The Microbiome in Celiac Disease

Suneeta Krishnareddy, MD, MS

KEYWORDS

• Microbiome • Celiac disease • Probiotics • Prebiotics

KEY POINTS

- The microbiome is vital for normal immune cell function and development.
- Alterations in the microbiome, or dysbiosis, is associated with celiac disease, as well as many other diseases.
- The microbiome is an important target for therapeutic potential in celiac disease.
- Prebiotics and probiotics are being studied as potential therapies in celiac disease, but further studies need to be done to elucidate their role.

INTRODUCTION

The human gastrointestinal tract is a complex and dynamic environment, sheltering a vast number and variety of commensal microorganisms.[1] This balanced microecosystem provides a natural defense against invasion of pathogens. Recently, much research has focused on the role of the human microbiome in health and disease, and the ability to harness the power of the human microbiome for treatment of these diseases.

Celiac disease (CD) is a complex multifactorial disorder involving both genetic and environmental factors. For many years, the only securely established genetic factors contributing to CD risk were various genetic variants located within the HLA region (those encoding the HLA-DQ2/DQ8 heterodimers).[2] With the introduction of genome-wide association studies and the immunochip study, an additional 39 non-HLA regions of susceptibility have been associated with CD development, some of which share with other autoimmune diseases.[3] Interestingly, most of the chromosome regions associated with CD predisposition contain genes with immune-related functions, and some CD susceptibility genes play a role in bacterial colonization and sensing. Studies also have shown an altered expression of nonspecific CD-risk genes involved in host-microbiota interactions in the intestinal mucosa of patients with CD,

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Digestive and Liver Diseases, Columbia University, 180 Fort Washington, New York, NY 10032, USA
E-mail address: Sk3222@cumc.columbia.edu

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such as those of toll-like receptors and their regulators.[4] Disturbances in the host-microbiota interaction and shifts in the immune balance in subjects with CD might propagate the inflammatory response by gluten, which is pathognomonic to CD.[5]

NORMAL COLONIZATION AND ITS IMPACT ON HUMAN DEVELOPMENT

Initial colonization of the infant gut by microbes sets the stage for the lifelong, relatively stable adult microbiome. Infants rely on colonization to complete development of the immune system and gastrointestinal tract. The first days and weeks of life represent a crucial window of opportunity for shaping the development of the gastrointestinal tract and immune system, as well as the future adult microbiome. "Normal colonization" likely affords protection of developing childhood and adult diseases. The evidence of the contribution of the microbiome to healthy immunity and defense against multiple diseases is growing, as the list includes many nonintestinal diseases, such as obesity, food allergies, and diabetes mellitus, in addition to intestinal-related autoimmune disorders, such as inflammatory bowel disease and CD.

In vaginal birth, the infant is inoculated as he or she passes through the birth canal. This inoculum is a mixture of gram-negative and gram-positive bacteria, aerobes, and anaerobes. This initial colonizing species has been shown to be important in establishing a "pioneer microbiome" that in turn educates the developing immune system and provides favorable conditions for colonization by subsequent microbes.[6]

Beneficial infant colonization is dependent on the maternal microbiota, which in turn is influenced by maternal genetics, environmental exposures, and diet before and during pregnancy as well as during breast-feeding. Once the infant has been inoculated, compounds already present in the infant gut as well as from breast milk act as prebiotics and encourage growth of commensals. Priming for microbial colonization begins in utero. The vernix caseosa, the waxy skin coating of a fetus, is shed into the amniotic fluid as the fetus approaches term. While still in utero, the fetus swallows amniotic fluid containing pieces of vernix. The vernix is made up of short-chain fatty acids (SCFAs) and lipids. Although these SCFAs and lipids are indigestible by human enzymes, they provide a rich medium for growth of bacteria. These prebiotics continue to be administered to the infant in the case of breast-feeding. Colostrum contains especially high concentrations of human milk oligosaccharides (HMOs), which are indigestible by human enzymes, but like the vernix SCFAs, promote growth of intestinal microbes.[7] The HMOs selectively promote growth of commensals, such as *Bifidobacterium longus* subspecies *infantis*, and suppress growth of pathogens, like *Escherichia coli* and *Clostridium perfringens*. Furthermore, growth of these bacteria on HMOs alters their activity, making *Bifidobacterium infantis* more bound to intestinal epithelial cells, which then affords a stronger barrier promoting an anti-inflammatory effect.[8] These prebiotics give commensals an advantage over pathogens in the developing infant gut, which helps prevent newborn enteric infections, as well as laying the foundation for a strong immune system.[9]

NORMAL COLONIZATION MAINTAINS HOMEOSTASIS OF THE IMMUNE SYSTEM

The microbiota play a role in shaping the architecture of the immune system (such as Peyer patches), the development of specific immune cell populations (such as regulatory T cells) and the balance between immune cell types. The 2 features of the gastrointestinal immune architecture most affected by microbiota are the mucous layer and gut-associated lymphoid tissue (GALT), which includes Peyer patches.[10]

The mucous layer maintains spatial segregation between the bacteria-rich gut lumen and the intestinal epithelium. This bacteria-free zone (approximately 50-μm

thick) protects against otherwise continuous immune stimulation and inflammation. This segregation serves to augment the barrier function of the epithelial layer, which is only 1 single cell thick.[11] Intestinal microbes provide the stimuli for maintenance of the mucous layer. Germ-free animals have thinner mucous layer than conventional animals, with specific members of the microbiome contributing to production of mucous, such as *Akkermansia muciniphila* and *Lactobacillus* species.[12]

The innate immune system maintains the sterility of the mucous layer. Intestinal epithelial cells produce antibacterial RegIIIγ in an MyD88-dependent manner. RegIIIγ is an antibacterial C-type lectin that targets gram-positive bacteria. RegIIIγ knockout mice exhibited increased adaptive immune activation, increased fecal immunoglobulin (Ig)A and increased Th1 cells in the lamina propria. These increases were dependent on the intestinal microbiota. This physical segregation of the microbiota from the intestinal epithelium is an example by which the innate immune system maintains tolerance of the intestinal microbiota by limiting contact with the adaptive immune system.[13]

A healthy microbiome further protects the host by forming its own protection against colonization, a phenomenon known as colonization resistance. As mentioned previously, microbes stimulate the mucous layer and also stimulate the epithelium to secrete antimicrobial peptides into the mucous layer, providing a barrier against pathogens.[14] Commensals themselves can produce substances to prevent infections, such as acetate production by *Bifidobacterium*, which protects again enterohemorrhagic *E coli* O157:H7.[15]

In addition to the microbiome's effect on the physical barrier promoting immune tolerance, the microbiota also stimulates the formation of GALT. It has been shown that germ-free animals have dramatically reduced germinal centers in the GALT, and reduced secretory IgA.[16,17] IgA produced by the GALT acts in an immunomodulatory manner. Intestinal dendritic cells sample the intestinal lumen; when they come in contact with bacterial polysaccharide A (PSA), a component of the commensal *Bacteroides fragilis*, they then stimulate the adaptive immune response to secrete IgA, locally. This locally produced IgA then coats the bacterial antigen, resulting in decreased activation of the innate immune response.[18] In this way, GALT functions as a self-contained immune system, recognizing bacterial antigens and stimulating the immune response, but the response is contained to the mucosal compartment, thereby avoiding systemic inflammation. A study done in Swedish infants showed that increased diversity of *Bifidobacterium* species is associated with increased IgA,[19] which has been linked to protection against allergy and autoimmunity.[20]

These data support the role of the initial microbiome and "beneficial" bacteria in regulating the adaptive and innate responses.

MICROBIOTA BALANCE AND IMMUNE RESPONSES

The rapid colonization after birth shifts the perinatal immune system of that of hyperstimulation to that of tolerance. In this system, the neonatal gut allows colonization by microbes and a specific population of CD71+ erythroid cells dampens the innate immune response.[21] In addition to anti-inflammatory signals from the host, these signals also come from the colonized microbiota. SCFAs produced by host bacteria affect regulatory T-cell (T_{reg}) populations.[15] Butyrate (a commonly produced SCFA) increases the differentiation of progenitor cells to become T_{reg} cells, and SCFAs, in general, specifically expand the population of colonic T_{reg} cells.[22]

The microbiota also plays a key role in regulating the balance between populations of CD4+ helper T cells, Th1 and Th2 cells. During the perinatal period, the immune

system is skewed toward a Th2 cytokine milieu; however, persistence of this Th2 environment has been associated with atopic diseases. This shift is caused by a bacterially derived carbohydrate. Gut dendritic cells protruding through the intestinal epithelium sample commensal *B fragilis* from the gut lumen; this sampled PSA is transported to the systemic immune system where it restores the balance between Th1 and Th2 cells.[23] It has been considered that the onset of CD is mediated by a skewed Th1 response. Although the exact cause of this skewed response is unknown, it can be suggested that the balance of these CD4+ cell subsets depends on a balanced microbiome.[24]

Similarly, Th17 cells, which are mostly proinflammatory cells that protect against infection at the mucosal surfaces, are also regulated by the microbiota, specifically, Th17 cells are induced by segmented filamentous bacteria (SFB).[25] In mice, SFB act via major histocompatibility complex class II (MHC-II) on intestinal dendritic cells to increase differentiation of CD4+ T cells into Th17 cells in the lamina propria.[26] These Th17 cells, which have been shown to have critical functions in host defense against bacterial pathogens and the inflammatory response to deamidated gliadin peptides, are important in the pathogenesis of CD.[27]

Through appropriate colonization and the resulting "education" of the gastrointestinal immune system, infants develop more optimal gut function. This early priming of the immune system is critical for later life. Dysbiosis is abnormal colonization, or the imbalance of microbes inhabiting a certain part of the body. The 4 known categories promoting intestinal dysbiosis are (1) abnormal microbial exposures, (2) disruptions in diet, (3) antibiotic usage and other medications, and (4) influence of host genetics.

Abnormal microbial exposures can occur at time of delivery, such as cesarean versus vaginal delivery. A study done in 2010 showed that infants born via vaginal delivery closely resembled their mother's vaginal microbiota, whereas those born via cesarean delivery reflected the microbes present in the infant's environment (including Staph).[6] Infants born via cesarean specifically lack presence of and diversity within the *Bacteroidetes* phylum. Although it is unclear if this is the cause of increased CD seen in children born via cesarean, this association has been made.[28,29] Many studies have focused on the association between microbial colonization and disease later in life, such as obesity, asthma, and allergy, and have found that the timing of colonization is also important. These data demonstrate the importance of a "window of opportunity" for microbial education of the developing immune system, which results in persistent alteration in systemic gene expression and, potentially, persistent changes in microbial populations.[30,31]

A possible second window of development for the intestine and immune system, especially regarding oral tolerance, is the exposure to dietary antigens. Data regarding the timing of antigen introduction to reduce likelihood of an autoimmune or allergic reaction are not uniform. In the case of CD, it is often stated that gluten should not be introduced before 4 months of age and not after 6 months of age. However, a small study done showed that the delayed introduction of gluten from 6 months to 12 months resulted in a decrease in the incidence of CD, as well as the development of antigliadin IgG antibodies.[29]

The effects of antibiotics and infections on the intestinal microbiome provide further evidence for the importance of a diverse microbiome in maintaining homeostasis, particularly in the perinatal period. A recent study in mice demonstrated that early antibiotic usage had a lasting effect on immunity and metabolism, even though changes in the microbiome were transient. Mice treated with antibiotics early in life were seen to have elevated fat mass and decreased expression of immune-related genes despite

normalization of the microbiome.[31] In children and adults with HLA predisposition for CD, a gastrointestinal infection increased the risk of CD autoimmunity by 33%.[32] Another study done looking at the role of viral infections and initiation of Th1 cells, identified reovirus as a possible trigger for both the altered immune response seen in CD, as well as a factor in gliadin antigen tolerance.[33] These data highlight the importance of not only initial colonization but maintenance of "healthy" microbes in preventing disease development.

Studies of the role the microbiome in CD are evolving, and as with most studies of the microbiome, most studies have shown descriptive data, but lack cause and effect. Indeed, although CD is prevalent in both adults and children, most of the microbiome data in CD comes from studies done in children.[34–37] Studies characterizing the microbiota of adult patients with CD began only in 2012, and a single study of both children and adults reported a slight difference in the percentages of the main phyla between subjects and also a more diverse profile in duodenal biopsy specimens from adults.[38] The Firmicutes are the most abundant bacteria in adults with CD, whereas Proteobacteria are present mainly in children with CD. Other phyla shared between adults with CD and children with CD belong to the Bacteroidetes and Actinobacteria. Regarding bacterial genera, adults with CD harbor larger numbers of *Mycobacterium* spp and *Methylobacterium* spp, whereas *Neisseria* spp and *Haemophilus* spp are more abundant in children with CD. Although these studies have given us information about the general makeup of the microbiome of patients with CD, they do little to answer the questions if these changes precede disease onset, if they are a consequence of inflammation, or if the changes seen in the microbiome are associated with changes in immune cell phenotype. Future studies need to focus on causality, and possibly a specific bacterial group that could be pathogenic or protective in this group of patients, and that could be targeted for treatment.

Although it is unclear whether the altered microbiome is a cause of or consequence of disease, it is hypothesized that gram-negative bacteria in genetically susceptible individuals may contribute to the loss of gluten tolerance. If modified bacteria are a result of disease, the disrupted mucosa inundated with immature enterocytes could lead to conditions favoring gram-negative instead of gram-positive bacterial colonization. Although this theory has not been proven, early studies have shown a propensity toward higher gram-negative colonization in duodenal samples of pediatric patients with CD compared with healthy controls, in which case the dysbiosis seen seems to be of importance.[38] CD offers a unique disease in which to study the microbiome, as many other factors can be controlled for, including genetic makeup, environment, and triggers, as these are all known, and the effect of the microbiome on disease pathogenesis can be further explored. Also, because the genetic makeup can be determined before a subject acquires CD, it is possible to do longitudinal studies in these patients and observe the change in microbiome to see if the alterations noted are a cause of or consequence of disease.[39,40]

MICROBIOME AS A THERAPEUTIC TARGET

Although research into the effects of dysbiosis on the host abounds, the effects of the host on the microbiome are more limited. In a study done by Olivares and colleagues,[41] infants carrying the HLA-DQ2 haplotype influence the early microbiota composition, underlying the importance of host factors on microbial composition.

Genetic studies contribute to the concept recently described by Hooper and colleagues,[42] that the host exerts inside-out control over the microbiota, whereas the microbiota also exerts outside-in programming of host immunity and metabolism.

This cross-talk among the microbiota, host genetics, nutrition, immunity, and metabolism is initiated in infancy and continues throughout life. The window of opportunity to establish host immunity, and therefore inside-out control of the microbiome, depends on appropriate infant colonization through prenatal maternal exposures,[43] delivery mode,[6] breast-feeding,[44] and judicious use of antibiotics.

Although dysbiosis has been clearly associated with the development of autoimmunity, treatment strategies are still in their infancy. Ideally, in the future, treatments will be tailored to the cause of dysbiosis and will reflect knowledge of microbial-gut homeostasis. Once dysbiosis has already occurred, 2 main categories of treatment exist: (1) nutritional changes to encourage growth of normal endogenous microbes and (2) direct administration of live microorganisms.

To date, a gluten-free diet (GFD) is the only therapy for patients with CD; a GFD reduces symptoms and restores the well-being of the individual and heals the mucosal damage.[45] Several studies have compared the gut microbiota of patients with CD on and off a GFD and healthy controls. In patients with CD, even after following a GFD (for at least 2 years), the duodenal microbiota was not completely restored and showed a less abundant bacterial richness compared with healthy and untreated subjects, with a persistent imbalance of the ratio of potentially harmful/beneficial bacteria.[39] Species-specific analysis has shown that although *E coli* and *Staphylococcus* counts are restored after a GFD, *Bifidobacterium* counts remain lower in the feces of patients on a GFD compared with controls. A targeted study on Bifidobacterium composition from patients with CD on both a gluten-containing and a GFD and from healthy controls showed a correlation between the levels of total Bifidobacterium and Bifidobacterium longum species in the fecal and tissue samples. Moreover, a generalized reduction in these bacterial populations was found in patients with CD as compared with healthy children overall.[46]

Few studies have followed the same patients pre and post GFD to test the effect of gluten on the microbiome in the presence of CD. An Italian study showed that the *Lactobacillus* community was lower before than after a GFD and lower in patients with CD than in healthy controls. There was also a lower ratio of *Bifidobacterium* to *Bacteroides* and Enterobacteriaceae as compared with healthy controls.[45] Additional information comes from a study that evaluated the effect of a GFD on healthy subjects using fluorescence in situ hybridization and quantitative polymerase chain reaction.[34] In this study, it was noted that the GFD leads to a decrease in *B longum, Clostridium lituseburense, Lactobacillus,* and *Faecalibacterium prausnitzii* and an increase in Enterobacteriaceae and *E coli* strains. This was thought to be due to reduced production of proinflammatory and regulatory cytokines due to a generalized reduction in the total luminal bacterial load of the large intestine caused by the GFD. The main finding was that a GFD influenced gut microbial composition and immune activation (as measured by cytokine production) regardless of the presence of disease, and these effects were directly related to reduction in polysaccharide intake.

These studies show that a GFD only partially restores fecal microbiota balances in patients with CD. The reason is still unclear, although some suggest that genetic influences in those predisposed to CD affect the colonization of the microbiome, which persists despite a GFD; furthermore, because gluten has a prebiotic action, its absence in the GFD induces a different gut microbiota even in healthy individuals.[47]

In theory, probiotics represent a tempting fix to complex dysbiosis: identify the missing bacteria and replace them; in practice this has proved more difficult. Prebiotics are substances that induce the growth or activity of microorganisms (eg, bacteria and fungi) that contribute to the well-being of the host. Dietary prebiotics are typically nondigestible, fiber compounds that stimulate the growth of advantageous bacteria,

although they do not target a specific bacterial group. Several foods are rich in prebiotics, including raw garlic, leeks, chicory root, and whole wheat (although not relevant to patients with CD). However, the ideal daily serving is not agreed on. Current research is ongoing as to the possibility of altering gluten-free products with prebiotics. Some early evidence has suggested that adding prebiotic inulin-type fructans to gluten-free breads can provide benefits for patients with CD, as these are ingredients that can increase calcium absorption and possibly other nutrients as well.[48]

Although prebiotics refer to the nutritional components found in food sources, probiotics are microorganisms that are believed to provide health benefits when consumed.[49] Live probiotic cultures are available in fermented dairy products and probiotic-fortified foods. Tablets, capsules, powders, and sachets also contain the bacteria in freeze-dried formulations. According to the Food and Agriculture Organization/World Health Organization, a probiotic is defined as a"live microorganism, which when administered in adequate amounts confers a health benefit on the host."[50] Probiotics have been found to be effective in some diseases, such as irritable bowel syndrome and pouchitis, but effects in other diseases, such as CD, have been less than conclusive. Some probiotics have been found to digest or alter gluten polypeptides. De Fallani and coworkers analyzed the potential role of the specific probiotic preparation VSL#3 (a cocktail of 8 strains that belong to the species *Bifidobacterium breve, B longum, B infantis, Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus delbrueckii bulgaricus,* and *Streptococcus thermophiles*) in decreasing the toxic properties of wheat flour and found that VSL#3 was highly effective in hydrolyzing gliadin peptides. However, this ability was not noted with other probiotic preparations.[51] Specific *Lactobacillus* and bifidobacterial strains have been found to improve gut health. De Palma and collaborators[52] evaluated in vitro immunomodulatory properties of *Bifidobacterium bifidum* strain IATA-ES2 and *B longus* strain ATCC15707 versus *B fragilis* strain DSM2451, *E coli* strain CBL2, and *Shigella* spp on peripheral blood mononuclear cells under the effects of gliadin. This study found that *B bifidum* and *B longum* were able to induce lower levels of interleukin (IL)-12 and interferon (IFN)-γ production compared with *E coli* and *Shigella*. These bacteria were more likely to induce production of proinflammatory cytokines, which in turn contribute to development of disease. Lindfors and colleagues[53] found that *Bifidobacterium lactis* exerted a protective effect on epithelial cells against cellular damage induced by gliadin incubation. Recently, a study using a gliadin-induced enteropathy animal model was developed to observe whether *B longum* CECT 7347 could provide beneficial effects. The administration of this probiotic enhanced villus width and enterocyte height, which partially restored alterations in animals sensitized with IFN-γ and fed gliadin. It also decreased the levels of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and increased levels of anti-inflammatory IL-10.[54]

Another study evaluating the effect of *B longum* CECT 7347 for 3 months in addition to a GFD in children newly diagnosed with CD showed a decrease in CD3 T cells, improving symptoms, and greater height percentile in those on a probiotic and GFD compared with those on the diet alone.[55]

Studies evaluating the role of probiotics and CD in humans are scarce. In a randomized, double-blind, placebo-controlled study, *B infantis* and its effects on gut permeability, occurrence of symptoms, and the presence of inflammatory cytokines in untreated patients with CD were evaluated. In this study, it was noted that probiotic administration was unable to modify gut barrier function; however, there was a marked improvement in digestion and a reduction in constipation. Abdominal pain and diarrheal symptom scores were also diminished, although not significantly. There was no difference in inflammatory markers.[56]

A study in children, studying the effect of *Bifidobacterium breve* BR03 and B632 on serum cytokine production, showed a decreased production of proinflammatory cytokine production after administration of probiotics compared with diet alone. The effect on proinflammatory cytokine TNF- α was seen only while receiving the probiotic, whereas anti-inflammatory cytokine IL-10 levels were undetectable throughout the study period, suggesting that continuous probiotic supplementation is necessary and intermittent administration does not affect microbial milieu.

Alternatively, members of the Firmicutes phylum, specifically lactobacilli, are thought to play a role in CD pathogenesis as well. A study identified a significant lack of *Lactobacillus* in symptom-free children with CD. Thus, the investigators isolated 5 different lactobacilli in the stool of healthy children, and proposed *Lactobacillus rhamnosus* and *Lactobacillus paracasei* as potential targets.[57]

Although preliminary research has suggested a possible role for probiotics in the treatment of CD, the relatively poor regulation of these supplements makes this treatment relatively hard to monitor. A study done testing 22 of the top-selling probiotics, labeled gluten-free, and using chromatography to check for presence of gluten showed that 12 (55%) of the 22 probiotics contained more than 20 ppm of gluten, the acceptable cutoff for labeling a food product as gluten-free.[58,59]

To date, the evidence regarding the use of probiotics in patients with CD is still insufficient to justify their use in clinical practice, and until the Food and Drug Administration places stricter regulations on these supplements, their use can be considered dangerous for patients with CD. The recent evidence that probiotics do not alter the fecal microbiome of healthy subjects adds to the question of their applicability to widespread use.[60]

SUMMARY

In recent years, as evidenced by the growing number of publications, an increasing amount of attention has been paid to the microbiome in health and disease. Although most publications on the microbiome in CD have been conducted using different models, study populations, and small sample sizes, most of the studies have seen differences in the populations of *Bifidobacterium* and *Lactobacillus* in the gut microbial concentrations of patients with CD. In addition, patients with CD seem to have an increased number of gram-negative bacteria, specifically *Proteobacteria.* In vitro data have suggested that dysbiosis in CD can lead to modification of the mucosal barrier, and persistent immune activation or sensitization to activation by gliadin causing clinical symptoms. Additional studies dissecting out the role of the microbiome in immune cell activation and T-cell priming will help further clarify the role of the microbiome in autoimmune disease pathogenesis and possibly the role of microbiome manipulation as treatment for CD.

As far as the GFD diet is concerned, it is currently the only accepted treatment for patients with CD. However, as evidenced by several studies, with regard to the microbiome, complete “normalization” is not achieved with this diet. In this setting is where probiotic therapy might be beneficial. Treatment with *Bifidobacterium* and/or *Lactobacillus* might be helpful in restoring altered gut microbiota and dampening immune activation, although further studies are needed to understand the dosing and proportion in which these bacteria need to be given for this to be achieved.

Finally, if considering the microbiome as a possible environmental activator for CD pathogenesis, it is possible to consider probiotics as a modulator of risk in those with high-risk factors, such as the DQ2 or DQ8 phenotype. In these subjects, probiotic administration might have a role in primary prevention; however, no study has been

conducted using probiotics for this purpose, so much research needs to be done in this area before any conclusions can be made.

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