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RESEARCH ARTICLE

REINFORCEMENT OF INTESTINAL EPITHELIAL BARRIER BY PROBIOTICS AND THEIR EXTRACELLULAR PROTEINS

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Abstract

The intestinal epithelium acts as the first line of defense in our body that restricts harmful pathogen to proliferate and disseminate. Any consequence to this defense leads to altered gut environment as well as the immune system. Probiotics have long been used as an alternative to traditional medicine with the goal of maintaining enteric homeostasis and preventing disease. Probiotics increase barrier function in terms of increased mucus, antimicrobial peptides, and sIgA production competitive adherence for pathogens, and increased tight junction integrity of epithelial cells. The mechanism of action is an area of interest. Surface-associated and extracellular components produced by probiotic bacteria are able to directly interact with the host mucosal cells, promote the stabilization and enhancement of the mucosal barrier function. Compared to the other bacterial components, the interactive ability of extracellular proteins/peptides has been less extensively studied. This review mainly highlights the abilities of probiotics and their extracellular proteins to strengthen the intestinal epithelial barrier function.

Introduction

The intestinal epithelium acts as an intrinsic defense against dissemination and proliferation of potentially harmful agents which it constantly confronts in the luminal compartment. To achieve this, the epithelium has developed multiple innate defense mechanisms that restrain bacterial growth, limit direct contact with the bacteria, and prevent bacterial dissemination into underlying tissue. Therefore, protect the intestine from microbial intruders. The intestinal barrier defenses consist of the mucous layer, antimicrobial peptides (such as defensins, cathelicidins and lysozymes), secretory IgA, and the epithelial cells linked through tight junctions (TJ). A breach in any one of these barriers can lead to diseases/disorders or altered functioning of gut immune system. Such disorders can be restored by using nonpathogenic organisms (Probiotics) as a dietary supplement.

Probiotics are able to reach the intestines in sufficient numbers and can effectively deliver beneficial effects including competitive exclusion of pathogenic bacteria, induction of defensin production, modulation of host immune functions and improvement of the intestinal barrier function (Beisner et al., 2010). However, the underlying mechanisms are still unclear. Nowadays, gut microbiologists are dealing with different extracellular components (exopolysaccharides, bacteriocins, lipoteichoic acids and surface-associated and extracellular proteins) of probiotic origin to unravel the mechanisms of probiotics functioning. Compared to the other bacterial components, the interactive ability of extracellular proteins/peptides has been less extensively studied. Also, the evidence of a direct interaction between bacterial extracellular proteins and the human immune system is mainly available for commensal and pathogenic species (Adams et al., 2008). Extracellular proteins include proteins that are actively

transported to the bacterial surroundings through the cytoplasmic membrane, as well as those that are simply shed from the bacterial surface. This review mainly highlights the abilities of probiotics and their extracellular proteins to strengthen the intestinal epithelial barrier function.

Probiotic and intestinal barrier function

Probiotics are the live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (Singh et al., 2012). Various *in vitro* and *in vivo* studies have supported that probiotics increase barrier function in terms of increased mucus, antimicrobial peptides, and sIgA production, competitive adherence for pathogens, and increased TJ integrity of epithelial cells.

Gastrointestinal (GI) mucin is a constituent of luminal barrier function and is the first line of host defense against invading pathogens. Probiotics may promote mucus secretion as one of the mechanisms to improve barrier function and exclusion of pathogens. Several evidences support that *Lactobacillus* species elevate the mucin expression in the human intestinal cell lines such as Caco-2 (MUC2) and HT29 (MUC2 and 3), thus blocking pathogenic *E. coli* invasion and adherence (Mack et al., 2003). However, this protective effect is dependent on *Lactobacillus* adhesion to the cell monolayers, which most likely does not occur *in vivo*. Conversely, Kim et al. (2008) showed that *L. acidophilus* A4 cell extract was sufficient to increase MUC2 expression in HT29 cells, independent of attachment. *In vivo* studies are less consistent, in part because of the fact that very few such studies have been performed. Therefore, further studies are needed to prove that mucus production may be increased by probiotics *in vivo*.

Defensins, host antimicrobial peptide, display antimicrobial activity against a wide variety of bacteria, fungi, and some viruses and are constitutively expressed to keep pathogens from reaching the epithelium. Schlee et al. (2008) have shown that hBD-2 expression and/or secretion was significantly upregulated in Caco-2 cells upon stimulation by several *Lactobacilli* species, or VSL#3. Schlee et al. (2007) also observed flagellin of *E. coli* Nissle1917 (EcN) as the major stimulatory factor since purified EcN flagellin induced hBD-2 mRNA expression, but flagellin-negative mutants or EcN incubated with flagellin antiserum did not. In addition to altering expression levels of host cell-derived antimicrobial peptides, probiotics can directly inhibit growth or killing of pathogens by production of antimicrobial molecules including organic acids, H_2O_2 short-chain fatty acids (SCFA) and bacteriocins. All of these mechanisms induce rapid bacterial death and thus contribute to maintaining a pathogen free intestinal barrier.

sIgA is considered to be the primary element of the mucosal immune response as it protects the intestinal epithelium against colonization and/or invasion by binding antigens on pathogens or commensals (Sabirov and Metzger, 2008). Probiotics have been shown to augment total and pathogen specific sIgA levels upon infection, while typically not inducing production of probiotic-specific sIgA. Galdeano & Perdigon (2006) found that mice given *L. casei* displayed significantly increased numbers of IgA^+- and IL 6- producing cells (which can stimulate B cell class switching to IgA) in the small bowel lamina propria. It was also found that antibodies against *L. casei* were not produced, indicating the non responsiveness of the gut immune system to this beneficial bacteria. However, Martins et al. (2009) found that mice mono-associated with *L. casei* did not have increased levels of sIgA. LeBlanc et al. (2004) showed that the peptides released by *L. helveticus* during milk fermentation can also increase the sIgA response. Rats treated with this bioactive peptide before challenge with EHEC displayed increased numbers of intestinal lamina propria IgA^+ B cells and levels of sIgA. Available evidence suggests that not all probiotics are equal in terms of their effects on sIgA production. Roller et al. (2004) found that a combination of *L. rhamnosus* and *B. lactis* did not increase sIgA levels in rats. But rats given prebiotics (oligofructose-enriched inulin) or synbiotics (*L. rhamnosus*, *B. lactis*, and inulin) did exhibit increased sIgA production, indicating the diversity of stimuli that can lead to enhanced immune exclusion in the gut. Ng et al. (2009) reviewed the available evidences and suggested that probiotics also have many other immunomodulatory effects in the human intestine, including promoting tolerogenic dendritic cell and regulatory T cell phenotypes, inhibiting inflammatory cytokine production, and enhancing natural killer cell activity.

Many studies have shown that pretreatment with probiotic bacteria can directly alter epithelial barrier function by influencing the structure of TJ and thus reduce the injury conditions resulting from stress, infection, or proinflammatory cytokines (Ewaschuk et al., 2008). A study by Resta-Lenert and Barrett (2003) found that *S. thermophilus* and *L. acidophilus* independently increased Transepithelial Resistance (TER) and decreased permeability of HT-29 and Caco-2 cells. These bacteria also induced activation of occludin and ZO-1, as shown by increased levels of phosphorylated proteins without a significant change in the total levels. It was also found that bacterial cultured medium and killed bacteria (by antibiotics or heat) failed to elicit any of the same responses,

suggesting that live *S. thermophilus* and *L. acidophilus* are required for enhancement of barrier function. Whereas, Ewaschuk *et al*. (2008) studied that conditioned medium from several bacterial strains found in VSL#3 can independently increase TER of T84 cells after 4h of incubation. *In vivo* studies by Ukena et al. (2007) demonstrated that colonization of gnotobiotic mice with EcN causes an increase in ZO-1, but not ZO-2, expression. In conventionally colonized mice challenged with DSS to induce colitis, probiotic pretreatment lessened disease and induced ZO-1 expression. Conversely, Zareie et al. (2006) found that *L. rhamnosus* and *L. helveticus* did not alter ileal or colonic permeability in rats. It has also been concluded that studying colonization effects of probiotics in only germ-free mice is a concern because these mice display numerous morphological and immunological differences that could affect results. Specific pathogen-free mice or antibiotic-treated and mono-associated with a specific bacteria would be an important complement to germ-free models and perhaps more clinically relevant.

Extracellular proteins/peptides of probiotics and intestinal barrier function

Scientific evidences suggests that extracellular proteins secreted and released into the environment by probiotic bacteria might mediate certain interactions, since they would be able to interact directly with mucosal cells, such as epithelial and immune cells. These interactions could be responsible for some of their mechanisms of action (Sa´nchez et al., 2008). Therefore, it is assumed that certain extracellular proteins secreted by probiotic bacteria might also reach the gut mucosa, acting as molecular effectors responsible for downstream responses in mucosal cells. Different studies suggest that extracellular proteins secreted by probiotic lactobacilli might promote the stabilization and enhancement of the mucosal barrier function by increasing the production of human defensins, secretion of mucus and the concentration of Heat Shock Proteins (HSPs) in epithelial cells.

Neutrophils are recruited in the intestinal mucosa from the blood vessels by means of the secretion of inflammatory cytokines, and are, therefore, involved in the inflammatory episodes. Ivanov et al. (2006) identified, the first extracellular protein, serine protease inhibitor (serpin) from *Bifidobacterium longum* subsp. *longum* NCC2705 that interacted directly with the host factors. It has been shown that serpin efficiently inhibits pancreatic and neutrophil elastases (Ivanov et al., 2006). Also, bifidobacterial serpin, acting on enzymes directly involved in the inflammatory response, might thus mediate some of the anti-inflammatory effects of bifidobacteria (Ivanov et al., 2006).

Tight junctions in epithelial cells are the key components for the regulation of the mucosal barrier function, selectively affecting the movement of solutes and water through the epithelium. Ewaschuk et al. (2008) studied uncharacterized extracellular proteins secreted by *B. longum* subsp. *infantis*, isolated from IL-10-deficient mice, which lack the ability to produce sufficient quantities of the anti-inflammatory cytokine IL-10. The mechanism of action of these extracellular proteins is mediated by the modulation of two cytoplasmic mitogen-activated protein kinases (MAPKs): an increase in the levels of extracellular regulated kinase (ERK) together with a decrease in p38 MAPK (p38).

Hoarau et al. (2008) studied interaction between extracellular proteins secreted by *B. breve* C50 with the TLR-2 present on the surface of immature human DCs, inducing different functional and physiological changes through different pathways. Prolonged DC survival was mediated by the PI-3K pathway, DC maturation by the p38 and PI-3K pathways, increase in IL-10 production by the MAPK (p38, ERK) and PI-3K pathways, and finally an increase in IL-12 production was mediated by means of the p38 and GSK-3 pathways.

Schlee et al. (2008) studied uncharacterized extracellular proteinaceous compounds secreted by *Lactobacillus acidophilus* PZ 1138, *Lactobacillus fermentum* PZ 1162 and *Lactobacillus paracasei* subsp. *paracasei* LMG P-17806 with an ability to induce production of the antimicrobial peptide human β-defensin 2 (hBD2) in epithelial cells. The signal of these extracellular proteins was shown to be transduced to the nucleus through the MAPKs, ERK, p38 and c-Jun terminal kinase (JNK), where hBD2 synthesis was increased through the modulation of nuclear factor kB (NF-kB) and activator protein 1 (AP-1), ending finally in an increase of IL-8 production. Lu et al. (2009) identified two antimicrobial peptides, NPSRQERR and PDENK, present in *L. rhamnosus* GG conditioned media, which possessed antimicrobial activity against *E. coli* EAEC 042, *Salmonella enterica* serovar *typhimurium* and *S. aureus*.

Caballero-Franco et al. (2007) studied that extracellular protein secreted by the lactobacilli contained in a probiotic formula (*L. plantarum, L. acidophilus, L. casei* and *L. delbrueckii* subsp. *bulgaricus*) that induced mucin secretion through muc2 gene expression in murine colonic epithelial cells, although the possible signal transduction pathways involved are not yet known. Further, Tao et al. (2006) observed that extracellular proteins present in growth media

conditioned by *L. rhamnosus* GG had an ability to increase the production of the heat-shock proteins HSP25 and HSP72 in young adult mouse colon (YAMC) cells. While on one hand HSP overproduction was due to the transcriptional regulation mediated by heat-shock factor 1 (HSF-1); on the other hand, the MAPKs p38 and JNK were also shown to be involved in the signaling pathway leading to HSP increase.

L. rhamnosus GG extracellular proteins, p40 and p75, are perhaps the best studied extracellular proteins. p40 is homologous to an uncharacterized surface antigen of *Lactobacillus casei* ATCC 334 (gi|116493594), whereas p75 is homologous to a cell wall-associated hydrolase of the same bacterium (gi|116493849) (Yan et al., 2007). Yan and co-workers have shown that both p40 and p75 are efficient growth promoters, as they induced the proliferation of YAMC cells by activating protein kinase B (AKT) through a PI-3K-dependent pathway. In the same study, these two proteins were also able to reduce the colon injuries induced by tumour necrosis factor alpha (TNF-α) in murine colon tissue explants (Yan et al., 2007). Moreover, the two proteins completely inhibited TNF-α-induced apoptosis in the KSRI2/2 MCE cell line, which undergoes apoptosis following TNF-α treatment (Yan et al., 2007). Also, p40 and p75 were capable of attenuating the TER decrease induced by hydrogen peroxide, and preventing the rearrangement of several TJPs (occludin, zonula occludens-1, E-cadherin and b-cathetin) through a protein kinase C- and MAPK-dependent signaling pathway (Seth et al., 2008). p40 and p75 thus appear to be important secreted proteins for GIT homeostasis, involved in both cell proliferation and apoptosis, and in the maintenance of the mucosal barrier.

Conclusions

Reports on probiotic health benefits have exaggerated the interest of gut microbiologist to unravel the molecular mechanism underlying these beneficial effects. Several surface associated and extracellular components can be targeted to explore the underlying mechanism as these molecules can interact directly with mucosal cells, such as epithelial and immune cells. Probiotic extracellular proteins could be linked to some of the beneficial effects ascribed to the corresponding strains. To date, only few proteins have been identified and characterized. Therefore, the understanding of the interaction between host epithelium and extracellular proteins may lead to development of novel strategy to reverse some of the processes involved in the initiation, or perpetuation, of various gastrointestinal disorders.

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