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On probiotic integration in the management of inflammation and the maintenance of the intestinal epithelial barrier's integrity

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Abstract

Inflammatory bowel disease epidemiology has grown dramatically in recent years, particularly in developed and developing Western countries. Many factors, including stress, diet, and medications, cause and exacerbate inflammatory conditions. Inflammation is closely related to the concept of intestinal barrier integrity. When integrity is compromised, toxins and pathogens can enter the bloodstream. In recent years, there has been a growing interest in using probiotic bacteria to prevent or treat a variety of pathologies, including inflammatory bowel disease. Some studies have looked at the effectiveness of multi-strain probiotic supplements in preventing intestinal barrier dysfunction in *in vitro* models of lipopolysaccharide-induced inflammation. To mimic the intestinal barrier, human colon adenocarcinoma cell lines were established in Transwell co-culture models. The epithelium permeability was assessed by measuring the transepithelial electrical resistance. The expression of individual proteins involved in barrier function was assessed. The immunomodulatory effects of probiotic formulations were studied in both human macrophage cell lines and *ex vivo* human peripheral blood mononuclear cell-derived macrophages. The intestinal epithelial layer was also interfaced with a human mast cell line. Selected probiotics have demonstrated high potential for use in maintaining intestinal barrier integrity and possessing anti-inflammatory properties.

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Introduction

The epidemiology of intestinal inflammatory states, such as Inflammatory Bowel Disorders (IBD), has shown a strong increase in the incidence of their development, particularly in Western countries, especially among children and the elderly, contributing to their increased prevalence.[1] In 2019, there were about 4.9 million cases of IBD in the world, with China and the USA having the highest number of cases (911,405 and 762,890; 66.9 and 245.3 cases per 100,000 people, respectively).[2] In Italy, a prevalence of IBD was 80.9 per 100,000 inhabitants in 1990 (56,469 cases) and increased to 93.8 per 100,000 inhabitants in 2017 (76,581 cases).[3]

There is not a single well-defined cause for IBD, and in fact many studies have shown that genetic and environmental factors, lifestyle, diet, stressors and medications all play a role. All these factors may result in damage to the intestinal barrier. When the integrity of the intestinal epithelial barrier decreases, it allows the passage of toxins and pathogens into the bloodstream and there is a significant increase in the immunological response.[4,5]

An attempt has been made to identify the causes of this growing epidemic of intestinal inflammatory states, as well as to identify increasingly targeted tools for controlling and potentially preventing these conditions.

Gut microbiota

The intestinal barrier performs fundamental tasks of digestion and absorption, establishing tolerance or immunity based on the antigens it encounters. The intestinal microbiota forms the first level of the intestinal barrier. It plays a critical role in the regulation of host immunity, as it is important for the development of the immune system and for the modulation of the immune response as well.[6]

In this regard, recently, the expression "muco-microbiotic layer" has been proposed to identify the innermost layer of the intestinal wall; this layer includes not only the intestinal microbio-

ta, but also the mucous membranes that constitute its natural "microenvironment" as well as the nanovesicles, such as exosomes, critical for trafficking between human and bacterial cells.[7]

The gut microbiota is an extremely delicate ecosystem. There is a constant physiological dynamism in the state of eubiosis of the microbiota. The gut microbiota is constantly changing due to changes in people's lifestyles. Stress, aging, the use of antibiotics and poor nutrition pose a danger to the composition of the microbiota. These factors destabilize the homeostasis of the microbiome as well as the homeostasis of the whole organism.[8]

The intestinal microbiota performs several important functions. The gut microbiota supplies vitamins to the host, such as folic acid, vitamin K, biotin, riboflavin (B2), cobalamin (B12), and possibly other B vitamins.[9,10] One of the fundamental contributions of the intestinal microbiota is the extraction of energy from food. Generally non-digestible fibers, commonly found in vegetables, can be digested by specific species of *Bacteroides*, *Lactobacillus* and *Bifidobacterium*, such as *Bacteroides thetaiotaomicron* and *Bacteroides ovatus, Lactobacillus acidophilus, Bifidobacterium adolescentis* and *Bifidobacterium longum.*[9,11] Carbohydrates are fermented by saccharolytic bacteria, resulting in the production of Short-Chain Fatty Acids (SCFAs) such as acetic, propionic, and butyric.[12]

The intestinal microbiota and its metabolites can regulate the innate and adaptive immune responses.[11] Intestinal Epithelial Cells (IEC) are an integral component of the innate immune system and influence the intestinal microenvironment through, for example, the identification and uptake of SCFAs. Innate immunity includes intestinal mucosal barrier function, antibacterial proteins, acidic stomach pH to limit microbial growth, innate immune cells, such as neutrophils, macrophages, dendritic cells, natural killer T cells and mast cells (MCs). In this context, MCs, which play an important role in both innate and adaptive immunity, are equipped with a large repertoire of receptors.[14-16] MCs recognize harmful antigens by associating with Pathogen-Associated Molecular Patterns (PAMPs) on their surface. The common receptors found on MCs are Toll-Like Receptors (TLRs) and complement receptors, useful to recognize a wide variety of pathogens, environmental toxins, allergens, neurotransmitters, neuropeptides and hormones, and to respond to these requests through the release of preformed or newly synthesized molecules, including cytokines, chemokines, histamine, proteases, growth factors.[14,16] All this suggests that the body's defense mechanisms to protect itself from pathogens consist of an apparent redundancy of systems. However, despite all the protective mechanisms present at the level of the gastrointestinal mucosa, occasionally enteric pathogens can penetrate through the "mucus layer" and microbiota, adhere to or invade the IECs, causing an inflammatory cascade that leads to an imbalance between protective intestinal bacteria, symbiotic and harmful bacteria, pathogens, and activation of immunoregulatory mechanisms.[17] These events have the ability to initiate or contribute to the development of a number of chronic inflammatory conditions that are collectively known as IBD. Numerous studies have demonstrated that the two main forms of IBD, Crohn's Disease (CD) and Ulcerative Colitis (UC), are associated with a reduced complexity of the commensal microbiota and lead to continuous shifts towards a dysbiotic state.[18-20]

The health of the intestinal microbiota is fundamental for the appropriate modulation of the immune response, for the state of integrity of the intestinal epithelial barrier, for the dialogue and interactions with the central nervous system, in what has been outlined to be the "microbiota-gut-brain" axis.[21-23]

Bacteria can produce most of the neurotransmitters and some strains can affect stress responses and cognitive abilities.[24,25] A growing body of evidence indicates that the gut microbiota affects brain function and may be altered in neurological diseases including stress, anxiety and depression, as well as in addiction conditions, in learning and memory phenomena and sexual behavior.[26-30]

The intestinal epithelium can be enhanced, protected and repaired by growth factors and cytokines. A perturbation of the composition and any change in the content of the intestinal microbiota constitute another important factor in the alteration of intestinal homeostasis.[31] Last but not least, a breakdown of the epithelial barrier function induces a pathological response.[32]

Many researchers have tried to understand which microorganisms cause intestinal inflammation. It is known that the intestinal microbiota also varies in healthy subjects because there are many factors that can influence it.[33] Overall, it was found that Bacteroidetes levels in CD patients are increased compared to healthy people, while *Lactobacillus* spp. and *Bifidobacterium* spp. levels are decreased, and if *Lactobacillus* spp. and *Bifidobacterium* spp. are exogenously introduced, IBD is prevented.[34] In the intestinal microenvironment, cell-cell interactions regulate microbial multiplication and preserve intestinal homeostasis, leading to a variety of host responses against commensal organisms and pathogens.[35]

The microbiota is an extremely dynamic ecosystem influenced by genetic, metabolic, food, geographical, pharmacological factors.[36]

Probiotics and their roles

Various studies have suggested the use of probiotic formulations as a tool for the prevention and treatment of many ailments, from non-infectious to infectious. It was emphasized that the use of probiotics is beneficial. The FAO/WHO definition of a probiotic is a "live microorganisms which, when administered in adequate quantities, confer a health benefit to the host".[37,38]

A role played by probiotics is their ability to stimulate the host's immune and inflammatory response, and regulate immunomodulation against pathogenic microorganisms. This task is carried out by specific bacterial strains that strengthen and help maintain the integrity of the intestinal barrier, increasing the number of intraepithelial leukocytes and goblet cells and stimulating the production of proinflammatory, including Tumor Necrosis Factor α (TNFα) and interleukin IL-1β, and regulatory (Transforming Growth Factor β, IL-10) cytokines. Studies have reported that certain multi-strain probiotics can stimulate the production of anti-inflammatory cytokines.[39,40]

Administration of a multi-strain probiotic (containing *Lactobacillus acidophilus, Lactobacillus fermentum, Lactobacillus plantarum* and *Enterococcus faecium*) has been reported to be effective in reducing interferon gamma (IFN-γ) in *Salmonella enterica*-infected chickens.[41]

Supplementation with *Lactobacillus casei* and *Bifidobacterium lactis* was able to reduce intestinal mucosal damage in a model of 2,4,6-trinitrobenzensulfonic acid-induced inflammation.[42] Metabolites produced by *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactococcus lactis*, *Lactobacillus reuteri*, and *Saccharomyces boulardii* can negatively modulate the production of inflammatory cytokines from human macrophages.[43]

Probiotics can stimulate mucus production and strengthen tight junctions of the intestinal epithelial barrier. Selected strains of *Lactobacillus* spp. and *Bifidobacterium* spp. increase mucin expression in human intestinal cell lines and increase tight junction protein

(zona-occludens 1).[44] A protective mechanism performed by probiotics is the competitive shift against pathogens for adhesion and colonization of the mucous membranes. The adhesion of proteins to the intestinal mucosa is very important in the presence of intestinal inflammation. Some probiotic microbes share binding sites with enteropathogens and this could limit their adhesion to host cells. It is therefore essential to use probiotics in the initial stages of inflammation with the possibility of temporary colonization.[45]

Further studies have also highlighted anti-genotoxic, as well as antioxidant effects carried out by specific probiotic bacterial strains.[46-48]

Probiotics can produce antimicrobial molecules. Among the various bioactive substances produced by bacteria we find hydrogen peroxide, lactic acid, and antimicrobial peptides, but some probiotic strains also produce antibacterial peptides, chitinase, and dextranase, which inhibit other bacterial pathogens. Additionally, antibacterial proteins, such as bacteriocins, have been isolated that act by binding to surface receptors or by invading host cells or forming pores on target cells. This leads to degradation of cell DNA and inhibition of bacterial cell wall peptidoglycan biosynthesis. Some probiotic bacterial strains can stimulate the production of enzymes that hydrolyze bacterial toxins and modify the toxin receptors in the host. Species of lactic acid bacteria produce substances that inhibit the growth and adhesion of pathogenic microorganisms, increasing the immune response.[49]

Relevance of multi-strain probiotics

It has been reported that multi-strain probiotics have often shown greater benefits than the use of a single bacterial strain, this probably thanks to the synergy of the strains and their additive effect. Multi-strain probiotics result in high mucosal adhesion and inhibition of pathogens in the intestinal canal. On the other hand, knowledge of the genetics of the species or constituents of multi-strain probiotics is fundamental to understanding the mechanisms underlying the interactions between intestinal microbiota and host. Recent studies reported a careful comparative genomic analysis of the multi-strain probiotic VSL#3 which revealed genes encoding various bioactive substances associated with the health benefits of probiotics.[50]

A mechanism by which multi-strain probiotics exert their effects is likely via downregulation of the nuclear factor-kappa-B (NFκB) signaling pathway. It is known that this pathway constitutes a crucial system of the inflammatory response.[51]

As stated before, the intestinal microbiota maintains a high stability. This stability is dependent on a process known as Quorum Sensing (QS), which is how bacteria communicate and control the expression of various genes. It is a cell-cell communication system that is based on the production, secretion and detection of signal molecules of bacterial origin, called Auto-Inductors (AIs). The concentration of AIs is closely related to the cell density of the organisms that secrete them in the local microenvironment. When bacterial density reaches a certain threshold, AIs bind to their receptors on the high-density bacterial surface. The next step is the internalization of the receptors and the binding to specific gene domains for the regulation of physiological functions, leading to a self-inductive feedback mechanism for the synchronized development of bacterial populations.[52,53]

Various studies have been conducted with probiotic combinations to test any effects in managing inflammatory states.[42,54,55]

In this context, a large study was carried out to evaluate the efficacy of a commercially available multi-strain probiotic formulation containing *Lactobacillus rhamnosus* LR 32, *Bifidobacterium lactis* BL04, and *Bifidobacterium longum* BB 536 (Serobioma®, Bromatech, Milano, Italy) in the prevention of intestinal epithelial barrier dysfunction in an *in vitro* model of lipopolysaccharide-induced inflammation.[40] The formulation was also analyzed for its ability to prevent inflammatory states, as well as to intervene in the dialogue between the intestinal barrier and immune cells.[56,57] To replicate the intestinal barrier, human colon adenocarcinoma cell lines HT-29 and Caco-2 were grown in monolayer on a permeable membrane using the Transwell model (Figure 1). In particular, the Caco-2 cell line can form a monolayer of cells that spontaneously differentiate into polarized enterocytes joined by junctional protein complexes (*i.e.* tight junctions consisting of transmembrane proteins, including occludins, claudins, and junctional adhesion molecules).[58] Under these conditions, the permeability of the reproduced intestinal epithelium was assessed by measuring the Transepithelial Electrical Resistance (TEER).[56]

The expression of individual proteins involved in barrier function was evaluated by Real Time-PCR and Western Blotting.[56] The probiotic formulation under investigation was able to counteract the increase in permeability induced by Lipopolysaccharide (LPS). Serobioma promoted the expressions of *ZO-1*, cadherin, claudin 1 and occludin, while it reduced claudin 2 expression at both mRNA and protein levels. Claudin 1 is known to form continuous sealing filaments, while claudin 2 forms pores, regulating paracellular permeability. Thus, the study highlighted a protective effect of the formulation against epithelial barrier dysfunction. In conclusion, the probiotic formulation has been shown to have a high potential for its use in controlling the integrity of the intestinal barrier.[56] The immunomodulatory effects of the same multi-strain probiotic formulation were studied in the human macrophage cell line THP1 and macrophages derived from *ex vivo* human peripheral blood mononuclear

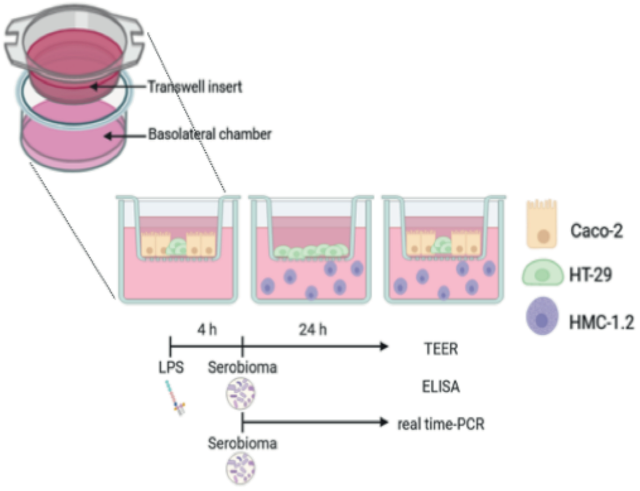

Figure 1. The *in vitro* transwell model was employed and Caco-2 and HT29 human colon adenocarcinoma cells in single cultures and in co-culture, with or without mast cells (HMC1.2) were used to reconstitute the intestinal epithelial barrier. The cells were challenged with the lipopolysaccharide (LPS) proinflammatory stimulus and treated with the multi-strain probiotic formulation (Serobioma®, Bromatech S.r.l. Milano, Italy) (created by BioRender).

cells.[40] Serobioma was able to induce an increase in IL-10 production and to decrease the secretion of the major proinflammatory cytokines IL-1β and IL-6. In addition, the ability of Serobioma to modulate macrophage phenotypes, M1(pro-inflammatory role) or M2 (tissue-regenerative, anti-inflammatory activity), and the polarization of human monocytes from healthy donors through the epithelial cells was determined. The study was the first to describe the switching of macrophage phenotype M1/M2 induced by the passage of probiotic supplementation metabolites across the human HT-29 cell line epithelium.[40]

Finally, the probiotic formulation on co-cultures of intestinal epithelial cells and mast cells was also investigated. Co-cultures of intestinal epithelial cells interfaced with the human MC line HMC-1.2 in the basolateral chamber were challenged with LPS, and then the cells were treated with probiotics (Figure 1). In the HT29/HMC-1.2 co-culture, the multi-strain probiotic formulation was able to counteract the LPS-induced release of interleukin 6 from HMC-1.2, and it was effective in preserving the epithelial barrier integrity in the HT29/Caco-2/HMC-1.2 co-culture.[57] In conclusion, these studies show that adequate probiotic integration can effectively fight inflammatory states and promote the integrity of epithelial barriers.

Conclusions

The synergistic and/or additive action of multi-strain probiotic supplementation have shown great potential and are promising for the use of probiotics in the treatment of various diseases.

Probiotic organisms can secrete various bioactive substances, such as lecithins and bacteriocins, capable of inhibiting the multiplication of pathogens, possibly even those resistant to antibiotics.

To optimize the benefits associated with the consumption of probiotics, future studies will be aimed at identifying the mechanisms underlying the actions of specific probiotic strains and their combinations. This will allow for more specific applications in different pathological conditions.

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