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Novel perspectives in probiotic treatment : the efficacy and unveiled mechanisms of the physiological functions

Fujiya, Mikihiro ; Kohgo, Yutaka

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Mikihiro Fujiya, Yutaka Kohgo

Division of Gastroenterology and Hematology/Oncology, Department of Medicine, Asahikawa Medical College

Running title

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Correspondence

To whom correspondence should be addressed:

Yutaka Kohgo, MD, Ph.D.

Division of Gastroenterology and Hematology/Oncology, Department of Medicine, Asahikawa Medical College, Asahikawa, Japan

2-1 Midorigaoka-higashi, Asahikawa, Hokkaido 078-8510, Japan

Tel: +81-166-68-2462

Fax: +81-166-68-2469

e-mail: yk1950@asahikawa-med.ac.jp

Abstract

Probiotics are defined as “live microorganisms which confer a health benefit on the host” when administered in adequate amounts, and have potential effects for maintaining intestinal development, nutrition, and treating intestinal inflammations, functional disorders, and other extra-intestinal diseases. Although the benefits of probiotics for human health were first noted over 100 years ago, the analysis of probiotic functions began in earnest only 20 years ago. Probiotics, such as some strains of Lactobacillus, Bifidobacterium, Escherichia coli, and Bacillus subtilis, inhibit the growth of pathogenic bacteria, induce competitive effects for the adherent of pathogenic bacteria and their toxins to intestinal epithelia, induce cytoprotective heat shock proteins, enhance the intestinal barrier function and modulate the host immune responses. The crosstalk between the host and the probiotics appears to be mediated by bacteria-derived effectors, which can be sensed with multiple systems, including the Toll-like receptors and cell membrane transporters. Future analyses will identify more probiotic-derived effectors, the recognition mechanisms of these effectors, and the subsequent changes of the intestinal epithelia and immune cells for each probiotic treatment. For clinical use, a procedure that objectively evaluates the ability of each probiotic effect will help establish a standard for choosing the most valuable strain and

its proper dose for each individual patient.

Introduction

The mammalian intestine continuously comes in contat with prokaryotes, which are indispensable partners for developing intestinal tissue and maintaining its homeostasis. The International Human Microbiome Project (1) (2) has provided a tremendous amount of information on commensal bacteria and has ushered in a new era for the field of gastroenterology. It is clear that more than 2,000 species of commensal bacterial organisms exist in our bodies, and the majority is located in the gut, but most of these bacteria are not pathogens (3). Recently, new ribosomal RNA- and whole genome-based technologies have highlighted that the populations of microbial species differ between individuals, regions of the gut axis (4), and different mucosal layers at a single anatomical site, thus illustrating the diverse and complicated role of "the partner" in our biological activities (5) (6).

Probiotics, which are defined as "live microorganisms which confer a health benefit on the host, when administered in adequate amounts" (7), have potential effects for human health as well as the treatment of intestinal disorders. Indeed, many etiological studies have proposed the beneficial effects of probiotics since 100 years ago (8) (9) (10). Several recent meta-analyses and systematic reviews suggest that probiotics are potentially useful preventive or therapeutic strategies for multiple gastrointestinal

diseases, while the data from these studies were based on various subjects using different genera, species, strains, and doses of probiotics (11) (12) (13) (14) (15) (16) (17) (18). However, data from probiotic treatments remain preliminary thus far, and are influenced by the diversity of the microbiota, diets, and genetic backgrounds of the individual subjects. It is crucial to elucidate the mechanisms of certain probiotic functions. Early mechanistic investigations involved the remodeling of microbial communities to suppress the growth and activity of pathogens, particularly in acute infections. Subsequently, probiotics were found to cause various effects, including the upregulation of anti-inflammatory factors, immunomodulation via the suppression of pro-inflammatory mediators, enhanced immunity, epithelial cell differentiation and proliferation, and promotion of intestinal barrier function. In the present review, the clinical and physiological effects of probiotics and novel perspectives in the mechanistic studies of the probiotic effects are discussed.

1. Clinical benefits of probiotics

There is increasing evidence that probiotics are required for the development of a healthy intestinal system, the promotion of host nutritional status, and the treatment and prevention of intestinal infections, and functional disorders of the gastrointestinal

tract.

1) Intestinal development

It is known that the intestinal microbiota are important for the development of the normal gut, particularly in maintaining normal immunological reactions and cytoprotective responses, and also in regulating apoptosis (19) (20) (21). Indeed, LGG decreases chemically induced apoptosis and increases the expression of genes involved in cytoprotective responses in the developing mouse small intestine (22). The administration of Lactobacillus casei DN-114001 appears to ameliorate the gut immune response through the stimulation of macrophages and dendritic cells (23). These studies demonstrate that the probiotics may thus play a significant role in intestinal development.

2) Nutrition

Intestinal microbiota are heavily involved in the regulation of human and other host nutritional status, including the promotion of polysaccharide digestion and nutrient uptake and the regulation of the energy balance by cooperation with intestinal epithelial cells (24) (25). Intestinal microbiota regulate energy balance by using and

storing the calories harvested from the host's diet (26) (27). Furthermore, a recent study has shown the deviations from a gut core microbiome, which shares the genes involved in various metabolic functions, to be associated with aberrant physiological states, such as obesity (28). Supportive evidence concerning the probiotic effects on the prevention and treatment of obesity has been recently addressed by clinical trials (29) (30).

3) Intestinal disorders

A) Infectious diseases

There are many studies which reveal the therapeutic effects of probiotic treatment for several types of the infectious enteritis (31) (32). Travelers' diarrhea is one of the common diseases among travelers to foreign countries, particularly in Africa and Southeast Asia. From the data of more than 10 randomized controlled studies, probiotic treatments have been shown to be safe and effective for the prevention of travelers' diarrhea (33) (34). However, this effect appears to be dependent on the population of each study, the type of probiotics used, the duration of treatment, the trip destination, and the compliance with treatment. Otherwise, the therapeutic and preventive effects of probiotics such as VSL#3, a mixture of 8 probiotic bacteria, and *Lactobacillus rhamnosus GG (LGG)* for rotavirus diarrhea have also been proposed in randomized

controlled studies (35) (36).

B) Inflammatory bowel diseases

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the gastrointestinal tract. The most common types of IBD are ulcerative colitis (UC) and Crohn's disease (CD), with the prevalence of IBD estimated to be 150,000 patients in Japan. Although the precise etiology of IBD is still obscure, the alterations in the intestinal microbiota are thought to play an important role in the pathogenesis of IBD. Many trials were therefore conducted to manipulate the intestinal microflora using probiotics, including VSL#3, LGG, and E. coli Nissle 1917, and several potential therapeutic applications for UC have been conducted for the induction of remission and the relief of clinical symptoms (37) (38) (39) (40), maintenance of the clinical remission (41) (42), and prevention of the onset of pouchitis (43) (44) (45) (46). Although controversial results have also been reported (47), the administration of probiotics appears to be a promising modality for the treatment of UC. Conversely, the potential use of probiotics in the prevention or treatment of CD remains unclear. Several clinical studies have been performed to analyze the effects of probiotics on inducing clinical remission or maintaining surgically-induced remission using LGG or Lactobacillus johnsonii LA1. While one study found a modest reduction in the recurrence rates of

patients after a surgical resection by supplementation with Lactobacillus johnsonii LA1, other studies revealed no benefit of probiotic administration for CD treatment **(Table 1)**. The reason that no benefits were obtained by the administration of probiotics may be linked to a poor understanding of both the mechanisms of probiotics and the pathogenesis of CD.

C) Irritable bowel disease

Irritable bowel disease (IBS) is a functional gastrointestinal disorder with low-grade inflammation and immune responses as well as changes in the fecal microflora (48), suggesting potential probiotic benefits for its treatment. Indeed, various probiotics including Lactobacillus, Bifidobacterium, and Saccharomyces are shown to ameliorate IBS symptoms such as a decrease in flatulence, abdominal pain, and bloating (53) (54) (55) (56) (57) (58). Zeng et al. proposed that the fermented milk containing Streptococcus thermophilus, Lactobacillus bulgaricus, L. acidophilus, and B. longum decreases small bowel permeability in patients with diarrhea–predominant IBS and improves mucosal barrier function for IBS patients (54). Other systematic reviews on basic and clinical studies have also emphasized the effectiveness of probiotics on IBS treatment (55) (56) (57) (58). Standardized species and strain selections and the optimal dose of probiotics for IBS therapy will likely be established for clinical applications in

future.

D) Necrotizing enterocolitis

Necrotizing enterocolitis (NEC) is an inflammatory necrosis of the intestine, typically observed in premature infants. A number of studies have shown that probiotic treatment reduces the incidence as well as the severity of NEC (59) (60). A meta-analysis demonstrated that probiotics supplementation, including Bifidobacteria (B. infantis, B. bifidum, and B. breve), Lactobacillia (LGG, L. acidophilus), S. thermophilus and S. boulardii, and E. coli Nissle exhibited beneficial effects for NEC treatment (61). However, in general, probiotic treatment is regarded to be less effective for reducing the risk of sepsis and the number of days on total parenteral nutrition (62) (63). The mechanisms responsible for probiotic effects in the treatment of NEC patients are still poorly understood.

E) Antibiotic-associated enteritis

Antibiotic-associated enteritis is frequently observed in patients administered antibiotics, which can lead to the suppression of the normal gut microflora, thus causing a change in the gut microflora and the overgrowth of pathogenic bacteria. Typical antibiotics-associated enteritis includes acute hemorrhagic enteritis and pseudomembranous enterocolitis. Various meta-analyses have shown that probiotics

successfully prevent antibiotic-associated enteritis (64) (65) (66). Probiotics aid the recovery of the gut microflora following antibiotic impairment. Antibiotics-associated enteritis is one of the diseases that benefit the most from probiotic treatment.

4) Extra-intestinal disorders

A) Helicobacter pylori infection

Recent clinical trials revealed the beneficial effects of probiotics with antibiotic treatment for the eradication of Helicobactoer pylori (H. pylori) infection (67) (68) (69). Almost all of the studies utilized Lactobacillae for these treatments. The mechanisms of H. pylori infection eradication were mediated by the antibiotic functions of lactic acid, bacteriocins, or other active components, competition for H. pylori adhesion to the gastric epithelia, enhancement of mucosal barrier function, and the modification of inflammatory mediators (70) (71) (72) (73) (74) (75) (76) (77) (78) (79). Probiotics can also relieve the side effects for the eradication of H. pylori infection (80) (81) (82) (83) (84) (85) (86) (87). However, no evidence has yet shown that probiotic treatment directly eradicates H. pylori. The clinical indications of probiotics for the treatment and eradication of H. pylori infection still remain unclear.

B) HIV infection

It is known that early HIV replication and $CD4^+$ cell destruction frequently occur in the gastrointestinal tract. HIV-associated enteropathy is one of the critical outcomes of AIDS. A pilot study proposed that yogurt containing L. rhamnosus GR-1 and L. reuteri RC-14 improved both diarrhea and $CD4^+$ cell number in HIV-infected patients (88) (89). Furthermore, the combination of L. rhamnosus GR-1, L. reuteri RC-14, and antibiotics increase the cure rate of bacterial vaginosis (90), thus suggesting a beneficial probiotic effect in both the management of HIV enteropathy and in reduced disease progression.

B) Others

It has also been reported that probiotic treatment is useful for curing urinary tract infections (91) and atopic diseases (92).

2. Mechanisms of probiotic functions

As mentioned in the previous section, probiotics are gaining widespread inclusion as a new preventive or therapeutic strategy for multiple gastrointestinal and extra-gastrointestinal diseases. However, these data are collected from an epidemiological analysis with the empirical selections of probiotics. In order to objectively determine how to choose the suitable strain for each disease, the mechanisms of probiotic functions must be clarified. In this regard, the mechanistic

analyses of probiotic functions for pathogenic bacteria as well as the host intestine are now under investigation **(Figure 1)**.

1) Growth inhibition of pathogenic bacteria

Probiotics exhibit direct antibacterial effects for pathogenic bacteria through the production of antibacterial substances, including bacteriocins and acid (93) (94). Gram-positive bacteria such as Lactococcus lactis produce small antimicrobial peptides, or lantibiotics (93) (95), which have been found to be an inhibitor for pathogenic bacteria by targeting the lipid II component of the bacterial cell wall (96). Bacteriocins are other small antimicrobial peptides produced by Lactobacilli including L. plantarum, L. acidophilus NCFM, L. johnsonii NCC 533, and L. sakei (97)(98)(99)(100). Bacteriocins exert an antimicrobial effect with a narrow spectrum and toxicity for Gram-positive bacteria such as Streptococcus, Staphylococcus, Lactococcus, Listeria, and Mycobacteria. Bacteriocin acts by forming pores in the cytoplasmic membrane and inhibiting the essential enzyme activities of sensitive bacteria.

Strains of Lactobacilli produce acetic, lactic, and propionic acids that lead to inhibited growth of a wide range of Gram-negative pathogens, such as *Salmonella enteric,* by lowering the local pH (101). Moreover, other unknown bactericidal substances derived from the Lactobacillus strain exhibit antibacterial effects in a

pH-dependent manner (101) (102).

2) Inhibition of the adherence of pathogenic bacteria or their toxin to intestinal epithelia

Probiotics, including several strains of Lactobacilli and Bifidobacteria, competitively decrease the adhesion of both pathogenic bacteria and their toxins to the intestinal epithelium (103) (104) (105) (106). Pathogenic bacteria are thought to bind to intestinal epithelia through the interaction between bacterial lectins and carbohydrate moieties of glycoconjugate receptors on the epithelial surface. Probiotics have lectin-like adhesion and proteinase-like components, which mediate the inhibitory effect of probiotics for the adherence of pathogenic bacteria to the intestinal epithelium (107)(108)(109). Probiotics also function to block bacterial enterotoxin binding. A recombinant E. coli generated to express the glycosyltransferase genes and produce chimeric lipopolysaccharide, which neutralizes enterotoxins, has been reported to reduce mortality by virulent V. cholerae infection in mice (110) (111).

3) Inductions of cytoprotective heat shock proteins

Heat shock proteins (Hsp) are essential for the maintenance of intestinal homeostasis, by protecting colonic epithelial from injury and foreign stress (112) (113). Hsps expression is changed in the intestinal epithelia of IBD patients, suggesting the

association of Hsps in the pathogenesis of IBD (114) (115). Tao et al. has shown that soluble factors in LGG-conditioned media induced Hsps in intestinal epithelial cells, which was mediated by the activation of the p38- and JNK/MAPK pathway (116). We have also demonstrated that Bacillus subtilis (B. subtilis) and its conditioned media induced Hsps in Caco-2/bbe cells and mouse intestine. In addition, we found that the B. subtilis-produced factor, competence and sporulation factor (CSF) mediates the Hsp induction and protective effect of B. subtilis against oxidant stress (117), thus illustrating that the probiotic effects, at least in part, can be mediated by the induction of the cytoprotective Hsps.

4) Enhancement of intestinal barrier function

The intestinal barrier function is an important mechanism for defending epithelial cells against the attack of foreign virulence. The impairment of the intestinal barrier function causes the disruption of the intestinal integrity. Probiotics including *E. coli* Nissle 1917, LGG, L. casei DN-114 001, VSL#3, L. acidophilus, and Bacteroides tetaiotaomicron augment the intestinal barrier function following pathogenic bacteria, hydrogen peroxide, or cytokine-induced disruption of epithelial tight junctions (118) (119 (120) (121) (122). This improvement in intestinal barrier function is mediated through the expression and translocation of zonula occludens (ZO)-2, protein kinase C

(PKC), and/or the activating mitogen-activated protein kinases (MAPKs). Our recent study also demonstrated the protective effect of B. subtilis and its soluble factor CSF against oxidant stress-induced intestinal injury by promoting epithelial barrier function (117).

5) Modulations of host immune responses

Probiotics have the potential to modulate both innate and adaptive immunity. Recent reports suggest that fecal sIgA is increased by Bifidobacterium animalis (123) as well as nonviable LGG, which augments interleukin (IL)-6 secretion (124). Escherichia coli Nissle 1917 and VSL#3 induce β-defensin (125) (126), which is a pivotal antibacterial peptide preventing bacterial adherence and invasion. B. tetaiotaomicron also stimulates the release of an antimicrobial peptide, angiogenin 4 from Paneth cells, suggesting the significant role of probiotics in host innate immunity.

Probiotics also exert an inhibitory effect on the production of pro-inflammatory mediators. LGG or its conditioned media inhibits lipopolysaccharide- (LPS-) or Helicobacter pylori-stimulated TNF production by murine macrophages (127). E. coli Nissle 1917 suppresses TNF, IFN-γ, and IL-2 release, and increases IL-10 expression by T-cells (128). L. casei Shirota decreases IL-6 and IFN-γ production induced by LPS in peripheral blood mononuclear cells of normal and colitis mice (129). LGG and L.

rhamnosus GR-1 also induce granulocyte-colony stimulating factor (G-CSF) from macrophages, which inhibits E. coli- or LPS-induced TNF production (130). On the other hand, it is proposed that VSL#3, E. coli Nissle 1917, Lactobacillus reuteri, and L. casei suppress excess inflammation through inducing IL-10 (128) (131) (132) (133). Conversely, probiotics enhance the host immune response when the host suffers from pathogenic bacteria infection. It is reported that dendritic cells take up a polysaccharide (PSA) of Bacteroides fragilis, which induces the maturation of dendritic cells and the production of Th1-type cytokines (134). Therefore, probiotics regulate the host immune responses for each condition through the modulation of immune cells such as dendric cells, and by inducing production of pro- and anti-inflammatory factors.

NF-κB signaling is a pivotal pathway for epithelial interactions with immune cells. The excess activation of this pathway was believed to cause intestinal injury by inflammatory disorder. Probiotics, including L. reuteri, LGG, B. infantis, and L. salivarius, have been reported to downregulate the expression of NF-κB-induced pro-inflammatory mediators such as IL-8 in intestinal epithelial cells (135) (136) (137) (138). Bacteroides thetaiotaomicron and Enterococcus faecalis inhibit NF-κB function through the promotion of the nuclear export of the RelA subunit of NF-κB by the activation of peroxisome proliferators activated receptor (PPAR)-γ (139) (140). Therefore,

probiotics may exhibit an inhibitory effect on NF-κB signaling, and thereby regulate excess inflammation. However, a recent study has proposed that the deficiency of NF-κB leads to the promotion of intestinal epithelial apoptosis, impairment of antimicrobial peptide expression, and subsequent chronic inflammation (141). It is still unclear whether probiotics affect the regulation of the NF-κB-related pathways and subsequent intestinal inflammation.

3. How do we sense probiotics?

As mentioned above, probiotics exhibit competitive effects for pathogenic bacteria as well as beneficial effects for host intestinal tissues, thus indicating the significance of the crosstalk system between the host and the probiotics. Some probiotic supernatants exert the beneficial effects similarly as probiotics themselves. The bacterial components or soluble factors released by bacteria in the supernatants are sensed by intestinal epithelia by some mechanism.

Toll-like receptors (TLRs), which belong to the family of pattern recognition receptors (PRRs), are expressed in both intestinal epithelial and dendritic cells. TLRs are candidates that recognize the factors or bacterial components, including microorganism-associated molecular patterns (MAMPs), flagella, lipoteichoic acid,

peptidoglycan, lipopolysaccharide, and cell wall-associated polysaccharide (CPS), originated from probiotics. It has been proposed that the physiological effect of E. coli Nissle 1917 for improving the intestinal inflammation of the mouse enteritis model is diminished in TLR2- or TLR4-deficient mice (142). Conversely, the VSL#3 function for relieving mice colitis has also been reported to decrease in TLR9-deficient mice, but not in TLR2- or TLR4-knockout mice (143), thus suggesting that specific TLRs are involved in the probiotic functions. However, the target PRR of each probiotic is unidentified, and it is unclear how TLRs distinguish the probiotic-derived molecules from those of pathogenic bacteria.

Our recent study shows that the B. subtilis-derived peptide CSF is imported by the epithelial cell membrane transporter protein (117), a novel organic cation trasporter 2 (OCTN2), whose gene polymorphism is susceptible to Crohn's disease (144). Furthermore, the protective effects of B. subtilis and its soluble factor CSF on the intestinal epithelia are reduced by inhibition of OCTN2 function (117), suggesting that OCTN2 transport is essential for the effects of B. subtilis and CSF. This suggests a novel mechanism for sensing probiotics through the uptake of the soluble factors produced by bacteria. Consequently, multiple systems may be involved in recognizing and discriminating bacteria, including commensals, pathogens, and probiotics **(Figure**

2).

4. Future outlook

While more than 100 years have elapsed since the probiotic benefits on human health were noted, the mechanistic analysis of probiotic functions has been given recognized only in the past 20 years, and many of the mechanisms of probiotic effects are still unclear. It should be established the standard for selecting appropriate strain for the treatment of individual disease through clarifying the physiological function and their mechanism of each probiotics. While controversial findings have also been reported, the proper strain and dose of probiotics are essential for treating intestinal disorders, even neoplasms (145) (146) (147) (148) (149) (150) (151) (152). The identification of effective molecules produced by probiotics, which mediate the probiotic functions, is essential for providing clear mechanisms. This enables the development of novel therapeutic strategies for intestinal disorders using bacteria-derived effectors. Furthermore, the procedure to objectively evaluate and quantify the ability of each probiotic effect will help to establish a standard for choosing the most valuable strain and its proper dose for individual cases.

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Figure legends

Figure 1 Mechanisms of the probiotic functions

Probiotics can exert physiological functions through the inhibition of pathogenic bacteria growth and the adherence of pathogenic bacteria or their toxin to intestinal epithelia, the induction of cytoprotective heat shock proteins, the enhancement of the intestinal barrier function, and modulations of the host immune responses.

Figure 2 Bacterial components or soluble factors released by bacteria are sensed by the intestinal epithelia in some manner (proposal).

Probiotics release bacterial components and effectors which are possibly sensed by TLRs or transported by cell membrane transporters such as OCTN2. Activated downstream of TLRs led to the modulation of host immune responses, while probiotics-derived effectors imported by cell membrane transporters are thought to activate some cell signaling pathway and induce Hsps, thus leading the protection of the intestinal epithelia.

CSF, competence and sporulation factor; LPS, lipopolysaccharide; PG, peptidoglycan; OCTN2, novel organic cation transporter 2; TLR, Toll-like receptor; MAPK, mitogen-activated protein kinase; TRAF6, TNF receptor-associated factor 6; Hsp, Heat

shock protein; NF-κB, nuclear factor-κB;