biota present in the intestine, transitory and external to the intestinal ecosystem but without adhesion capacity to intestinal mucosa, are allochthone bacteria [9]. Clinical and experimental studies of these bacteria have demonstrated their interaction with the host that favors health through various mechanisms. The intestinal bacterial community can act favorably due to antibacterial, immunomodulatory and / or metabolic-nutritional action (Table 1) [10]. The events that occur principally in the first three weeks of life [11] are of particular importance to the development of a microbial community capable of conferring an immune response [12]. The establishment and maintenance of intestinal microbiota constitute a complex process, being influenced by diet, age, use of antibiotics, use of probiotics, environment, maternal microbiota, route of birth, microbial interactions, microorganism-host interactions and the presence of certain genes and receptors [13; 14; 15]. According to Nicoli [16], this microbiota forms an immensely complex ecosystem, which can be compared to a functional entity or to an “organ” within the host.

Table 1 Principle functions attributed to intestinal microflora.

Function	Mechanism
Antibacterial	Competition for adhesion sites
	Competition for nutrients
	Production of a physiologically restricted environment
	Production of antimicrobial substances
Immunomodulator	Stimulation for the immune system
	Development of immunological tolerance
	Energy savings
	Colonocyte nutrition
Nutritional/metabolic	Conversion of cholesterol into coprostanol
	Conversion of bilirubin into urobilin
	Inactivation of trypsin
	Synthesis of vitamin K

Tannock GW, 1999.

Among the many other observable benefits of intestinal microbiota are the promotion of lactose digestion, relief of constipation, increase of mineral absorption and production of vitamins. Although still unproven, other possible effects include: diminutions in the risks of colon cancer and cardiovascular disease, diminishment of cholesterol, anti-hypertensive effects, reduction of *Helicobacter pylori* ulcerative activity, control of *Clostridium difficile*- and rotavirus-induced colitis and the prevention of urogenital infection, besides inhibitory effects on mutagenicity [17; 18; 19; 20; 21; 22]. According to Bravao et al. (2011), probiotics are also related to behavior as shown by improved well-being and the diminution of stress and anxiety [23].

3.1. Protection against pathogens

Resistance to colonization is the principal mechanism exercised by intestinal microflora in controlling pathogens. It occurs in two “habitats” of the intestinal tract: the mucosal surfaces and luminal contents. On mucosal surfaces occurs

the occupation of the adhesion sites utilized by pathogenic agents, thereby impeding their binding to specific receptors [12]. In luminal contents the pathogens are inhibited through the production of antibacterial substances produced by this microbiota, which not only reduce the number of viable cells of the microorganism, but also affect the bacterial metabolism or the production of toxins [24; 25]. These substances may be organic acids, hydrogen peroxide, bacteriocins, antibiotics and unconjugated biliary acids [26]. In addition to competition for the adhesion sites in intestinal mucosa with pathogens, the microbiota also competes for available nutrients [26]. In addition, the immune system is frequently stimulated through the action of intestinal microbiota, being highly important in the inhibition of pathogenic bacteria in the intestine. By interacting with intestinal epithelial cells of the host, this microbiota provokes a continuous response of the immune system [27]. An interaction between the immune system and commensal microbiota is initiated immediately after birth and leads to a low-grade inflammation characterized by elevated expression of interleukin 8. This results in infiltration of heterophils and lymphocytes in the *lamina propria* of the intestinal epithelium and normalization of the local immune system [28]. These cells may develop later, depending on the composition of the resident microbiota, in different cellular lineages with receptors that are also variable [29]. This response generally causes the immune system to respond with production composed primarily of immunoglobulins such as IgA, activation of T and B lymphocytes and defense cells, thus augmenting phagocytic activity [30; 31; 32]. M cells of lymphoid tissue associated with the intestine have the capacity to actively engulf particles ranging from small proteins to bacteria and protozoa [33]. The B lymphocytes that reside in the *lamina propria* produce secretory IgA [34] by the intermediation of helper T lymphocytes, which supply two distinct signs: the first, involving direct contact between B and T cells and the second, mediated by cytokines [35]. Cytokines are proteins or peptides that perform relevant control of the immune system. They determine the type and degree of the immune response generated after a vaccination or infection. Depending on the combination of cytokines produced, an immune response can be mediated by antibodies or by cells [36]. In the intestine, the production and secretion of IgA depends on mechanisms that are involved since the sampling of antigens by M cells, processing by antigen-presenting cells, activation of T and B cells on Peyer plates, until the action of multiple cytokines, including IL-4, TGF- β , IL-5, IL-6 and IL-10 [37]. The immune system of mucosa, together with the local lymphoid nodules, form the lymphoid tissue associated with the mucosa that is quantitatively the most important organ of humoral immunity [38]. The mediation of adaptive immunological defense at these sites is initiated for the activation of lymphocytes and local secretion of IgA, which acts in the process of immune exclusion, interfering in the establishment and colonization of pathogens [39; 40]. Immunomodulation is a function that the microbiota exerts in the host which consists of immunostimulation against possible microbial aggressors and immune-acceptance of its own normal microbiota present in the intestine [41].

3.2. Probiotics

Probiotics are previously identified live microorganisms, belonging to normal intestinal microbiota [32] that, when ingested in adequate quantities, produce a beneficial health effect, as well as benefits to basic nutrition [42; 43]. The increase in the use of bacterially resistant antibiotics in immunosuppressed patients, of opportunistic infections and the appearance of new pathogens, are factors that determine the necessity of developing new strategies in the treatment and prevention of infections. Due to the characteristics of probiotics, it has been suggested that the microorganisms that compose them can be useful in infectious processes [44]. Probiotics must be viable in different microenvironments of the intestinal tract, in order to perform their protective function [45]. The utilization of probiotic cultures stimulates the multiplication of beneficial bacteria to the detriment of the proliferation of potentially harmful bacteria, reinforcing the natural defense mechanisms of the host [46]. A healthy microbiota is defined as a normal microbiota that conserves and promotes well-being and the absence of diseases, especially in the gastrointestinal tract. The correction of the properties of unbalanced autochthonous microbiota constitutes the rationale of probiotic therapy [47]. It is possible to augment the number of microorganisms that promote the health of the gastrointestinal tract by introducing probiotics via the diet, which would selectively modify the composition of microbiota, favoring the competition against other bacteria of the ecosystem [48]. In humans the genus *Lactobacillus* is the dominant or subdominant population in the small intestine, as is the genus *Bifidobacterium* in the colon [49]. Studies *in vivo* and *in vitro* indicate that *Lactobacillus reuteri* influences the cellular and humoral immune system by stimulating the production of cytokines and of immunocompetent cells, thus increasing the production of antibodies [50]. There is substantial evidence that *Lactobacillus reuteri* influences the expression of cytokines in intestinal mucosa [51].

The immune response of the host encompasses non-specific and specific, or adaptive, defenses, that are active solely against the pathogen that stimulates the response, and whose development requires several days [52]. Strains of *Lactobacillus* induce the formation of a more effective mucosal barrier with a certain degree of specificity [53; 54]. The safety of the bacterial strains utilized as probiotics is a very important question. The employment of *Lactobacillus* spp. and *Bifidobacterium* spp. does not cause concern due to the fact that these bacteria are normally present in a healthy gastrointestinal tract, with no damage involving these microorganisms being reported [55]. The *Lactobacillus* spp. are commensal bacteria predominant in the gastrointestinal tract of animals [56], representing the principle group of lactic-acid bacteria that have been widely studied and utilized as probiotics in recent years [57]. The capacity of *Lactobacillus* to inhibit and prevent intestinal colonization by enteropathogens was already observed in different studies. Santini et al. (2010) [58] verified, via *in vitro* tests, the inhibition of *Campylobacter jejuni* by *Lactobacillus plantarum*. Similarly,

Murry et al. (2004) [59] demonstrated that *L. plantarum* and *L. salivarius* can reduce infections by *Escherichia coli*, *Salmonella* Typhimurium and *Clostridium perfringens*, as a consequence of a high lactic-acid concentration produced and available in the microenvironment.

The adhesion of probiotic strains to enterocytes establishes and prolongs the intestinal residence of these microorganisms [60], contributing to the performance and efficacy of probiotic samples [61], thus ensuring the colonization of mucosal surfaces of the intestinal epithelium, though transiently, to ensure interaction with immune system cells of the host to inhibit intestinal colonization by pathogens [62]. The use of underdoses of antibiotics in animal feed, so-called growth promoters, is a widespread practice since it raises zoo-economic indices [63]. According to Cromwell (1991) [64] antimicrobial agents are compounds that, at low concentrations, reduce or inhibit the growth of microorganisms. This class of compounds includes antibiotics produced naturally by yeast, fungi and other microorganisms as well as chemically synthesized chemotherapies. The constant exposure of animals to these products can lead to the selection of a resistant biota. Starting in the 1980s, researchers began to note that determinate bacterial strains had become resistant to the antibiotics utilized in birds and that the continued use of antimicrobial growth promoters, was serving to expand the “pool” of genes of resistance in nature. The great concern is that resistant bacteria in production animals may contribute to a resistance to antibiotics in humans [65]. It is known that resistance genes are easily transferred to antimicrobials among enterobacteria. For example, *in vitro*, *Salmonella* Enteritidis can become resistant to determinate drugs employed against it, simply by concomitant culturing with another bacterium known to be resistant [66].

The problem of antimicrobial resistance is very serious and presents many clinical implications. Therefore, one of the greatest challenges in the area of animal production is to locate alternatives that would reduce the use of antimicrobials as growth promoters in rations. This challenge is a consequence of the growing pressure imposed by laws in countries that import products of animal origin, such as in the European Community, which prohibits the inclusion of antimicrobials in the diets of broiler chickens and other animal species [67]. Since the discovery of antibiotics, doubts have arisen about the true safety of their use as growth promoters. The principle doubt concerns the use of antibiotics in animal diets contributing to resistance of enteric bacteria capable of transferring their resistance to pathogenic bacteria (*Salmonellas*, *Campilobacter*, *Clostridium perfringens*, etc.), thereby posing a risk to public health [68; 69]. In this context, many research studies have aimed to develop alternatives whose action mechanisms would not be directed toward eliminating biotas and the consequent appearance of resistance, but rather toward competitive action that would favor the multiplication of microorganismos that produce antimicrobial substances capable of adhering to intestinal mucosa and impeding the fixation of enteropathogenic bacteria [70]. In recent years in the animal production industry, the employment of probiotics containing *Lactobacillus* has increased greatly [66] due to its proven efficiency in preventing diseases and weight gain, in improving feed conversion and in diminishing mortality in production animals [71]. *Lactobacillus* are commensal bacteria of the animal gastrointestinal tract that represents the principle group of bacteria that has been widely studied and utilized as a probiótico in recent years [57]. The inhibition potential of *Lactobacillus* can be observed in many animal studies, both *in vitro* and *in vivo*. The inhibition against *Salmonella*, an agent that causes gastroenteritis in humans, is well proven (Figure 1) [72; 73; 74], as well as against other pathogenic bacteria. And the benefits that accompany the improvement of performance in animal production are also well defined, despite the continuation of many gaps that still need to be filled in relation to the true action of these bacteria [30; 75].

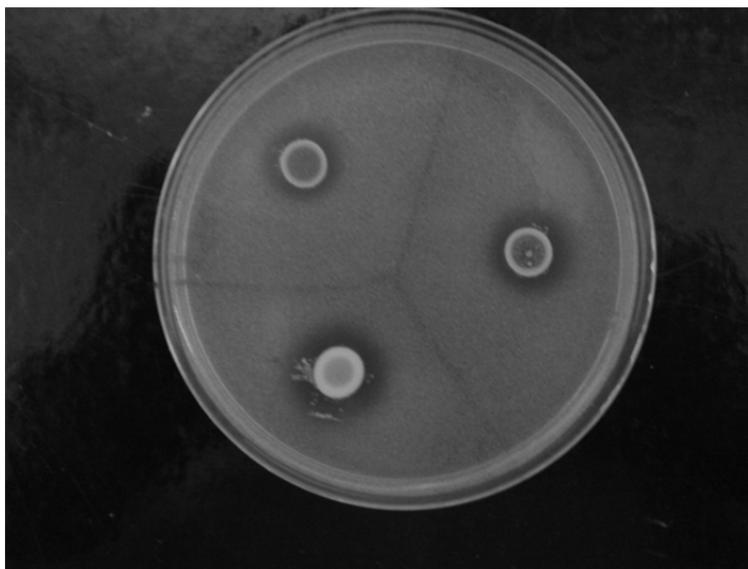


Fig. 1 Petri dish containing nutrient agar highlighting the inhibition halo of *Salmonella* Enteritidis, at spots cultivated with *Lactobacillus* spp. Modified antagonistic method “spot on the lawn” [76].

The potential of normal intestinal bacteria, such as probiotics, has also been widely researched in the human food industry, performing beyond production and preservation of some foods to include modulation of normal intestinal microbiota, acting against diverse enteropathogenic agents [77; 78; 79; 80]. The development of lactic products containing probiotic bacteria is an important general focus of the food industry, namely the technological challenge posed by the production of foods containing specific probiotic strains with suitable concentrations of viable cells during their shelf life [81; 82]. Shirota, in Japan in about 1930, focused his research on the selection of strains of intestinal bacteria that could survive the passage through the intestine and on the use of these strains to develop fermented milks for distribution in his clinic [83]. Among the various types of food products, yogurts and similar products have become the most popular vehicles for incorporating microorganisms of normal intestinal microbiota [84]. Application of bacteria from the *Lactobacillus casei* group into new types of yogurts has increased progressively [85]. Although fermented lactic products constitute a substantial market share of prebiotics and probiotics, the number of non-lactic products is growing, particularly those based on soy [86; 87; 88]. The mechanisms by which normal intestinal microbiota exert their beneficial effects on hosts remain largely unknown [89]. Previous studies have predominantly employed culturing methods to identify the components of resident microbiota, thereby leaving a large number of bacteria to be identified due to lack of knowledge about appropriate culture media [90].

Biochemical assays were the primary method utilized to identify microorganisms, but require time, skill and rectified technical standards [91]. Furthermore, it is impossible to identify new bacterial species and strains by employing known commercial media [92]. There is still a lack of detailed studies on microbiota composition [93]. Until very recently, the identification methods have utilized this microbiota with culture media [94], but they include only culturable bacteria of the microbiota [95]. It was stipulated that only 60% of intestinal bacteria can be detected by culturing. Therefore, a large number of bacteria remain unidentified due to a lack of knowledge on appropriate culture media [93]. Research data suggest that normal intestinal microbiota contain innumerable species [96] belonging to diverse genera of which only 10% are known [97]. Molecular techniques have been employed to investigate intestinal microbiota by staining bacterial DNA and thus generating a more detailed vision of the subject [98]. These methods demonstrate greater sensitivity and specificity for detection, essentially because bacterial DNA remain practically unaltered throughout their life cycle, even when exposed to stress [99]. For some years, the sequencing of ribosomal 16S rDNA has been utilized as a principle tool due to its specificity that permits the detection and identification of bacteria [99]. During the last decade there has been notable progress in the use of methods based on 16S rDNA with the aim of determining the diversity of the bacterial population [100], thus detecting a much greater diversity in the bacterial population than that achieved by other culturing methods [101]. An analysis of clones originated from PCR of 16S rDNA demonstrated that normal intestinal microbiota is highly diverse and complex [93]. These methods are based on the behavior of specific denaturing of fragments generated by PCR of 16S rDNA, including the method *temperature gradient gel electrophoresis* (TGGE). The TGGE technique was developed specifically for the analysis of microbial communities in a rapid and accurate manner [102], since it does not require prior knowledge about the constituent organisms composing the microbial population to be studied. This method is highly specific in separating mixed DNA fragments of the same size that differ by merely one single base pair, and is also sufficiently sensitive to detect bacteria that represent a small percentage of the total population [103]. But although some bacterial species are probably inhabitants common to many animal species, such as *Bifidobacterium*, *Pseudomonas* and *Lactobacillus*, at least part of the bacterial population of the enteric tract of different animal species may be unique [104]. The roles of microorganisms are critical in the interactions between host and diet, which makes a more comprehensive vision of normal intestinal microbiota essential to enable their manipulation [105] and development, for example, of products that more effectively promote the health of the host [106]. More studies are needed to confirm the relationship between microbiota and gastrointestinal diseases, an aim whose fundamental goal is to ascertain why the bacterial population changes as a consequence of disease or vice-versa [92].

A detailed understanding of these responses can permit the modification of intestinal microbiota [12] through commercial products, to favor the health and well-being of the host. Healthy microecologically equilibrated intestinal microbiota results in normal performance of physiological functions of the host, which would ensure improvement in the quality of life of the individual. This result is of crucially important, particularly in the current context of exponential growth in life expectancy. The direct role of probiotic microorganisms and indirect role of prebiotic ingredients, in the sense of providing, in the field of preventive nutrition, this healthy equilibrated intestinal microbiota to the host, are already well established. The effect of probiotic microorganisms and of prebiotic ingredients can originate, through their association, symbiotic functional foods. Only a small fraction of the mechanisms for the occurrence of probiotic and prebiotic effects has been elucidated. Nevertheless, studies in this research line are increasing in intensity. Better comprehension of the interaction between non-digestible vegetal compounds, their intestinal metabolites, the intestinal microbiota and the host would open up new possibilities to produce new ingredients for food products nutritionally optimized to promote the health of the host through microbial reactions in the intestine [107].

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