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***REVIEW ARTICLE***

# PROBIOTICS – IDENTIFICATION AND WAYS OF ACTION

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**Abstract**

Probiotic bacteria are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. While this beneficial effect was originally thought to stem from improvements in the intestinal microbial balance, there is now substantial evidence that probiotics can also provide benefits by modulating immune functions. In animal models, probiotic supplementation is able to provide protection from spontaneous and chemically induced colitis by down-regulating inflammatory cytokines or inducing regulatory mechanisms in a strain-specific manner. In animal models of allergen sensitization and murine models of asthma and allergic rhinitis, orally administered probiotics can strain-dependently decrease allergen-specific IgE production, in part by modulating systemic cytokine production. Understanding the how probiotic bacteria exert their beneficial effect is crucial for the establishment of definitive selection criteria.

**Keywords:** Probiotics; Mechanisms of action

## Introduction

The term probiotic (opposite of antibiotics) is relatively new and is currently used when we refer to bacteria associated with beneficial effects on humans and animals. It was invented in the early twentieth century by Nobel Prize winner, Eli Metchnikoff, and introduced in his study The prolongation of Life. Optimistic Studies proposing the interesting idea that microorganisms may have beneficial effects on human health and especially on digestive disorders (Metchnikoff, 1907). Metchnikoff has shown since 1907 that *Lactobacillus bulgaricus* is able to eliminate pathogenic bacteria from the intestinal microflora.

The actual introduction of the concept belongs to Lilly and Stillwell in 1965, after which probiotics are characterized as "microorganisms that promote growth of other microorganisms (Lilly *et al.*, 1965). In 1974, Parker talks about a food supplement for livestock and improve name of probiotics as "organisms and substances that helps the microbial ecosystem" (Parker, 1974). Their

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importance was highlighted by Fuller in 1989 who described probiotics as live microorganisms with beneficial effects on host body, improving intestinal microbial balance (Fuller, 1989). Today the universal meaning of the term "probiotic" was established by the World Health Organization and the Food and Agriculture Organization of the United States. These two organizations defined probiotics as "live microorganisms which when administered in adequate amounts, have a beneficial effect on health of the host organism"

Probiotic bacteria are known to be promoters of the host body's defence mechanisms. In addition to these effects probiotics influence the defence mechanisms in the intestine, which is characterized by stabilization of local microflora, involvement in triggering a humoral immune response and to promote the construction of a barrier to protect against immunological disorders.

Therapeutic potential of probiotic bacteria is immense and the steps taken so far are quite small due to the diversity of existing niches in the intestines. Researchers consider that probiotic microorganisms can shape the immune system both local and at systemic level which will allow future probiotics as treatments for many diseases in humans and animals. The immune system of mammals consists of a complex of cells and molecules that interact to protect the body against various pathogens. Interaction between microorganisms and the host is of great importance especially in neonatal period.

In this review we will attempt to present the mechanisms of probiotic action in the human and animal intestinal tract and also insisting in the first instance on the probiotic selection criteria.

**Selection of probiotic strains**

The basis for assessing probiotic efficacy in humans requires the understanding of probiotic strains, each of which is unique and different. Novel methods to select and characterise the probiotic strains are therefore needed.

Regarding the isolation and characterization of probiotic bacteria is of special interest the criteria used for strain selection, physiological characteristics, tolerance to conditions of the digestive tract, multiplication and operating capacity in the intestine, the effect on the immune system, antibacterial factors, the ability to colonize, resistance to industrial processing, their efficacy and safety. All bacteria used as probiotics are now selected on the basis of these criteria in particular *Lactobacillus* and *Bifidobacterium*.

An effective probiotic product requires proper identification and characterization of a bacterial species used. This is very important because there is now a wealth of information from food industry, in regards to probiotic bacteria, which not always corresponds to reality (Temmerman *et al.*, 2003). Traditional cultivation and microscopy have been improved by the introduction of genotypic studies. The latest techniques involves analysis of ribosomal RNA, specifically to a subunit, called 16S and 32S rRNA for bacteria (Wilson *et al.*, 1996). This sequence contains hypervariable subunits that are specific to each species. By using universal primers I and with Polymerase Chain Reaction (PCR) we can now determine and identify bacterial species. Bacteria generally have 5.7 copies for each gene rRNA. Currently there are about 16,000 sequences 16S rRNA in the databases associated with new bioinformatics techniques and in parallel with genetic and molecular biology techniques and this are used very easy to identify and characterize strains of probiotic bacteria (Thornton, 2001). There are less time-consuming techniques such as distortion in gradient electrophoresis (DGGE) (Yang *et al.*, 2009). However the most efficient method is to use species-specific primers that allow direct identification of probiotic organisms.

There are now methods available to investigate physiological characteristics of probiotics. Of these the most used are fermentation of carbohydrates and enzymatic activity. There are more specific tests such as the ability to hydrolyse bile salts (Lim *et al.*, 2004) or to produce antimicrobial substances (Toure *et al.*, 2003).

The viability of probiotic strains is considered crucial to ensure optimal functionality. This is explained by the fact that after ingestion these bacteria have to survive the inevitable three biological barriers such as salivary lysozyme, the acidic environment of the stomach and to the bile

acids in the duodenum (Saarela *et al.*, 2009). Therefore to ensure their survival during passage through the gastrointestinal tract, the probiotic strains are tested in terms of resistance to pH and bile acids. These tests were conducted on several strains and the results were different depending on the species (Tuomola *et al.*, 2001). In general resistance in the digestive environment is low as a result currently investigated novel approaches such as those based on mechanisms to stress adaptation of probiotic bacteria (Collado *et al.*, 2005).

Multiplication in the digestive tract will lead to the development of probiotic population and therefore to an increase of their metabolic products and thus implicitly an increase of the beneficial effects. It is not yet clear whether probiotics can multiply in the gastrointestinal environment. This arises from the fact that so far none of the known probiotics permanently colonize the intestine. There are few studies in this direction that shows that probiotics have the ability to colonize the intestinal mucosa since they could be isolated by biopsy (Alander *et al.*, 1999, Zoetendal *et al.*, 2004). It is also important to assess the activity of probiotics in situ, in this context, the new techniques of molecular biology open new directions for evaluation (Bron *et al.*, 2004). The bacteria produce acids and peroxide which are directly correlated with growth and development.

In the gut probiotic bacteria will make contact with the lymphoid tissue causing an immune reaction. The immune response in these situations is of major importance in combating gastroenteritis in humans. Similar beneficial effects have been detected in bladder and colon cancer (El-Nezami *et al.*, 1998, Aso *et al.*, 1995). Bacteria of the genus *Lactobacillus* are secret key factors affecting the health of individuals by shaping an immune response to pathogenic bacteria.

Lactic acid bacteria generally produce a variety of factors such as bacteriocins, antibiotics, lactic acid and peroxides. These substances help in the colonization of intestinal mucosa by probiotic bacteria preventing in this way the attachment of pathogens. In broilers bacteria of the genus *Enterococcus* produces bacteriocins substances with inhibitory effect on pathogenic bacteria of the genus *Clostridium* and *Listeria* (Shin *et al.*, 2008).

It is important to be able to easily manipulate the probiotic bacteria especially in food industry. These strains must be stable in continuous culture which gives them a high industrial applicability (Lee, 1995).

Live microorganisms are used as supplements for restoring microbial balance in case of intestinal dysfunction. It is absolutely necessary that extensive research studies are performed in order to develop a probiotic product is considered beneficial to human and animal health. It is important to understand that each probiotic microorganism is unique in its own way requesting a good knowledge of their properties and characteristics. Knowledge of the role of each strain, the target area and their biomarkers is crucial for their possible therapeutic role.

Using probiotics without adverse effects in human and animal health is an important issue. Lactic acid bacteria in general have quite positive history in this regard. Cases of infection have been reported with some strains that are currently abundant in human intestinal mucosa (Salminen *et al.*, 1998) . It is therefore important that the probiotic strains used are tested and meet safety standards set by the EU.

## MECHANISMS OF PROBIOTIC ACTION

**Immune modulation.** The intestinal lymphoid tissue is the largest in size compared with other areas of the body. It is well known that bacteria are critical for the development and functioning of the immune system at this level, being actually the defence mechanism against infection by pathogens (Cebra, 1999, Falk *et al.*, 1998). Intestinal lymphoid tissue makes contact with the food components, the antigens and with the beneficial or pathogenic bacteria. Antigens, substances that can trigger an immune response, enter the body through the intestinal mucosa that is essential in controlling immunity to invasion of pathogenic bacteria. The adaptability to various antigens is extremely important if we consider that the composition of intestinal mass change very frequently. Most of the antigen is released from first contact with the intestinal mucosa (Sanderson

*et al.*, 1993). After crossing the epithelial barrier by transcytosis, they are restructured by a lysosomal degradation processes. A further screening is in the presence of M cells (cells of follicular epithelium associated with lymphoid tissue) followed by the T cells (lymphocyte cells belonging to the group of white blood cells) which are then differentiated as cells that mediate an immune response and promotes cell differentiation and secreting IgA (immunoglobulin A) (Strober *et al.*, 1998). IgA is an antibody that plays a crucial role in mucosal immunity. In figure 1 (Corcionivoschi *et al.*, 2009) we are presenting the hypothetical effect in modulating and immune response. Through TLR receptors (Toll Like Receptors), dendritic cells (DC) and T cells, probiotics, leads to reduced secretion of TH1 (lymphocyte involved in an enhanced immune response), IL12 (interleukin which is naturally produced by dendritic cells), TNFα (inflammatory cytokine) and IFN-γ (cytokine that is critical for innate and adaptive immunity) which are responsible for the onset of inflammatory processes in the intestines.

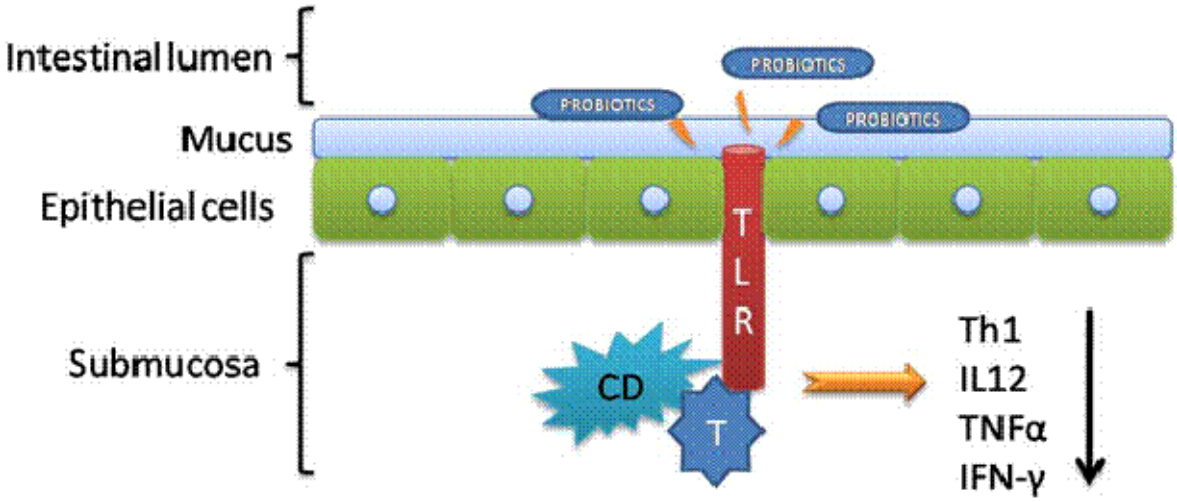


Fig. 1. The effect of probiotic bacteria on the immune system (Corcionivoschi *et al.*, 2009).

The mechanisms by which epithelial cells are making the difference between probiotic and pathogenic microorganisms appear to be different. Pathogenic bacteria induce a pro-inflammatory response in epithelial cells by activating transcription factor NF-kB. Compared with these bacteria, non-pathogenic species may alleviate to the side of pro-inflammatory response by blocking this factor (Neish *et al.*, 2000).

It was found that the administration of *L. rhamnosus* HN001 and *Bifidobacterium lactis* HN019 stimulates activity of cytotoxic lymphocytes. Similar experiments show that administration of these probiotics reduced the activity of these lymphocytes (Gill *et al.*, 2001a, Sheih *et al.*, 2001). Stimulation of cytotoxic lymphocytes activity is correlated with the secretion of IL-12, another cytokine involved in their activities when *L. casei* Shirota is administered (Takeda *et al.*, 2006). These studies suggest that probiotics can play an important role in stimulating the activity of cytotoxic lymphocytes having a direct role in preventing the development of malignant tumours. It also appears that the role of probiotics in phagocytosis and the activity of cytotoxic lymphocytes is vital especially in the elderly, who have a compromised immune system (Arunachalam *et al.*, 2000, Gill *et al.*, 2001c, Gill *et al.*, 2001b).

Quality and dose of probiotic preparations influence the IL-8 secretion via the enterocites. IL-8 is associated with the development of intestinal inflammation. Recent data shows that when incubated with *L. rhamnosus* GG the CaCO-2 cells

(intestinal epithelial cell) reduces the amount of IL-8 produced (Zhang *et al.*, 2005). In many cases was shown that enterocites produce IL-8 and other cytokines in the presence of probiotics such as IL-6 (Ruiz *et al.*, 2005). IL-6 stimulation was achieved by administering *L. casei* CRL431 and *L. helveticus* R389 (Vinderola *et al.*, 2005).

In conclusion studies to date show that each probiotic is characterised in regards to its influence on the immune system. In other words, bacteria have immunomodulatory qualities characteristic of each one. Next objective would be to determine the exact components of each probiotic strain that are or may be directly involved in triggering an immune response. Probiotics can influence the immune system by different metabolites, the cell wall components and DNA.

**Inhibition of pathogenic bacteria.** The gastrointestinal environment contains a wide range of contents ranging from harmless beneficial dietary and microbial flora to harmful pathogenic bacteria. The mammalian organism fights against these pathogenic bacteria through various ways: blocking pathogenic bacteria effects by producing bactericidal substances and competing with pathogens and toxins for adherence to the intestinal epithelium; regulation of the immune responses by enhancing the innate immunity and modulating pathogen-induced inflammation via toll-like receptor-regulated signalling pathways; regulate intestinal epithelial homeostasis by promoting intestinal epithelial cell survival, enhancing barrier function, and stimulating protective responses (fig. 2) (Corcionivoschi *et al.*, 2009). The strategy is based on the ability of probiotic bacteria (B) to bind pathogens (C) in intestinal epithelial tissue (A). Anti-pathogenic action of probiotics consists in production of lactic acid (D) which decreases the pH, interacts with the toxins produced by pathogens (E), with the production of hydrogen peroxide (F) and synthesis bacteriocine (G).

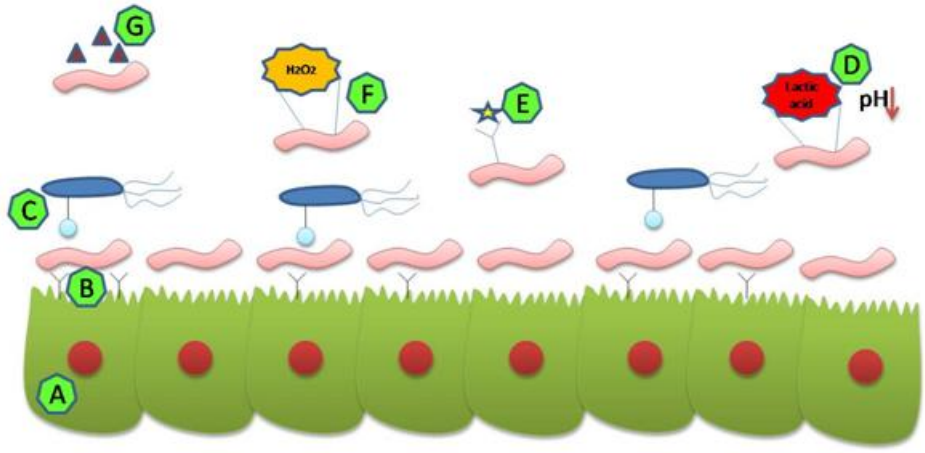


Fig. 2. Schematic representation of the mode of action of probiotics in the intestines (Corcionivoschi *et al.*, 2009).

Production of antimicrobial substances (bacteriocins), in situ in the intestine can be improved by increasing the ability of probiotic bacteria to adhere to the intestinal mucosa. Bovine colostrum contains substances that can triple the capacity of *Lactobacillus casei* species to adhere to intestinal cell line Caco-2. However, in situ production of microbial substances adversely affect intestinal microflora beneficial to the host organism (Sanders, 1993). Ruminal bacteria can also produce such bacteriocins which by their presence are able to modify the ruminal ecosystem. Some studies even recommend using ruminal bacteriocins as an alternative to antibiotics in cattle (Russell *et al.*, 2002).

In vitro studies have shown that strains of lactic acid bacteria are effective in removing or stopping the activity of pathogenic bacteria. Studies in vitro with human cell lines have helped to investigate how probiotics adhere to the intestinal epithelium. These cell lines have different phenotypic characteristics and they have been widely used especially in humans (Louvard *et al.*, 1992). Their use has its explanation in the fact that mimics the

intestinal barrier that pathogenic microorganisms must pass in order to infect and then systemic circulation to reach various parts of the body (Cereijido *et al.*, 1998).

Production of certain metabolites such as lactic acid lowers the pH with a decisive role in inhibiting the development of pathogenic bacteria. But there are also cases where pathogen inhibition (*Shigella*) is due not only to pH but also to some antibacterial substances secreted by lactic acid bacteria (Apella *et al.*, 1992). Secretion of hydrogen peroxide is also an important factor and was identified as having inhibitory effect on growth and development of *E. coli* 0157: H7 (Brashears *et al.*, 1998). Supernatants derived from *L. rhamnosus* Lcr35 cultures had an inhibitor effect on nine types of pathogenic bacteria: *E. coli* (ETEC), *E. coli* (EPEC), *Klebsiella pneumoniae*, *Shigella flexneri, Salmonella typhimurium, Enterobacter cloacae, Pseudomonas aeruginosa, Enterococcus faecalis and Clostridium difficile* (Forestier *et al.*, 2001).

Competitive exclusion of pathogens can be used efficiently to farm animals after treatment with antibiotics to prevent infection with *Salmonella* during especially because the host microflora is in recovery. This concept involves administration of non-pathogenic bacterial cultures (one or more strains) in order to reduce colonization or presence of pathogenic bacteria in the intestine (Steer *et al.*, 2000).

There is therefore sufficient evidence to demonstrate the use of probiotics in maintaining control of *Helicobacter pylori* colonization of gastric mucosa. Clinical studies and experimental animal models have shown that *L. acidophillus* can affect growth and development of this pathogen both in vitro and in vivo (Felley *et al.*, 2003). However to date there is insufficient data to suggest the use of probiotics in the absence of antibiotics to prevent infection with *H. pylori*.

Administration of probiotics (*L. rhamnosus* HN001) in animals, under experimental conditions, resulted in an improved immune response following *Salmonella enterica* infestation (Gill *et al.*, 2001c). It is also interesting that the animals who were artificially infected with *Salmonella* and which received probiotics have synthesized high levels of serum antibodies leading to increased survival to infection but also to a decrease in the presence of these pathogens in liver and spleen. The same effects have been identified when *L. salivarius* CTC2197 is administered to Leghorn birds (Pascual *et al.*, 1999). Given this we can say that probiotics help the digestive tract by competing with pathogenic bacteria for the adhesion sites. If they manage to cross the epithelial barrier will trigger an immune response and antibody production, a process that is mediated by the probiotic and will lead to pathogenic bacteria eradication.

Prevention of infections by *Listeria monocytogenes* is a topic of great interest particularly for poultry. Bacteriocins produced by *Enterococcus faecium* SH528, SH632, *Pediococcus pentosaceus, Enterococcus faecium* SH740 were proven to be effective in combating *Listeria monocytogenes* (Shin *et al.*, 2008). Studies on rats artificially infected with *Listeria* show that administration of *L. casei* lead to reduced presence of pathogens in particular the liver (Sato, 1984).

Efficacy of probiotics was also proven in urogenital infections and was tested by studies performed on healthy patients or female patients who were diagnosed with uro-vaginal infections. Results from these studies suggest beneficial effects of the use of probiotics in preventing urinary tract infections (Petricevic *et al.*, 2008).

However clinical research should be expanded, especially for commercial products to increase their effectiveness and in particular to accurately identify their spectrum of action.

So far have been suggested several mechanisms by which probiotics are involved in preventing the harmful effect of intestinal pathogens such as competition for nutrients, inhibition of interaction between pathogens and intestinal mucosa, production of antimicrobial substances and stimulation of mucosal immunity (Steer *et al.*, 2000). However, there are many aspects of interaction between pathogens and probiotics which are of great interest for many researchers in the field aiming to understand the anti-pathogenic mechanism of probiotics.

**Probiotic effects in gastro-intestinal diseases.** Probiotics are well known to be effective in treating two types of diarrhoea: those caused by antibiotics and travelers' diarrhoea. *Clostridium difficile* is the etiologic agent of pseudomembranous colitis and one of the most frequent causes for the outbreak of diarrhoea after antibiotic treatment. The infection is associated with ecosystem disruption allowing microbial colonization of *C. difficile* and toxin production characteristic of this microorganism. The best known side effect is diarrhoea, occurring in approximately 20-39% of patients due to changes in the microbial balance in the colon (Gismondo *et al.*, 1999, Marteau *et al.*, 2001, McFarland, 1998). In this context there have been some clinical trials to determine efficacy of probiotics in patients with diarrhoea occurred after treatment with antibiotics (Gorbach, 2000). It was found that oral administration of *Saccharomyces boulardii* significantly reduces the occurrence of diarrhoea following administration of antibiotics (Brown *et al.*, 2004). The same effect was found when *Lactobacillus rhamnosus* GG was (Gismondo *et al.*, 1999, Gorbach, 2000) administered in patients who have received treatment based on erythromycin, penicillin and ampicillin. Studies conducted in order to determine the probiotic strain with the greatest impact on diarrhoea treatment with antibiotics, suggested almost that all probiotics had a direct effect (Cremonini *et al.*, 2002). Unfortunately, at this point the literature is still ambiguous in terms of dose and mode of their administration.

**Mechanistic activities of genetically modified probiotics**. It is known that intestinal microflora plays an essential role in maintaining inflammation in the intestinal mucosa. Because of this manipulation of the fraction of intestinal flora involved in inflammation may represent a new therapeutic option in treating this disease. There are many strains of probiotic organisms with an enormous phenotypic diversity that can have multiple beneficial effects on human and animal health. In this context the use of new techniques provided by the molecular biology offers the possibility that genetic screening may lead to identification of new probiotic strains to demonstrate multiple beneficial effects in difficult environmental conditions. To create new strains of genetically modified probiotic is essential to know all their possible mechanisms of action.

Although genetic modification (GM) of probiotic bacteria can bring significant improvements, formidable barriers were imposed which lead to restrictions for their use commercially. GM bacteria are facing significant repulsion from the general public mainly due to the effects they might have upon their arrival in the uncontrolled environment. If we were to compare with the success that the plant biotechnology industry had in the U.S. then we can expect public acceptance for probiotics to occur mainly due to progress in microbial biotechnology that are beneficial and bring real benefits especially in medicine. There are already studies showing that genetically modified probiotics are starting to be accepted by consumers particularly when evidence of their beneficial effect on human health are shown (Verrips *et al.*, 1996). Genetic progress in the case of *Lactobacillus and Bifidobacterium* is still insignificant but a more rapidly development in the area with increased knowledge will prove their applicability. In this respect there are clear research directions leading to a better understanding of their role: correlation of phenotype and genotype characteristics influencing their functionality, molecular detection methods and systems for genetic transfer. Other lines of research focus on genetic modifications to improve existing characteristics of probiotic bacteria. For example expression of a gene that encodes an amylase in *L. amylovorus* in a strain used in the manufacture of silage, as *L. plantarum*, resulted in enhanced ability to degrade starch (Fitzsimons *et al.*, 1994). Another example is to introduce a gene that encodes a glutamate dehydrogenase involved in catabolism of *Peptostrptococcus asaccharolyticus* in *L. lactis* in order to allow these microorganisms to produce α-Ketoglutarate from glutamate, an amino acid present in high quantities in cheese (Rijnen *et al.*, 1999).

## Conclusions

This review covered the knowledge of probiotics in regards to their mechanistic abilities. Some of the developments in the field of probiotic actions are provided in this review. Scientists continue to work on elucidation of the mechanisms of the most common probiotic strains. The results that might arise from could be extremely important because the use of probiotics to maintain health must be considered promising, although much remains to be elucidated. The universal use of some strains seems less reasonable from an ecological point of view than selection of strains from their natural habitat were they are adapted to the ecological niche. It is important to understand that all probiotic strains are unique and different and their properties and characteristics should be well defined. Knowledge of the mechanisms is an important factor, complemented with target functions and biomarkers that are accepted as relevant to the state of health and well-being or reduction of risk of disease.

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