

The human microbiome and its interactions with the immune system

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Abstract:

Lately, the field investigating the immense quantity of microbe-inhabitants covering the human body, known as the human microbiome, has been growing rapidly. Especially the gut microbiome has revealed an array of functions related to human health and disease. In this review a general introduction to the human microbiome is given with focus on model system limitations, health effects and the microbiome structures of individuals considered healthy and diseased. Next, the complex interactions between the immune system of the host and the gut microbiome are explored. Subsequently, different environmental factors are reviewed regarding their potential to modulate the composition and function of the gut microbiome. These factors include antibiotics, infant feeding strategy, birth mode, probiotics and diet. This review shows that there is a great potential for therapies dealing with diseases and increasing health and wellbeing by targeting the human microbiome. Many of these relatively simple interventions, could probably be carried out independently of health professionals and might act as preventive measures that can be performed at home.

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Abbreviations used frequently:

FMT, Fecal Microbiota Transplant	MAC, Microbiota-accessible carbohydrate
FOS, Fructo-oligosaccharides	MAMPs, Microbe-associated molecular patterns
GF, Germ free	MC, Medium-carbohydrate
GOS, Galacto-oligosaccharides	MyD88, Myeloid differentiation primary response gene 88
HC, High-carbohydrate	NLR, NOD-like receptors
IBD, Inflammatory bowel disease	RS, Resistant starch
IBS, Irritable bowel syndrome	SCFA, Short-chain fatty acids
IL, Interleukin	SFB, Segmented filamentous bacteria
ILCs, Innate lymphoid cells	TLR, Toll-like receptor
LC, Low-carbohydrate	Treg, Regulatory T
LPS, Lipopolysaccharides	T _H , helper T

The human microbiome - microbes in and on us all

Introduction - what the human microbiome is and where it is found

Since the development of next generation sequencing methods for in depth analysis of microbial environments, the extensive microbial life in and on the human body has been studied intensively¹. As it turns out there is a vast range of microbes associated with the human host organism termed the *microbiota*. Another term, the *microbiome*, is the total amount of microbes of the microbiota, their milieu for interaction as well as their associated genetic material². These microbes live in symbiosis with the host and serve a range of purposes most likely originating from the long co-evolution of host-organism and microbe². Even though viruses, archea and unicellular eukaryotes inhabit the human body, the most studied microbes of the microbiota are bacteria¹. The taxonomic composition of the bacterial populations vary among body sites and to a lesser degree between individuals, with the phyla present being mainly Actinobacteria, Firmicutes, Proteobacteria and Bacteroidetes². The microbes are found all over the human body including skin, hair, GI tract, oral cavity, airways and vagina. This review will be focusing on the intestinal microbiota - the microbiota with the largest bacterial load though there is some controversy about the precise number of bacteria present^{3,4}. Different compositions of the microbiota have been associated with a range of different disease states⁵ as discussed later. The human microbiome is often studied in mice, which produces a variety of limitations.

Limitations of using mouse models

When studying the gut microbiota in humans it is difficult to reach conclusions regarding mechanism and causal relationship. Often it is inappropriate to perform trials that control for most confounding variables such as diet, microbial exposure or genetic factors⁶. Other trials would simply be too invasive to carry out in human beings. In these cases animal models are highly relevant in order to reach a deeper understanding. The most used microbiome animal model today is the mouse model. Mouse models are most likely among the most used models because of our advanced understanding of their genetics and the wide variety of available gene modified mouse strains⁶. Furthermore they are very inexpensive and fast to produce, due to their relatively cheap basic requirements, short generation time and fast reproduction pace.

By using mice, researchers are able to manipulate the variables in microbiome experiments such as host genetics, composition of the microbiome and environmental factors including diet and other factors⁶.

There is, however, a range of considerations mentioned below that one must take into account before translating mouse model results to humans.

Mouse housing, diet and genetic makeup compared to humans

Mice in the laboratory are generally exposed to a restricted amount of microbes due to the nature of experimental settings, contrasting the vast microbe exposure of humans⁶. The chow diet of mice is mainly plant-based and differs greatly from what is considered a common human diet. This, the uniformity of the chow diet and the inter-individual dietary difference of humans may impact the observed difference in composition of the microbiota. Lastly the genetic uniformity of inbred mice eliminates the between-individual variations and their impact on experimental outcomes⁶. On the one hand the restricted circumstances mentioned above control for confounding variables, which is of great importance in experimental settings. On the other hand they may fail to mimic the reality of the vast inter-individuality in diet, genetic makeup and microbial exposure seen in humans.

Anatomy

Anatomy differs quite a bit between humans and mice even though a lot of similarity is also observed⁶. These anatomical differences include cecum and colon size, compartmentalization of the gut and distribution of goblet and Paneth cells. This may alter the bacterial nutrient extraction proficiency and local immune responses⁶. Both of these effects are likely to lead to a natural dissimilarity in intestinal microbiota diversity and composition between mouse and human.

Compositional resemblance

Another thing to consider when using mouse models is the difference in bacterial taxonomy naturally found between humans and mice. One study found that 85% of analyzed mouse gut microbe sequences represented genera that had not been detected in humans⁷. Another more recent study found that only 4% of the sequenced mouse gut microbe genes were also represented in the human gut microbial gene catalogue used in the comparison⁸. This seemingly big difference is worth taking into account when considering the long co-evolution between host and microbiota². If the difference is species dependent it calls into question whether a similar microbiota will have the same impact on a mouse host as a human host. It could also be caused by dissimilarities in microbial exposure and food intake between the two systems. One last concern is the difference in sampling. When looking at the composition in humans, fecal matter is often analyzed whereas cecum samples are used in mice⁶. This could also influence results.

The composition of the microbiota differs significantly between healthy and sick individuals in both humans and mice as will be discussed later. To consider how well the mouse model represents the human system one might ask whether or not we see the same shift in composition when investigating diseased and healthy subjects. Nguyen et al. looked into this problem in obesity and inflammatory bowel disease (IBD)⁶. They found a varying degree of solid mouse to human translatability for these two conditions. Generally obesity studies in mice changed the microbiota composition of mice more

rapidly than in humans. This may reflect the drastic diet change that is possible in mouse studies which is completely controlled by the researchers⁶. It could also be explained by the difference in ability to control other parts of the experimental setup (e.g. genetics) as discussed previously. Moreover, the ratio between Firmicutes and Bacteroidetes, which is often used as a marker in obesity studies, seemed to be somewhat inconsistent. Even though microbiome alterations solely happening in mice were observed, Nguyen et al. saw more similarity in the overall compositional shift of the microbiota in IBD⁶. These two cases confirm that lines drawn between mouse model results and humans should be made with care and different illnesses may have different translatability.

Limitations of genetically modified mice and gnotobiotic mice

Gene modified mouse models are often used to simulate certain human diseases in mice. A shortcoming is that these models cannot fully mimic the exact disease state of humans due to the fundamental difference between the two organisms⁶. As an example in IBD the interactions between the immune system and the gut microbiome might not be the same for the two species.

Another model is the humanized gnotobiotic mouse. These models are created by artificially constructing the microbiota of mice by exposing germ-free mice to bacteria of choice⁶. This allows researchers to look at a “human” microbiome while still appreciating the benefits of studying mice. As with other models this approach too has its inadequacies. By transplanting a microbiota between organisms and assessing outcomes one must not forget the long co-evolution between host and microbiota as previously mentioned. This could mean that the microbiota’s influence on the host is simply different when transplanted to another host species⁶.

The mouse models mentioned here both have their limitations. That said, they are very potent strategies to explore mechanisms and causal relationships for different microbiome related diseases otherwise not possible in humans.

In summary, mouse models are convenient because of their qualities making them cheap and effective in a laboratory and our advanced knowledge of their genetics. As with all models there are dissimilarities with the system being modeled. For the lab mouse this comprises diet, genetics, microbial exposure, anatomy and microbiota composition. In the case of interpreting results one should consider the translatability of the disease, dissimilarity with true human disease and difference in interactions between host and microbiome. In spite of this the advantages greatly outweigh the limitations. Mouse models are essential for deepening of our understanding of mechanisms and causal relationships regarding human microbiome and health.

Health effects commonly associated with the human gut microbiome

Colonization resistance

A healthy microbiota can directly prevent the colonization of pathogenic bacterial species. This is done by competing for nutrition, secreting soluble factors such as antimicrobial peptides and organic acids, and simply taking up available intestinal space⁹. For example *Bacteroides thetaiotaomicron* both inhibits enterohaemorrhagic *Escherichia coli* toxin secretion by producing a soluble factor and consumes the food source required for *Citrobacter rodentium* growth⁹. With the rise of antibiotic resistant pathogens a healthy well functioning mi-

crobiome could be a way of combatting infections that are otherwise becoming harder to treat.

Fermentation/nutrient metabolism

Part of the food eaten by the human host organism ends up in the large intestine where microbe fermentation takes place¹⁰. A chief source of nutrients for the microbes comes from carbohydrates or fibers that were not absorbed in the small intestine such as indigestible oligosaccharides. By fermenting these food substances the microbiota produces short-chain fatty acids (SCFA) that can be taken up by the host¹⁰. Besides carbohydrates the gut microbiota can utilize a range of dietary components including proteins, primary bile acids and polyphenols. Examples of amino acids converted in the gut are histidine and glutamate to histamine and γ-amino butyric acid, respectively¹⁰ - both with essential functions in human biology.

Gut barrier integrity

The integrity of the gut barrier is crucial in limiting translocation of different components¹¹ such as undigested food substances and microbiota-derived antigens. This integrity is in part maintained by interactions with the residential microbiota. Examples of this are the tight junction preservation mediated by TLR signaling with bacterial peptidoglycan and bacterial secretion of soluble proteins in preventing apoptosis of epithelial cells¹⁰. Epithelial barrier dysfunction plays an important role in many disease states¹¹ and the mechanisms are still being unraveled. The microbiota could potentially be a principal piece of this puzzle.

Immune development

The gut microbiota has been shown to be very important in mice regarding development of the immune system. Germ free (GF) mice live but exhibit a diverse set of abnormalities¹. The spleens and lymph nodes of GF mice develop irregularly and they express a decreased number of IgA secreting plasma cells. Also the composition of the different cytokines and their quantities are altered and the T cell profile is abnormal. Some of these alterations seem to be eradicated by introducing microbe-derived components or incubating with microbes¹. The influence of microbes on immune development in humans is harder to assess directly. However, the hygiene hypothesis, as introduced later, links the growing incidence of autoimmune disorders to a decline in exposure to microbes and infections in our environment¹². Though this is not the same as being GF it implies a possible dysfunctional immune development when “deficient” in microbes as seen in mice.

Regulation of immune system

Besides its role in the development of the immune system the gut microbiome also seems to greatly affect and regulate the fully developed immune system¹³. This is one of the major topics of this review and will be discussed in more detail.

Composition of the gut microbiota in health and disease

Quantifying composition of the microbiota

In discussing the composition of a given microbiota and how it relates to other microbiotas certain terms are frequently used. When examining a microbiota sample one will often assess the *species richness* and *species evenness*. Species richness is a measure of the total unique species found in a sample, whereas species evenness is a parameter quantifying the

relative abundance of the species present¹⁴. Alpha-diversity, a term used when analyzing the diversity within samples, is a combination of evenness and richness¹⁴. As an example alpha-diversity is often measured when one wants to know whether a certain disease state is associated with higher or lower microbiota diversity. A commonly used measure of alpha-diversity is Shannon's index, which displays the unlikelihood of predicting the next species taken from a sample¹⁵. Another very used diversity is beta-diversity. This is a measure of the difference found between samples¹⁴. An example where this could be used would be to evaluate the difference in diversity between individuals with various disease states in a cohort. An example of one such measure is weighted UniFrac¹⁴. In microbiome research 16sRNA gene sequencing is often performed¹⁵, with a species usually defined as >97% sequence similarity. Since this is a somewhat arbitrary definition of a species many prefer using the term Operational Taxonomic Unit (OTU) when grouping the microbes according to sequence similarity¹⁴.

Composition

The healthy microbiota

As stated earlier the normal human microbiota consists of only a few different bacterial phyla. In the gut, Bacteroidetes and Firmicutes are the main phyla present but also Actinobacteria, Proteobacteria and Verrucomicrobia are observed. At the species level, however, a tremendous diversity is found, with estimates of the total amount found in the human population reaching more than 1,000¹⁶. Some of these species are pathogenic such as *Salmonella enterica* and *Vibrio cholera* and are also located in the healthy human intestine in minor proportions¹⁰. A way to classify the different individuals based on the composition of the microbiota is by the so-called enterotypes¹⁷. This categorizes the population into one of three groups: Type 1, consisting of high abundance of *Bacteroides*; Type 2, having an abundance of *Prevotella*; and Type 3 with a large proportion of *Ruminococcus*. The grouping of individuals in this way has proven to be feasible in some cohorts but others have not shown the same pattern⁵. This makes it appear as if numerous different configurations are distributed among the population and a simple classification like the enterotyping could be inadequate. Another factor complicating the classification of the healthy microbiota is its seemingly dynamic nature. However, samples taken from the same individuals over time do resemble each other more than samples from different individuals⁵. This may mean that rather than a stable composition one individual has a stable equilibrium state.

The diseased microbiota

Generally, individuals with different diseases seem to exhibit an altered microbiome. In IBD a much lower diversity of microbes is detected in Crohn's disease and ulcerative colitis as well as an overgrowth of Proteobacteria¹⁸. Also other species such as *E. coli*, *Yersinia*, and *Clostridium difficile* are witnessed more often in Crohn's disease¹⁸. Just as an overgrowth of inflammatory bacteria is observed, a diminishing of the beneficial bacteria correspondingly takes place. This is seen in the low abundance of the fermenting bacteria of the Clostridia class in IBD¹⁸.

Another case in which a shift of the normal microbiome happens is obesity, where a lower diversity is seen⁵. The altered ratio of Firmicutes to Bacteroidetes has been indicated as a marker¹⁹. However, some are of the opinion that the significant variation of this ratio even between healthy individuals has questioned its relevance¹⁰. Furthermore, in type-2 diabetes

a reduction in SCFA-secreting Clostridia as well as an increase in *E. coli* is detected¹⁸. Lastly, in rheumatoid arthritis and the mouse model for multiple sclerosis a significant alteration in the microbiome has also been revealed¹⁸.

Causality between microbiome and disease

Thus far the microbiome and its relation to disease in humans is mainly based on associations between diseased phenotypes and composition of the microbiota². One disease may harbor a certain composition of the microbiota. But determining whether the relation is causal as well as the direction of the causality is not easily done. From observations it is relevant to evaluate the following criteria² to establish a causal link: Strength of association - consistency, specificity, temporality and biological plausibility; whether biological gradients exist; whether experimental support exist; and whether support can be extrapolated from known causal relationships. Another approach, as discussed earlier, is the use of model systems such as the mouse. Causality can be established by observing a change in phenotype in a subject by exclusively altering microbiota content²⁰. One way of doing this is to transplant the microbiome of a diseased individual to a healthy recipient. If the disease is transferred between the subjects there is a high likelihood of the microbiome causing the illness.

In one study¹⁹ researchers found that the microbiomes of obese mice were significantly enriched in microbial genes coding for nutrient extraction. This included starch, sucrose, galactose and butyrate metabolism. Also the fecal caloric content was lower for obese mice and their caeca was enriched with butyrate and acetate indicating increased nutrient extraction. The transplant of obese cecum content to lean recipients resulted in significant fat gain compared to control recipients receiving cecum content from lean mice. This was not caused by difference in calorie intake but was possibly due to a transfer of the nutrient extraction proficiency from the obese donor mice¹⁹. This indicated that the microbiome could have a causal effect in the development of obesity.

In another study a team of investigators wished to investigate the influence of MyD88 on development of type-1 diabetes²¹. MyD88 is an important component of some of the TLR signaling pathways. Knocking out MyD88 attenuated development of diabetes in non-obese diabetic (NOD) mice. However, in GF NOD mice knocking out MyD88 did not have this effect. Furthermore, GF NOD MyD88 knocked out mice incubated with a microbe mix related to that normally found in mice partly regained the attenuation of diabetes development. Additionally, exposing GF NOD mice, otherwise developing diabetes, to the altered microbiome of the diabetes-free knockouts also offset development of diabetes²¹. Overall this suggested that the microbiome could directly affect the development of autoimmunity.

In studying ulcerative colitis, scientists induced its spontaneous development by knocking out the transcription factor T-bet as well as RAG2 - both important in immune regulation²². They termed these mice TRUC. By treating adult TRUC mice with broad-spectrum antibiotics they were able to eliminate the ulcerative colitis. This elimination was also observed in the untreated offspring. Having peaked their interest, this led them to experiments exposing healthy control progeny to TRUC mothers and cohousing TRUC mice with healthy control adult mice. In both cases the otherwise healthy mice developed ulcerative colitis once exposed to the TRUC microbiome, with the adult mice to a less severe degree. This revealed that an immune genetic defect could generate disease

driven by an unhealthy microbiome, which could then transfer disease to genetically immune-sufficient individuals²².

As considered earlier these kinds of controlled interventions are thus far limited to model systems rather than humans. However, a thing to consider when discussing the causation of the microbiome in disease is the closely related fecal microbiota transplant treatment gaining increasingly more interest²³. This is a method that has shown great promise in the treatment of *Clostridium difficile* infection and clinical trials have been carried out. In one trial 90% of *C. difficile*, patients treated with fecal microbiota transplant were cured compared to 26% treated with vancomycin²⁴ (a commonly utilized antibiotic in *C. difficile* infection). Today fecal transplants are also being explored in the treatment of IBD and irritable bowel syndrome (IBS)²³. Therefore, administration of microbiota from healthy individuals seems to have a positive effect in managing certain illnesses. This indicates a causal relationship between the microbiome and disease in humans.

Overall these studies suggest that not only is the composition of the microbiota altered in disease. They suggest that the microbiota can actively contribute to the development, level of severity and eradication of some diseases.

Microbial regulation of the immune system

So far this review has established a couple of points: The gut microbiome has a collection of health outcomes from nutrient metabolism to modulation of the immune system; the composition of the microbiome is altered in diseased individuals; and there is a causal relationship between some health disorders such as ulcerative colitis and the microbiome. The next area to examine is how the microbiome can affect the immune system of the host and through which mechanisms it does so. How do the microbes potentially aggravate, cause, cure or fight these conditions?

Reviewing the different mechanisms by which the microbiome affects the immunological state of the host reveals significant overlaps. Since the immune system is a complex dynamic system of systems it is difficult to make discrete sections focusing on specific pathways. As examined later one such example is *Bacteroides fragilis*, signaling via TLRs but yet interfering with T cell subset proportions as final outcome. As such, the headlines of this part are not absolute, but rather a simplified segregation to ease comprehension of the regulation of the immune system.

MAMP recognition

Certain molecular patterns specific to microorganisms are very conserved. These are often referred to as microbe-associated molecular patterns (MAMPs) and do not appear in the molecular structures of the mammalian host. To recognize these MAMPs the innate immune system has developed a range of receptors termed pattern recognition receptors (PRRs). Two major classes of these PRRs are known as Toll-like receptors (TLR) and nucleotide-binding oligomerization (NOD)-like receptors (NLR).

TLRs/MyD88

TLRs are membrane-bound receptors found either on cell surfaces or in intracellular endosomes where each recognizes a distinct MAMP. As an example TLR5 recognizes flagellin,

utilized for bacterial motility. Stimulation of TLRs activates different pathways important for initiation of inflammatory responses. A common transcription factor activated is nuclear factor κB (NFκB). Myeloid differentiation primary response gene 88 (MyD88) is a protein associated with several TLR pathways. It is often knocked out to test for TLR activation. Multiple TLR ligands activate MyD88 including lipoteichoic acid²⁵ and flagellin²⁶. Therefore MyD88-dependent TLR activating ligands will be discussed altogether.

Since the microbiome consists of microbes one would expect that the MAMPs of these commensals would activate TLRs. How such a massive load of bacterial products can exist in proximity with the PRRs of the host and maintain a non-destructive relationship is indeed interesting. It was believed that commensal bacteria did not activate TLRs because they were located on the luminal side of the intestinal epithelia, unlike pathogens²⁷. However, it has since been shown that commensal MAMPs in fact do activate TLRs²⁵.

Intestinal epithelial barrier function

Some evidence indicates the importance of proper barrier function such as in cases of IBD²⁸ and colorectal cancer²⁹ where bacterial translocation leads to increase in detrimental inflammation. Also the microbiome of mice is shown to determine the degree of permeability of the mucus layer of the gut³⁰. Transfer of the microbiome co-transfers the permeability phenotype. Clearly, the microbiome is implied to be able to direct intestinal barrier function.

The cell wall component lipopolysaccharide (LPS) is found in cell walls of Gram-negative bacteria. Stimulation of TLRs with commensal LPS has several beneficial effects including gut injury protection and optimized proliferation and differentiation of intestinal epithelium²⁵. These effects happen in a MyD88-dependent manner. Presence of commensals stimulate proliferation of the epithelium as a response to intestinal injury³¹ and seems to be facilitated by macrophage TLR stimulation³². However, even in the absence of breach of the intestinal epithelial barrier the microbiota can induce tissues renewal and mucus secretion by indirectly recruiting monocytes³³. This happens as a result of epithelial MyD88 activation and shows that epithelial cells can relay signals of the microbiome to the immune system. Furthermore, dendritic cells (that typically sample antigens to present to the adaptive immune system) can interact with the microbiome without breaching the barrier³⁴. They extend some of their mass as short “arms” across the intestinal epithelial barrier following epithelial cell TLR activation to sample antigens.

Cell proliferation is not always a good thing though. When it happens excessively it may result in tumors³⁵. For example alterations in the microbiota can cause changes in intestinal epithelial cell TLR signaling, triggering high expression of interleukin-17C (IL-17C)³⁵. In this setting IL-17C drives cell survival and tumor development in mice in a microbiota-dependent manner. Moreover, IL-17C is also expressed in human tumors³⁵.

Antimicrobial proteins

Antimicrobial proteins are one of the first defenses against infection and the mammalian immune system secretes various kinds. One of these is regenerating islet-derived protein 3 gamma (RegIIIγ). RegIIIγ is an antimicrobial protein³⁶ that along with others³⁷ is expressed by Paneth cells in the presence of a microbiota. RegIIIγ belongs to the carbohydrate binding proteins called lectins and it binds directly to gram-positive bacterial peptidoglycans. It is a part of the innate immune system. RegIIIγ is expressed in a MyD88-dependent way and is rapidly secreted into the lumen of the gut upon

epithelial TLR stimulation with commensals³⁸, LPS³⁷ or flagellin²⁶. It kills bacteria by forming a pore-penetrating membrane³⁹ and separates the microbiota from the intestinal epithelial surface while not affecting luminal microbes⁴⁰. Additionally RegIIIy may also be responsible for enhancing intestinal epithelial cell proliferation⁴¹. Recently it was shown that many bacteria of the commensal microbiome are highly resistant to RegIIIy⁴². This resistance is maintained in the microbiome by selection induced by intermittent escalations of inflammation. This suggests that the microbiome is able to induce RegIIIy to defend the host from infection and maintain homeostasis in the gut environment. Furthermore, commensals are resistant to RegIIIy, making the specific targeting of pathogens by innate immunity possible. This interaction between the microbiome and the host could be a reason why MyD88 signaling is so crucial during inflammation^{25,37,38}. Other factors such as RegIIIβ, CRP-ductin and RELMβ are also expressed via TLR signaling³⁷. These likewise play important roles in keeping commensals as well as pathogens from translocating across the epithelial barrier, but yet leave luminal microbes unharmed³⁷. Since these factors are provoked by commensals this is yet another way the microbiome helps the host to eliminate potential threats.

Effects on T cells

Another microbe component that works as a TLR ligand is genetic material. TLR9 recognizes unmethylated cytosine phosphate guanosine, which is frequently found in bacterial (and viral) DNA. One study showed that mice missing TLR9 had severe immune abnormalities⁴³. TLR9 signaling with microbiome-derived DNA induced lower levels of regulatory T (T_{reg}) cells and higher helper T (T_h) cell count. Meaning that

microbiome-derived DNA primes the intestinal immune system⁴³. T cells, which will be further introduced later, are also regulated by other TLR ligands produced by the microbiome. These include lipoproteins⁴⁴, LPS⁴⁵ and flagellin⁴⁶ with different effects on the outcome.

It is noteworthy to bear in mind that not only MyD88-dependent TLR activation is capable of inducing different immune effects. Tumor necrosis factor receptor associated factor 6 (TRAF6) is a protein in the TLR pathway with microbiome-dependent effects on the immune system⁴⁷. Deletion of TRAF6 leads to downregulated T_{reg} cell differentiation, T_h2 cell development and excessive intestinal inflammation as a consequence thereof⁴⁷.

MyD88 and short-chain fatty acids (SCFAs)

Cytokines is the major category grouping the signaling molecules associated with the immune system. Lactate and SCFAs, a bacterial fermentation product of the microbiota, can resolve inflammation caused by TLR4 and TLR5 signaling as measured by proinflammatory cytokine secretion⁴⁸. This further adds to the dynamic equation of a pro- or anti-inflammatory state as dictated by inputs from the microbiome. Components indicating the presence of bacteria (TLR ligands) are not the only things dictating inflammation; also byproducts indicating their activity (SCFAs) do so.

Lactobacillus plantarum

Lactobacillus plantarum is a species that can alter the immune response of the host and is of the phylum Firmicutes. *L. plantarum* is used as a probiotic because of its health benefits and has been shown to reduce IBD symptoms in a clinical trial⁴⁹. Also it delays detection and colonization of *Helicobacter pylori* while downregulating inflammation of the stomach

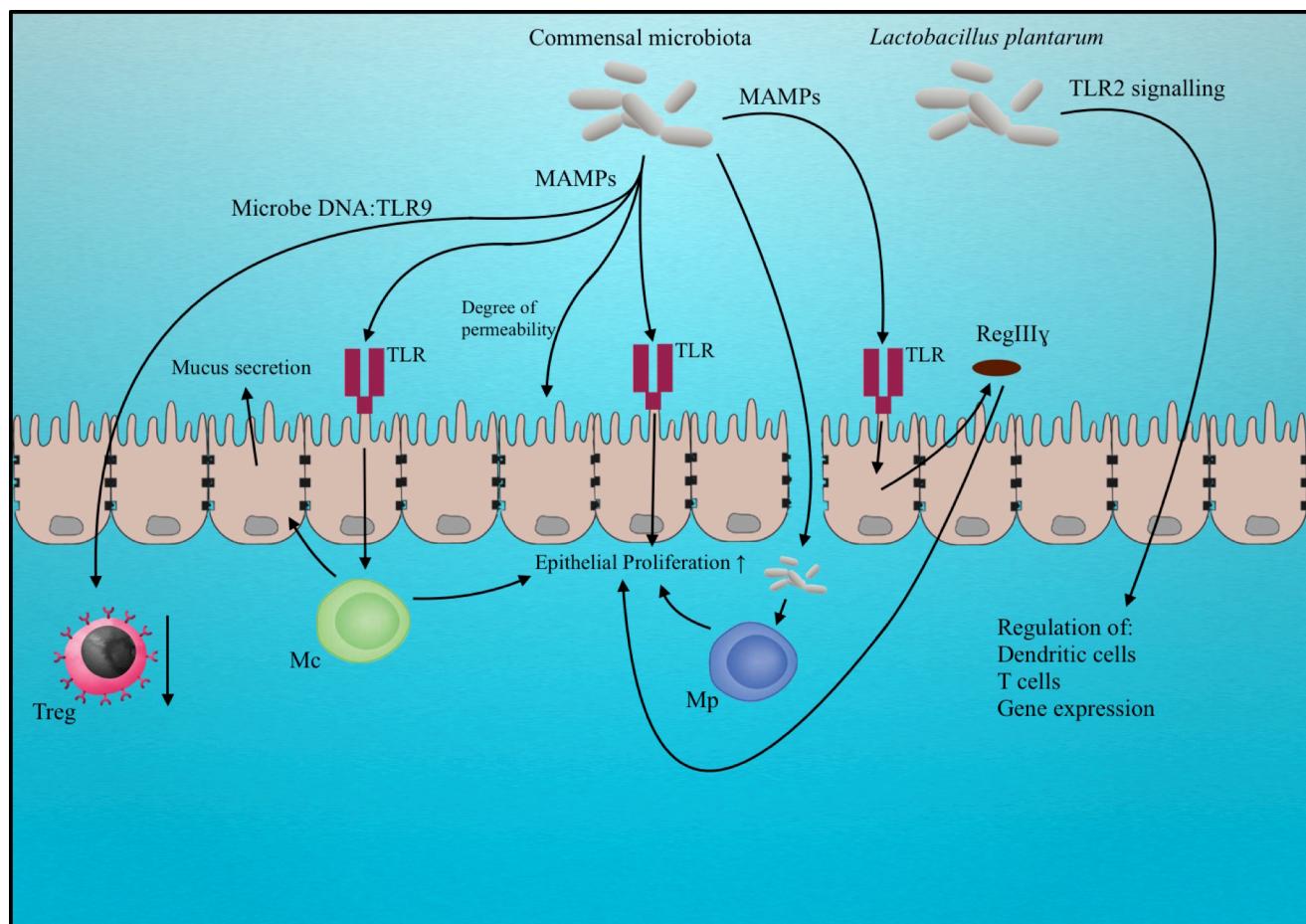


Figure 1 - Microbe-associated molecular pattern (MAMP) recognition by the immune system. The immune cells recognize and respond to the MAMPs of the microbiota in different ways.

Mc (Monocyte); Treg (T regulatory cell); Mp (Macrophage); TLR (Toll-like receptor).

in rats⁵⁰. The immune regulating properties have been attributed to the teichoic acids produced by the bacterium⁵¹ and its capability to signal through TLR2⁵². In this way *L. plantarum* is able to regulate T cell subsets as well as dendritic cell frequencies both towards an anti- and pro-inflammatory state. Additionally, *L. plantarum* was found to upregulate a series of genes associated with downregulation of inflammation to induce LPS tolerance⁵³. Another study revealed that colonization with *L. plantarum* in pigs increased expression of B cell transcription factors and genes that suppressed NFκB activation⁵⁴. Moreover, it lowered expression of adenosine deaminase, the enzyme that breaks down the anti-inflammatory adenosine. Overall this suggests *L. plantarum* is a distinct species of the microbiome that can singlehandedly modulate the immune response of its host.

NLRs

An alternative family of sensors of MAMPs is the NLR family. NLRs are not membrane-bound but positioned in the cytoplasm of the cell as soluble receptors. Like TLRs, they activate NFκB and as a consequence share a lot of the inflammatory responses upon activation.

The inflammasome

A subfamily of the NLR family is the NLRPs, named so because they contain a pyrin-domain in the amino terminal. Some of these receptors form a complex called the inflammasome upon activation, which acts to cleave and thereby activate pro-cytokines such as pro-interleukin-1β (pro-IL-1β) (*Figure 2*).

By providing mice treated with antibiotics with rectal LPS (a TLR ligand) it was possible to reestablish lung immunity against influenza. It was shown that the microbiota induces production of pro-IL-1β, pro-IL-18 and NLRP3, which was necessary for proper inflammasome activation⁵⁵. Besides priming the inflammasome (known as signal 1) the microbiome can also activate it under certain circumstances (known as signal 2). During intestinal injury some bacteria can activate the NLRP3 inflammasome of monocytes recruited to the site of damage to release active IL-1β. It was shown that *Proteus mirabilis* was the commensal bacterium mostly responsible and it activated the inflammasome through expression of HpmA hemolysin⁵⁶. *P. mirabilis* HpmA hemolysin activated the inflammasome through K⁺ efflux, which is the common trigger for NLRP3 activation⁵⁷. The ability of some pathogenic *Escherichia coli* to induce macrophage IL-1β release may be a common feature in IBD⁵⁸.

Whether NLRP3 inflammasome activation is necessarily bad in IBD is not carved in stone. IL-18, also released by the inflammasome, is shown to be beneficial for protection, whereas IL-1β is not notably secreted as a response to induced colitis⁵⁹. The effects of IL-18 were shown to be upregulated intestinal epithelial integrity and repair leading to better epithelial homeostasis. Others showed that fiber intake in mice was protective against induced colitis⁶⁰ and attributed this effect to IL-18 secretion. They showed that fiber fermented in the gut produced SCFAs that could activate the inflammasome. This activation was achieved through G protein coupled receptor 43 (GPR43) and GPR109A stimulation by SCFAs and as a result K⁺ efflux. Furthermore the fiber also shifted the compo-

sition of the microbiome. The fiber microbiome was highly efficient at inducing IL-18 expression compared to the conventional-housing microbiome⁶⁰. This shows that whether activation of the inflammasome leads to IL-1β or IL-18 production has great influence on the downstream effects. Moreover, these downstream effects seems to be controlled by the microbiome to an unknown extend.

A way in which the phagocytic cells of the intestine discriminate between pathogen and commensal is by the production of pro-IL-1β⁶¹. IL-1β is associated with a range of immune reactions including recruitment of immune cells⁶². By producing pro-IL-1β upon TLR stimulation instead of reaction as extra-intestinal phagocytes do, intestinal phagocytes cause an immune response only in the presence of pathogens. Since NLRC4 inflammasome (Another NLR-containing inflammasome) activation cleaves and activates pro-IL-1β pathogens, but not commensals, provoke inflammation⁶¹. This is among other things due to specific delivery systems of virulence factors required for activation that exist on pathogens. In this way the microbiome stimulates and primes phagocytes in a MyD88 dependent way to rapidly respond selectively to

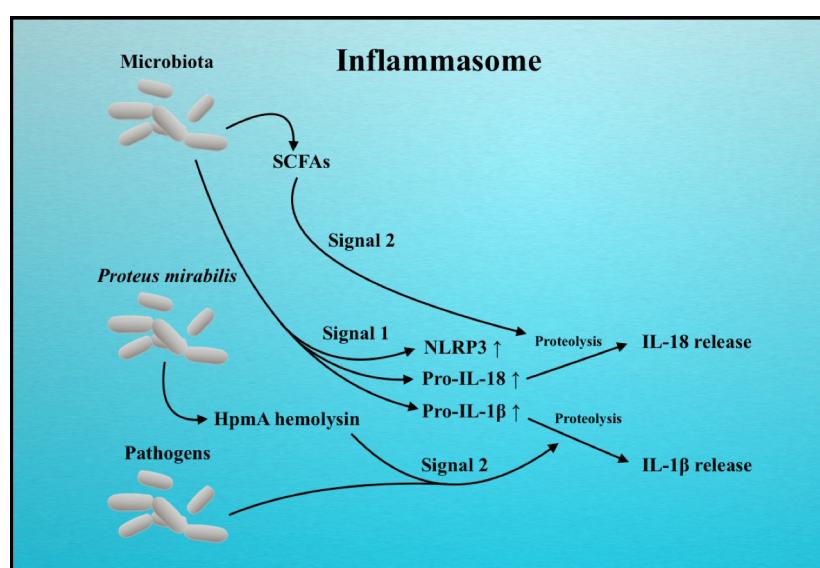


Figure 2 - Regulation of the inflammasome by microbes. Microbes can provide both signal 1 and signal 2 to promote secretion of either IL-18 or IL-1β.
SCFA (Short-chain fatty acid); IL (Interleukin); NLRP3 NOD-like receptor pyrin 3.

pathogens.

Effect on T cells

When knocking down *Nod2* in mice, aberrant intestinal T cell formation is seen⁶³. This implies that NLRs may have a plausible function in directing T cell proportions. Similarly, induction of early T_H17 responses requires NOD1 and NOD2 as well as a microbiota⁶⁴. Later it was shown that NOD2 signaling initiates microRNA (miRNA) expression of type miR29 in dendritic cells⁶⁵. miRNAs are short RNAs that target mRNA for cleavage and suppress expression⁶⁶. miR29 suppresses IL-23 expression⁶⁵, which is a cytokine important for T_H17 accumulation. This could perhaps explain the observed importance of NOD2 in intestinal T cell differentiation.

As illustrated here, the MAMPs of not just pathogens but also of the commensal microbiome supply the immune system with inputs (*Figure 1*). These inputs can be pro- or anti-inflammatory and all take part in determining the overall immune response of the host in any given instance.

T cell subset regulation

One way in which the gut microbiome regulates the immune system is by changing the proportions of the different T cell subsets. When a naïve T cell of the adaptive immune system is activated it differentiates into one of the several different effector T cell subtypes. These subtypes with different immunological functions are classified as helper (T_H), regulatory (T_{reg}) or cytotoxic T cells. The T_{reg} cells act mainly through down-regulation of the immune response. In contrast helper T cells act through activation of different components of the immune system. At least four main varieties of helper T cells have been described, namely follicular helper T (T_{FH}), T_{H1} , T_{H2} and T_{H17} cells. T_{H1} cells are known to induce immunity against intracellular pathogens (such as by activation of macrophages), where T_{H2} cells combat pathogens such as helminths. T_{H17} recruit dendritic cells and fight bacterial and fungal infections through cytokine secretion⁶⁷. By modifying the differentiation of T cells the gut microbiota is able to shift the immune response, as will be discussed.

Segmented filamentous bacteria

Members of the gut microbiota known as segmented filamentous bacteria (SFB) is a lineage of bacteria belonging to the family *Clostridiaceae*⁶⁸. Unlike other bacteria SFB typically adhere directly to the epithelium of the ileum in the small intestine, particularly on the surface of Peyer's patches⁶⁹. SFB have shown great capacity to modulate the immune response in mice^{67,69}. Colonization of GF mice and conventionally raised SFB-absent mice with SFB results in significant accumulation of T_{H17} in the lamina propria of the small intestine. SFB colonization regulates gene expression in the ileum, and specifically increases expression of serum amyloid A (SAA)⁶⁷. SAA induces T_{H17} differentiation *in vitro* in a dendritic cell-dependent manner. This indicates a potential mechanism, where SFB induce SAA that acts on dendritic cells, which drive T_{H17} differentiation. In agreement with this, SAA is necessary for production and proliferation of T_{H17} cells⁷⁰. However, it was later shown that macrophages and not dendritic cells were responsible for the T_{H17} proliferation⁷¹. Additionally, SFB is able to induce T_{reg} , T_{H1} , and T_{H2} responses⁶⁹.

SFB have been shown to provoke arthritis and experimental autoimmune encephalomyelitis in predisposed GF mice upon colonization by promoting T_{H17} accumulation^{72,73}. However, SFB seem to be protective in female type 1 diabetes mice⁷⁴. Many recent studies have worked towards understanding the mechanism by which SFB induce T_{H17} expansion. One such paper found that there was only a very limited resemblance in recognized antigen between intestinal T_{H17} cells and other intestinal T_H cells in SFB colonized mice⁷⁵. Moreover, most T_{H17} cell receptors in these mice recognized SFB-derived antigens and naïve T cells with receptors recognizing SFB-antigens almost exclusively became T_{H17} cells.

Major histocompatibility complex (MHC) class II is a molecule used to bind peptides for presentation to the immune system. The MHC-II of intestinal dendritic cells is necessary for SFB mediated T_{H17} differentiation^{76,77} and SFB is mainly recognized by T_{H17} through MHC-II. However, these studies did not examine the induction of T_{H17} by SFB and it has been shown that dendritic cells are in fact dispensable for this process⁷¹. On the contrary innate lymphoid cell (ILC) MHC-II regulates T_{H17} differentiation independent of SFB⁷⁶.

Notably, the induction of T_{H17} is carried out even in the absence of secondary lymphoid tissues⁷⁶⁻⁷⁸ (where T cell activation commonly takes place). This indicates that sampling of

antigen as well as priming of T cells happens directly in the lamina propria. This is surprising because of the observation that SFB adhere specifically to Peyer's patches⁶⁹ (which is a secondary lymphoid tissue). Finally, the ability of SFB to adhere to epithelial cells is crucial in inducing T_{H17} , since adhesion-defective mutants are unable to produce this outcome⁷⁹. All this considered SFB play a crucial role in regulating the immune response in mice. This likely explains why the helminthic parasite *Nippostrongylus brasiliensis* targets SFB to limit the immune response of the host⁸⁰. Recently SFB was cultured *in vitro* for the first time⁸¹. This provides new opportunities to further explore the mechanism by which it regulates the immune system. Since SFB also inhabit the human gut⁶⁷, understanding how it regulates the immune system of mice may have profound implications. This could lead to a greater understanding of the interaction between the immune system and the microbiome in humans.

Clostridium

Other individuals of the commensal microbiome of the gut have an effect on the immune response. Amongst these are some of the bacteria belonging to the genus *Clostridium*. *Clostridium* is of the phylum Firmicutes and is separated into clusters⁸², where some of these have been shown to induce T_{reg} accumulation^{83,84}.

When colonizing GF mice with *Clostridium* of primarily clusters IV and XIVa accumulation of colonic T_{reg} cells is observed. Particularly *induced* T_{reg} cells (produced from T_H cells exposed to transforming growth factor-β (TGF-β) in peripheral tissues and not the thymus) are detected⁸³. Of the T_{reg} promoting *Clostridium* species (spp.), 17 strains of clusters XIVa, IV and XVIII were specifically identified⁸⁴. These strains were unable to elicit a significant T_{reg} response individually. *Clostridium* spp. seem to prompt intestinal endothelial cells to produce TGF-β and other T_{reg} promoting elements⁸³. TGF-β appear to be secreted by intestinal epithelial cells as a consequence of *Clostridium* SCFA production. Furthermore, the *Clostridium* bacteria seem to also affect T_{reg} responses through the supply of bacterial antigens⁸⁴. This has also been proposed as a general feature of the residential microbiome since T_{reg} cells commonly recognize commensals⁸⁵, and a range of microbes may induce T_{reg} cell expansion⁸⁶.

A single bacterium *Faecalibacterium prausnitzii* from *Clostridium* cluster IV⁸³, significantly under-represented in IBD⁸⁷, has shown great anti-inflammatory abilities⁸⁸. Like previously noted about the other *Clostridium* spp., *F. prausnitzii* is likewise a producer of SCFA. Moreover, it produces a protein, microbial anti-inflammatory molecule, able to inhibit the activation of NFκB⁸⁹ (an important transcription factor in initiating innate immunity).

This suggests that bacteria of the genus *Clostridium* is able to regulate immunity in a multiple-strain interdependent manner. However, it also implies that single species have the potential to robustly regulate immunity.

Bacteroides fragilis

Another single species that has been found to be able to cause great immune regulation is *Bacteroides fragilis* of the phylum Bacteroidetes. It secretes a molecule, polysaccharide A (PSA), capable of modulating T cell subtypes observed⁹⁰, specifically by suppressing T_{H17} cells and promoting T_{reg} cells⁹¹. Purified PSA achieves this effect by activating T_{reg} TLR2⁹², of the very conserved TLR family of immune receptors. However, when PSA is secreted by *B. fragilis* surrounded by outer membrane vesicles they do not activate TLR2 on T_{reg} cells. In this case

dendritic cell TLR2 is needed to elicit the same response⁹³ and thereby indirectly induce T_{reg} cells.

SCFA

As seen in a previous section SCFAs are bacterial products with immune system-regulating properties. They are thought to act through either G-protein-coupled receptor (GPR) interactions^{60,94-98} or histone deacetylase (HDAC) inhibition⁹⁹⁻¹⁰². HDAC inhibition increases acetylations of histones to unwrap DNA and thereby upregulate transcription of certain loci. There has been some debate regarding which mechanism(s) it follows, probably because of the yet slightly unclear characterization of SCFA ligand-receptor pairs⁴⁸. As an example GPR43 is required for the SCFA acetate to decrease inflammation in induced colitis in mice⁹⁵. Propionate can reduce inflammation in allergic airway disease in mice through interaction with GPR41⁹⁷. Moreover, stimulation of GPR109A, of which butyrate is a ligand, causes the inhibition of NFκB⁹⁴. Also, GPR109A activation by butyrate stimulates dendritic cells and macrophages to induce T_{reg} accumulation⁹⁸. SCFAs can also interact directly with T_{reg} cells via GPR43 to modulate quantity of T_{reg} cells and associated cytokines. This seems to happen through HDAC inhibition, suggesting an overlap between the two mechanisms since it is still GPR43-dependent⁹⁶.

As mentioned before the other major mechanism for the influence of SCFAs on T_{reg} cells is HDAC inhibition. Butyrate induces T_{reg} cell expansion through HDAC inhibition and thereby upregulation of the expression of forkhead box P3 (*FoxP3*) (the transcription factor leading to T_{reg} differentiation). This was independent of GPR43 since acetate, a ligand for this receptor unable to inhibit HDAC, failed to elicit a similar response⁹⁹. Other results confirm these findings and add that dendritic cell GPR109A was found dispensable in inducing T_{reg} cells¹⁰⁰, sharply contrasting what was discussed earlier. It has since been added that SCFAs in addition may induce T_{H17} cells, depending on the cytokine milieu. It was also shown that there was no direct interaction with GPR41 or GPR43 on T cells¹⁰¹. The authors state that GPR stimulation may indirectly induce T_{reg} differentiation through other cells. However, it has also shown that macrophages are not affected

by butyrate through GPR109A, GPR43 or GPR41 but through HDAC inhibition¹⁰². This again shows some disagreements with what was mentioned before.

Generally more studies seem to be needed to fully elucidate how SCFA acts to regulate the immune system and specifically T_{reg} cells.

As illustrated, bacteria of diverse kinds are able to regulate the subpopulations of T cells through different mechanisms (Figure 3). Developing a better understanding of these different systems may have profound implications for autoimmune diseases.

Innate lymphoid cells

The innate lymphoid cells (ILCs) are a fairly new discovery that belongs to the lymphoid lineage. Like T_H cells they modulate immune responses through cytokine secretion but do not respond to antigens since they do not express T cell receptors¹⁰³. 3 ILC groups have been identified, namely group 1 (where ILC1 belongs), 2 (where ILC2 belongs) and 3 (where ILC3 belongs) producing cytokines resembling those of T_{H1}, T_{H2} and T_{H17}, respectively. In these groups natural killer (NK) cells and lymphoid tissue-inducer (LTi) cells are also found in group 1 and 3, respectively¹⁰³.

Figuring out whether or not the microbiome influences the development of ILC-populations has been problematic, since studies have shown the microbiota to be dispensable¹⁸. However, they still seem to be affected by microbiota since GF mice display altered ILC count^{104,105}. Whether or not the development is affected by the microbiota, the action of ILCs seems to be somewhat guided by it. The expression of IL-22 appears to be controlled by the microbiota in multiple ways (Figure 4). IL-22 is a cytokine also expressed by T_{H17} that prompts the secretion of antimicrobial peptides and is a signature cytokine of ILC group 3¹⁰³. One piece of evidence pointing towards this IL-22 regulating property is the fact that TLR2 ligands of the microbiota can stimulate IL-2 secretion by ILCs¹⁰⁶. This IL-2 has autocrine signaling properties resulting in the secretion of IL-22. Another way in which the microbiota determines ILC expression of IL-22 is through flagellin TLR-stimulation of lamina propria dendritic cells. Stimulated dendritic cells secrete IL-23 that in turn signals ILCs to produce IL-22¹⁰⁷.

As it turns out, the microbiome does not just stimulate IL-22 secretion. It is also capable of suppressing it. Intestinal IL-22 is constitutively expressed by ILCs but is repressed by the microbiome¹⁰⁸. This effect of the microbiota seems to be mediated by intestinal epithelial cell IL-25 expression. IL-22 is not the only soluble molecule of ILCs that the microbiota can regulate. IL-1β, the cytokine discussed earlier, secreted by macrophages and induced by the microbiome can stimulate ILCs. Upon stimulation the ILCs secrete granulocyte-macrophage colony stimulating factor to again promotes macrophages to induce T_{reg} cell accumulation in the intestines¹⁰⁹.

Aryl hydrocarbon receptor

The aryl hydrocarbon receptor (AhR) is a transcription factor located in the cytosol before activation¹¹⁰. ILC3s express this receptor, and their development as well as their secretion of IL-22 seems to be dependent on it¹¹¹. Upon activation the AhR interacts with ROR γ T¹¹², a

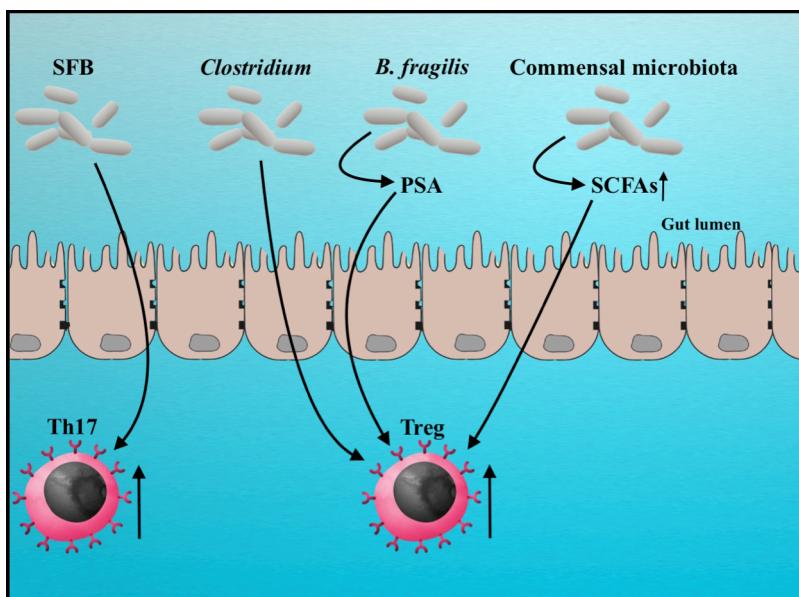


Figure 3 - Manipulation of T cell subtypes by the microbiota. Different microbes can upregulate different T cells of the immune system.
SFB (Segmented filamentous bacteria); PSA (Polysaccharide A); SCFA (Short-chain fatty acid); Treg (T regulatory cell); Th17 (T helper 17 cell).

transcription factor found in ILCs of group 3¹⁰³. RORyt then facilitates the binding of AhR to the *Il-22* gene to initiate IL-22 expression. Furthermore, ILCs not expressing AhR has increased apoptosis, explaining the low cell count observed¹¹². The AhR has several ligands. One of these ligands is indole[3,2-b]carbazole (ICZ) derived from indole-3-carbinol (I3C) found in cruciferous vegetables¹¹³. Another is indole-3-aldehyde (IAld), which can be derived from the amino acid tryptophan¹¹⁴. In mice fed high-tryptophan diets a vast expansion of bacteria of the *Lactobacillus* genus is observed¹¹⁴. These *Lactobacilli* are highly efficient at converting tryptophan to IAld and thereby provide high quantities of AhR ligands. As discussed before this AhR activation leads to increase in IL-22, which was sufficient to prevent colonization of *Candida albicans*¹¹⁴. Even administering IAld by itself could reproduce this effect.

IL-22 secreted by ILC3s may have an important role in maintaining a healthy symbiotic relationship between host and microbiome. In mice lacking AhR, IL-22 secretion is significantly decreased and as a consequence overgrowth of SFB is seen¹¹⁵. As discussed earlier IL-22 has a role in antimicrobial protein secretion and this could perhaps explain the overgrowth. SFB, as also reviewed earlier, drive T_H17 expansion and this overgrowth led to spontaneous colitis in some mice¹¹⁵.

This part of the review looked at how, and through what mechanisms, the microbiome affects host immunity. As it was revealed, the microbiome can greatly influence the immune system in a range of both beneficial and harmful ways. Through the MAMPs and SCFAs associated with the microbiome it can: alter permeability, proliferation and differentiation of the epithelial barriers as well as mucus secretion; affect antimicrobial protein secretion of the host; regulate the proportions of the different T cell subsets; change gene expression of genes associated with inflammation; And both prime

and activate the inflammasome to release different cytokines. Furthermore, different bacteria of the microbiota can regulate the T cells of adaptive immunity. They do so as individuals, by collaboration between a range of species and by secreting metabolites such as SCFAs. Lastly the microbiota can regulate the action of a group of immune cells known as ILCs. The result is mainly to control IL-22 expression through secretion of ligands and stimulation of TLRs. All this evidence points to the conclusion that the microbiome probably has a function in many inflammatory diseases such as inflammatory bowel disease (IBD).

Manipulating the microbiome

This part of the review will explore the different methods for modulating the microbiome to confer health benefits to the host. This is not necessarily composition-wise and can also refer to change in behavior and function of the microbiome. As the rest of the review there will be a main focus on immunity but the review will not be limited to the immune system. An attempt will be made to answer the following questions: What are some strategies by which one can improve health in a microbiome-dependent way? And how far reaching are these health benefits?

Antibiotics

Antibiotics are antimicrobials used to fight infection by directly killing bacteria or blocking their growth¹¹⁶. Broad-spectrum antibiotics have the potential to kill off many different types of bacteria in the gut. Given the great importance of these beneficial microbes, as reasoned earlier, it is intriguing to ascertain the possible effects of broad-spectrum antibiotics.

Some evidence indicates that antibiotics can shift the stable state configuration of the microbiota of the recipient. A seven-day treatment with clindamycin lead to disturbances in the microbial composition of subjects lasting the entire sampling period of 2 years¹¹⁷. Another trial used clarithromycin and metronidazole for 7 days in their subject group. They observed that some people had a substantial shift in their microbiome for up to 4 years following treatment¹¹⁸. The fact that individuals receiving antibiotics responded in different ways indicates various levels of resilience of the microbiome of different people. In subjects with high resilience to antibiotics one might wonder if a secondary intervention would have different impact on microbiota composition. This would indicate that in cases where composition does not change following antibiotic intervention resilience is in-

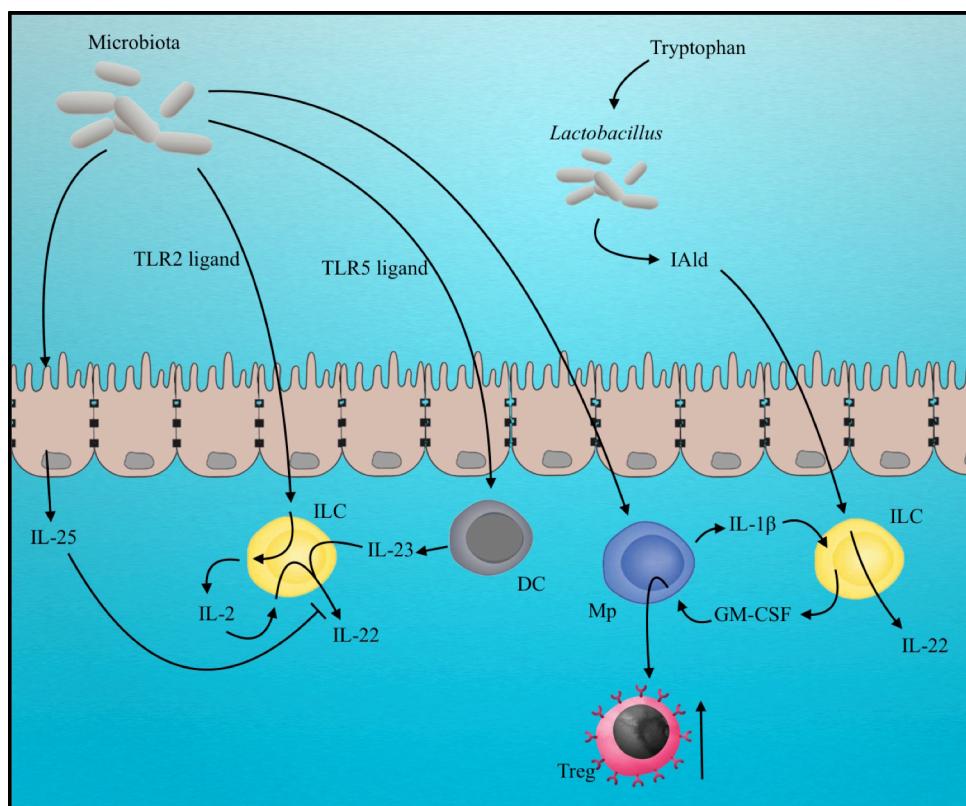


Figure 4 - Modulation of innate lymphoid cell (ILC) action by microbiota. The microbiota regulates ILC molecule secretion both directly and indirectly.

TLR (Toll-like receptor); IAld (Indole-3-aldehyde); IL (Interleukin); DC (Dendritic cell); Mp (Macrophage); Treg (Regulatory T cell); GM-CSF (Granulocyte macrophage colony-stimulating factor);

stead altered. In fact this was illustrated in another study where ciprofloxacin was utilized¹¹⁹. 1 week after treatment the composition of the microbiota began to return to its original state in recipients. However, the configuration reached at stable state dissimilar to that of the initial state. Furthermore, a second antibiotic intervention resulted in a composition differing from both pre-treatment stable state and stable state after first intervention. In all subjects the responses were different. Besides alterations in composition a participant subjected to ampicillin and cefazolin treatment also displayed marked changes in metabolic functions of the microbiota. The microbiome seemed to reduce its activity regarding metabolism of bile acids, cholesterol, hormones and vitamins¹²⁰.

These studies show that the composition of the microbiome in some individuals is altered long-term by antibiotics and that antibiotics influences the metabolic actions carried out by the microbiome. Given the vast influence of the microbiome on host immunity, antibiotics could have implications in inflammatory diseases. In line with this an observational study correlated antibiotic use with subsequent development of irritable bowel syndrome (IBS)¹²¹. Another study looked at antibiotic use in children and showed an increased risk of developing IBD, with more antibiotic courses corresponding to higher risk¹²². Finally, use of antibiotics has also been implicated in dysfunctional metabolism such as obesity. Exposure to antibiotics in the first six months of life significantly impacts body mass within first 7 years when other obesity-associated variables are controlled for¹²³. Although not very strong evidence, this suggests that antibiotics can influence health status at different stages of life.

Breast-feeding

A whole range of factors that can influence the development of the human microbiome is found in breast-milk. These include antimicrobial proteins, maternal immunoglobulin A (IgA), IL-10 and TGF- β ¹²⁴. Live bacteria are also detected in mother's milk likely in part as a consequence of the enteromammary pathway. This pathway allows bacteria of the maternal gut to reach the breast milk and as a result colonize the intestine of the infant^{125,126}. Furthermore non-digestible oligosaccharide carbohydrates are found in breast-milk¹²⁷. By escaping digestion they end up in the gut and specifically feed *Bifidobacterium* species (spp.)¹²⁸ and *Bacteroides* spp.¹²⁹. The microbiota of breast-fed infants is first populated by *Enterobacteriaceae* and *Staphylococcaceae* followed by a significant outgrowth of *Bifidobacteriaceae* and *Bacteroidaceae*. This change in composition corresponds to a substantial increase in breakdown of milk oligosaccharides in the gut¹³⁰. Besides functioning as a food substrate for bacteria, oligosaccharides can also prevent adhesion of pathogens such as human immunodeficiency virus¹³¹. They do so by binding the intestinal epithelium to take up available space. Also, they have been proposed to have a decoy-function¹³², letting pathogens bind to non-bound oligosaccharides instead of the enterocytes.

Breast-fed infants typically harbor a microbiome predominantly colonized by *Bifidobacterium*¹³³. In contrast the microbiota of formula-fed infants is colonized by a wider variety of microbes including *Atopobium*¹³³, *E. coli*, *C. difficile*, *Bacteroides* and *Lactobacilli*¹³⁴. This is possibly due to the presence and composition of different oligosaccharides in breast-milk. The breast-milk oligosaccharide composition varies greatly between mothers and even between different time points for the same individuals¹³². This makes it hard to replicate the compositions in infant formula. However, by adding a

mixture of 90% galacto-oligosaccharides and 10% fructo-oligosaccharides Boehm et al. obtained fecal matter from formula-fed infants closely resembling that of breast-fed infants¹³⁵. This was measured in terms of *Bifidobacterium* as well as SCFA amounts, which were significantly higher than in standard-formula-fed infants. However, not only oligosaccharide content and composition appears to have an important function in microbiota composition¹³⁶. A group of researchers showed the importance of overall resemblance to mother's milk. Standard cow's milk-based infant formulas typically have a high casein-protein and phosphate content. By giving infants a formula with 70% of protein from whey with low phosphate and low total protein amount the researchers observed a similar *Bifidobacterium* count to that in breast-fed infants¹³⁶. The study formula did not improve *Bifidobacterium* count by supplementing with *B. longum*. These studies suggest that multiple components of mother's milk contribute to its capacity to stimulate proliferation of beneficial bacteria. Feeding with infant formulas not enriched for optimal microbiota development may have detrimental health effects. Infants with atopic syndrome have skewed fecal microbial ratios with a higher amount of Clostridia and a lowered amount of *Bifidobacteria*¹³⁷. Additionally, longer breastfeeding duration and late introduction of formula feeding was associated with a lower incidence of type 1 diabetes in 760 kids younger than 5 years of age¹³⁸.

These findings imply that feeding method in infants has noteworthy effects on intestinal composition of the microbiome and potential health outcomes thereof. This is due to various soluble factors found in mother's milk and their likely absence or wrong ratios in infant formulas.

Delivery mode

The human fetus was originally believed to be sterile up until birth¹³⁹. However, since differences between pre- and full-term infant microbiomes were observed, this idea was questioned¹⁴⁰. Researchers reasoned that the fetus must be exposed to microbes in the uterus that would influence the microbiome. The difference between pre- and full-term infant microbiomes was explained by the dependence of colonization on time of gestation. It has since been shown that the placenta harbors a unique microbiome of its own¹⁴⁰.

Even though the fetus is exposed to a microbiome before birth, delivery method has been shown to still have a great impact on microbiota development. Microbiomes of infants born via caesarean section (CS) generally display lower abundance of *Escherichia-Shigella*¹⁴¹ and an absence of *Bacteroides*^{141,142}. They have an intestinal microbiome colonized with *Staphylococcus*, *Corynebacterium* and *Propionibacterium* resembling the skin microbiome¹⁴³. In contrast vaginally born (VB) infants are colonized with *Lactobacillus*, *Prevotella* and *Sneathia* spp. like that of the vaginal microbiome¹⁴³. Furthermore, overall lower diversity and richness is observed in elective CS births compared to VB and emergency CS¹⁴¹. Others have shown that in exclusively breast-fed infants many VB infants exhibit *Bifidobacterium* spp. whereas only few CS infants do¹⁴².

The altered development of the intestinal microbiome of infants can have long-lasting effects. One study showed that at seven years of age kids born via CS showed lower levels of Clostridia compared to VB children¹⁴⁴. A more recent study found that at two years of age CS resulted in lower overall microbial diversity. Also, CS children had lower abundance of Bacteroidetes¹⁴⁵. Regarding health effects it has been shown

that CS is associated with an increased risk of developing childhood obesity¹⁴⁶. Moreover, 2-year-old children born via CS display lower levels of T_H1 associated cytokines in the blood, indicating that birth method can affect immunity¹⁴⁵. Considering the reviewed evidence there are solid implications that birth method can influence the development of the microbiome. Furthermore, the altered development may impact immunity as well as disease risk.

Probiotics

The human intestinal microbiome has a great impact on several areas of human health. A way in which to take advantage of this is the administration of so called probiotics. The term probiotic is defined as: “live microorganisms which when administered in adequate amounts confer a health benefit on the host”¹⁴⁷. One might think that probiotics generally affect the host by modulating the composition of the microbiome. However, the beneficial effects may also be exerted without altering composition. As an example bacteria from a fermented milk product were unable to change the composition of the microbiome of human twins but changed the meta-transcriptomic profile¹⁴⁸.

Health effects of probiotics

Meta-studies of specific diseases

Probiotics have been demonstrated to have effects directly influencing the severity of some diseases. This has been shown in numerous instances as illustrated by the many meta-reviews on the effects on probiotics. In acute infectious diarrhea symptoms are improved by probiotics¹⁴⁹ as shown by a meta-analysis of controlled clinical trials including 8014 subjects¹⁵⁰. A wide array of probiotics, mostly from Lactobacillales or *Bifidobacterium* was used either alone or in combination to achieve these effects. Notably, no side effects were observed in any cases. In children a meta-review including 464 participants assessed randomized controlled trials and also showed shortening of diarrhea duration when using probiotics¹⁵¹ with no side effects. A specific organism that has been shown to cause diarrhea is *Clostridium difficile*. When antibiotics are administered occasionally *C. difficile* will take up available space and cause infection¹⁵². Following antibiotic treatment probiotics can limit *C. difficile*-associated diarrhea as shown by multiple meta-analyses in adults and children^{149,152–154}. Also, probiotics administered after antibiotic courses can limit the common antibiotic-associated diarrhea^{149,155}.

Besides diarrhea, symptoms probiotics have also been shown to limit symptoms of IBS in meta-reviews^{149,153,156}. Furthermore, the effect on severity of probiotics on the serious illness necrotizing enterocolitis of pre-term infants was assessed in a meta-review. In 2842 infants with a birth weight below 2500 g, probiotic administration significantly reduced both the incidence and death by necrotizing enterocolitis¹⁵⁷. All these meta-reviews show decent to limited improvement in symptoms across several diseases using many different strains of bacteria either isolated or in combination. It is highly noteworthy that no observations of adverse affects associated with using probiotics are documented in any of these studies.

Improved immunity

In addition to looking at specific diseases and their symptoms when administering probiotics it is also intriguing to consider effects in healthy subjects. Here improvement in disease-risk and physiological fitness is the goal. As assessed earlier in this review, microbes can influence the immunity of the host.

This leads to the appropriate hypothesis that probiotics can have a positive effect on human immunity. In support of this hypothesis a meta-review looked at probiotics and their effect on acute upper respiratory tracts infections (URTIs) such as the common cold¹⁵⁸. In 3451 participants they found that probiotics were better than placebo for reducing incidences of URTIs. Likewise, another meta-study found a reduction in incidences of the common cold in 2894 subjects¹⁵⁹. One systematic review looked at the effects of supplementing with strains of *Lactobacillus* and *Bifidobacterium*¹⁶⁰. The assessed outcome was duration of acute respiratory infection and in 20 randomized controlled trials they found a positive effect of probiotics. A significant reduction in days of illness, illness incident duration and absence from occupation was shown¹⁶⁰. As discussed earlier probiotics have a positive effect in treatment of acute diarrhea. However, whether or not it prevents its occurrence by lowering susceptibility has not been assessed. In a randomized clinical trial with a large cohort of 3758 Indian children a probiotic drink significantly reduced the frequency of acute diarrhea¹⁶¹.

An alternative way to assess the ability of probiotics to improve immunity is to examine their effect on vaccine responses in humans. Given the ability of probiotics to affect infectious disease severity and incidence it is plausible to think that probiotics could enhance this response. In infants there are slight indications that probiotics could improve immune responses to vaccinations in terms of vaccine-specific antibody production¹⁶². In adults the response is more well-defined, at least in IgA antibodies boosted by probiotics¹⁶². However, overall the evidence is not very strong and more clinical trials are needed to elucidate this effect of probiotics.

These things all support the role of probiotics in modulating the immunity in humans although some evidence only indicates a minor influence. Studies have tried to elucidate the mechanistic basis for the effect of probiotics. A major review has collected the available data in humans at its time and extracted some conclusions¹⁶³. They found that probiotics were able to affect phagocytosis, activity of natural killer cells and mucosal IgA production. These effects appeared to be specific for the strains used in the studies.

Health effects of “commensal probiotics”

Low diversity and the hygiene hypothesis

Generally a large fraction of studies of probiotics are done with bacteria of the genera *Bifidobacterium* or *Lactobacillus* as illustrated by studies cited in the previous section. However, many other genera are found in the human gut such as those belonging to the phyla Proteobacteria and Bacteroidetes². Low diversity of the intestinal microbiota is associated with diseases such as obesity¹⁶⁴ and IBD¹⁶⁵ as previously mentioned. This indicates that some health-promoting members of the commensal microbiome are missing in such cases. That members of the population are suffering from autoimmunity-related diseases from missing microbes due to lack of infections is the core of the hygiene hypothesis. The establishment of the hygiene hypothesis comes from migration studies. Individuals migrating from locations with low occurrence of autoimmunity to locations of high occurrence start developing autoimmune disorders in subsequent generations¹². Furthermore, exposure to siblings, day care, farming or parasite infection seems to be protective in some autoimmune states¹². In line with the hygiene hypothesis supplementing with some of these missing microbes could potentially reestablish the functionality of the microbiome in cases of autoimmunity. To support this case to some degree certain

commensal microbes can protect susceptible animals. As we have seen earlier in this review this includes protection from developing disease^{84,93} and from pathogen infection⁶⁷. This idea might push the boundaries of probiotics to include more different kinds of bacteria.

Fecal transplant

In keeping with the case for missing microbes, another angle that could support this is findings concerning fecal microbiota transplant (FMT), the procedure also mentioned earlier. However, it is generally not considered to be a probiotic due to the fact that unidentified microbes are being used¹⁶⁶. In fecal microbiota transplant the idea is to transfer stool from a healthy to a sick individual to restore health. Staying with the idea of an ill person being an individual missing certain commensal microbes, replacing these with the entire microbiota of a healthy donor could reestablish health. However as previously mentioned, so far only FMT treatment of *C. difficile* infection has shown true promise²⁴. In trials only vague effects have been established with ulcerative colitis¹⁶⁷ and modest with Crohn's disease¹⁶⁸. However, in subjects with metabolic syndrome FMT from lean donors increased insulin sensitivity and SCFA production of the microbiota¹⁶⁹. These studies indicate a decent potential but still with a long way to go. Regarding safety of the procedure very little evidence exists on long-term effects such as transmission of infectious pathogens and alterations in the microbiota leading to disease¹⁷⁰. However, it seems to be reasonably safe in the short term.

Fermented foods

Just as FMTs, fermented foods are not strictly considered as probiotics¹⁶⁶, but could have some of the same benefits as those observed for probiotics. Fermented foods contain live bacteria and are produced from milk products, vegetables and other food groups such as meat.

Fermented dairy

The most well studied types of fermented food are those from dairy like cheese, yoghurt and kefir (a fermented milk drink). Intake of fermented dairy products are associated with increased glucose homeostasis¹⁷¹, weight loss¹⁷² and lower risk of type 2 diabetes¹⁷³⁻¹⁷⁵. Furthermore, associations has been identified between fermented dairy intake and lower circulating triglycerides, blood glucose, systolic blood pressure, insulin resistance¹⁷⁶ and overall mortality¹⁷⁷.

In these cases it is difficult to recognize a health benefit from microbes in the food from health benefits of the food itself. However, in most of these associations fermented dairy is in a distinct group without unfermented dairy and yet shows individual features. This indicates that the fermentation itself and the bacteria it generates might have a benefit on its own.

Fermented vegetables

Kimchi refers to a Korean dish containing various salted and fermented vegetables, most often cabbage and radish along with other types such as leek and cucumber¹⁷⁸. These vegetables are generally fermented by Lactobacillales spp. of many kinds. Kimchi has been shown to promote health in several cases. For example adults display lowered cholesterol and fasting blood glucose in a kimchi-dose dependent manner¹⁷⁹. To make distinctions between the health effects of the fermentation process and the food itself some studies used unfermented kimchi as control. One of these studies found that both types of kimchi lowered body weight, body mass index and body fat¹⁸⁰. However, the fermented kimchi group was superior in all three of these measurements. Moreover, fermented kimchi had additional effects and significantly de-

creased waist to hip ratio, fasting blood glucose, blood pressure and total cholesterol¹⁸⁰. Another study found a reduction of body weight, body mass index and waist circumference in both groups¹⁸¹. Yet, only improvements in insulin sensitivity and blood pressure was found in the fermented kimchi group and also more participants showed enhanced glucose tolerance¹⁸¹. Interestingly, one study found a change in microbiota composition with intake of fermented kimchi compared to unfermented kimchi, which altered host gene expression¹⁸². With this distinction between fresh and fermented kimchi it is illustrated that the fermentation process itself has a unique impact. However, bacteria are known to produce components such as vitamins, essential to host health¹⁸³. Whether these components synthesized through fermentation or the colonization with the bacteria themselves confer the effects is not known.

In this section elucidation of the effect of probiotics have shown favorable effects in various diseases and overall health. Moreover, supplementation of commensal microbes may have unexplored potential as that seen in FMTs in fixing low-diversity microbiotas possibly caused by reduced microbe exposure. Lastly, fermentation of food products may improve their health effects by incorporation of beneficial bacteria and associated products.

Diet

Diet can greatly impact the microbiome in a short time-span¹⁸⁴. One study even showed effects of a diet-change on the microbiota composition within a 24 hour window¹⁸⁵. Just like affecting the microbiome short-term, diet also has a long-term impact. A recent mouse study even shows that the effect of diet can be multi-generational¹⁸⁶. They revealed that the offspring of mice fed a diet promoting a low-diversity microbiota were harder to rescue in terms of regaining diversity through diet. But what is more astounding, the third generation of offspring was even harder to rescue than the second¹⁸⁶. Carbohydrates seem to be the main component of the diet feeding the microbiota¹⁸⁷ and is the only one discussed in this review.

Defining microbiota-accessible carbohydrates

The term "prebiotics" traditionally refers to non-digestible food ingredients that travel to the colon and specifically stimulate growth and activity of health-promoting bacteria¹⁸⁸. However, this definition excludes a large group of fibers since their effect is not specific *per se*¹⁸⁹. Furthermore, even some well-known prebiotics such as fructo-oligosaccharides (FOS) are not specific in their growth-stimulation of the microbiota¹⁹⁰. Lastly, some claim that we do not know enough about the microbiome to specify what bacteria are exclusively beneficial or detrimental¹⁸⁸. Therefore this prebiotic definition loses its purpose. Instead of prebiotics this review will refer to dietary microbiota-accessible carbohydrates (MACs) when considering dietary components with influence on the microbiota. MACs refer to carbohydrates that are metabolically available to gut microbes from the diet and other sources such as host mucus and other bacteria¹⁹¹. The dietary MACs are not degraded and absorbed in the small intestine but act as an energy substrate for gut microbes. It can alter composition as well as metabolic output¹⁹¹. Different bacteria break down different components meaning that whether or not a compound is a MAC to one person is individual.

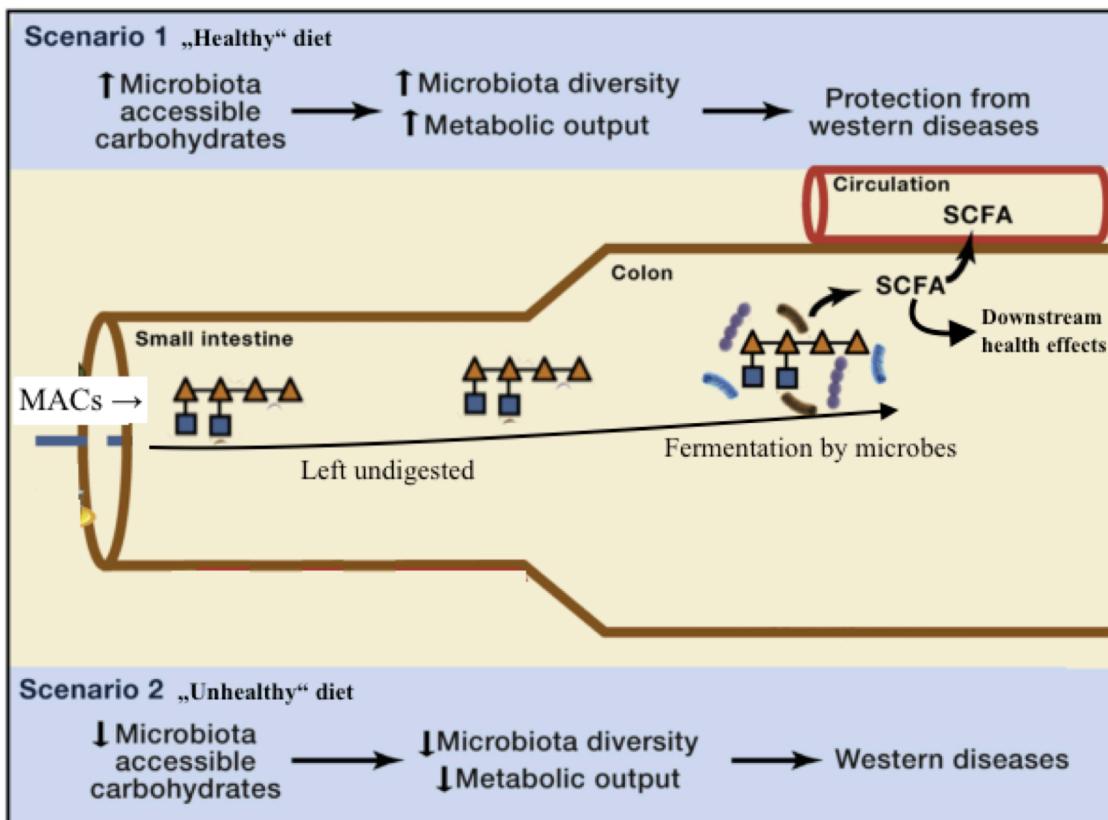


Figure 5 - Microbiota-accessible carbohydrates (MACs) are fermented in the colon. Fermentation of dietary MACs in the colon leads to increased diversity and metabolic output of the microbiota and seems to protect against disease. SCFA (Short-chain fatty acid).

Edited figure from source: Sonnenburg, E. D. & Sonnenburg, J. L. Starving our microbial self: The deleterious consequences of a diet deficient in microbiota-accessible carbohydrates. *Cell Metab.* **20**, 779–786 (2014).

SCFAs

As touched upon before a major fermentation product of the microbiota is SCFAs. Besides the big impact on immunity seen previously, SCFAs feed colonic epithelial cells¹⁹² and alter the pH of the colon to modulate the growth of different species¹⁹³. Additionally, the SCFAs produced in the colon enter the bloodstream and is utilized by the host as an energy substrate¹⁹⁴. However, one study shows that SCFAs can also promote intestinal epithelial proliferation leading to cancer in susceptible mice¹⁹⁵.

Different types of dietary MACs

MACs can include most of the traditional dietary fibers. These are: insoluble and soluble carbohydrates like cellulose, lignin and non-starch polysaccharides; non-digestible oligosaccharides where most “prebiotics” are found such as FOS and inulin; and lastly resistant starch (RS)¹⁹².

When the effects of MACs on the microbiome are quantified in the literature the “beneficial” microbes are sometimes restricted to the phyla *Bifidobacterium* and *Lactobacillus*¹⁸⁸. Other times SCFA production and overall diversity is measured¹⁸⁵. This indicates that there might not yet be a clear consensus on how to determine the health benefits of MACs.

MACs alter composition and function of microbiota

Epidemiological studies

A study on the fecal microbiota of European and rural African children showed a significant difference between the two populations¹⁹⁶. In the African children bacteria of the phylum Firmicutes were less abundant whereas those of the phylum Bacteroides were more abundant than in the European children. Specifically, only the African children inhabited bacteria of the genera *Prevotella* and *Xylanibacter* known as efficient metabolizers of dietary fiber. Furthermore, significantly more

SCFAs were found in the African children. The researchers attributed these differences to the high fiber content of the African diet that also protected them from inflammation and colonic disease¹⁹⁶. It is generally observed that more ancestral-like populations inhabit greater microbiota diversity. Healthy Amerindians and rural Malawians display higher diversity than Americans, and interestingly cluster together distant from Americans in Principal Coordinate Analysis¹⁹⁷. Their diet is rich in plant-derived polysaccharides contrasting the American diet of readily absorbed sugars¹⁹⁷. The Hadza of Tanzania display microbiotas that are more diverse than Italian ones¹⁹⁸. They show enrichment in metabolic pathways related to metabolizing complex polysaccharides¹⁹⁹, reflecting the difference in diet in the two groups. To further explore the cause and effect relationship between diet and microbiota on a population basis O’Keefe et al. changed the diets of two populations²⁰⁰. By giving African Americans a high-fiber diet, and rural Africans a low-fiber diet they decreased and increased the risks of cancer, respectively. This was illustrated with different biomarkers including upregulated fermentation and butyrate production levels in microbiotas of African Americans²⁰⁰.

Carbohydrate restriction

Diets low in carbohydrate are occasionally used to promote weight loss in overweight individuals²⁰¹. But since this necessitates the minimization of MAC intake one might question the effects of these diets on the microbiota. 91 overweight subjects were either given an energy-restricted high-carbohydrate (HC) or low-carbohydrate (LC) diet for eight weeks²⁰². After eight weeks the LC group displayed diminished stool weight with low concentrations of SCFA and low count of *Bifidobacteria* compared to the HC group. In another

study 19 obese participants first received a HC diet for three days followed by a medium-carbohydrate (MC) and a LC diet for 4 weeks each²⁰³. Some significant differences according to carbohydrate intake were observed at the end of each type of diet: Ranking of total SCFA and butyrate concentration of fecal matter of the three diets corresponded to their carbohydrate intake; bacterial count of *Roseburia*, *Eubacterium rectale* and *Bifidobacterium* decreased with carbohydrate intake; and decrease of *Roseburia* and *E. rectale* was proportional to butyrate decrease²⁰³. In a similar analysis, 17 obese men were assigned to a HC diet for 7 days followed by a LC diet and a MC diet for four weeks each²⁰⁴. The relative carbohydrate intake of the diets corresponded to the ranking of SCFA concentrations and butyrate ratio of total SCFAs in fecal samples. As in the other study a significant decline in *Roseburia* and *E. rectale* was observed with reduced carbohydrate intake.

FODMAPs (fermentable oligo-, di-, monosaccharides and polyols) is a group of fermentable carbohydrates that are often restricted in individuals with IBS and seem to alleviate symptoms^{205,206}. A series of studies has assessed the effect it has on the microbiome. 35 patients with IBS were given a low FODMAP diet or a control diet for four weeks²⁰⁷. There was no significant change in total bacteria, SCFA or *E. rectale* levels. However, there was a significant decrease in the proportion of *Bifidobacteria* in the low FODMAP group²⁰⁷. In 2015, 27 subjects suffering from IBS and 6 healthy individuals were assigned to a 21-day low-FODMAP diet or a control diet²⁰⁸. Afterwards the groups switched to the diet of the other group for 21 days with an in-between washout period of similar length (known as a crossover trial). The low-FODMAP diet increased overall bacterial diversity and the pH of fecal matter without affecting SCFA concentrations. In contrast the control diet increased the abundance of *Akkermansia muciniphila* and *Clostridium* cluster XIVa (where *Roseburia* and *E. rectale* belong) and decreased abundance of *Ruminococcus torques*²⁰⁸. Another study wanted to evaluate the baseline difference in microbiota composition between responders and non-responders to a low-FODMAP diet (as measured by symptom relief)²⁰⁹. They found that responders generally had a microbiome with upregulated carbohydrate-metabolism capacity. Since FODMAP-restriction is thought to lower symptoms by decreasing fermentation and gas production²⁰⁹, it makes sense that responders would have high fermentation potential.

Fiber supplementation

A variety of fibers that do not belong to the group of prebiotics or resistant starches (RSs) exist. Their effect on the microbiome has been evaluated in some studies. Gum arabic, found in acacia trees, significantly increases *Bifidobacterium* and *Lactobacillus* counts compared to control and even compared to the established prebiotic inulin²¹⁰. A study found that poly-dextrose supplementation decreased stool pH without affecting SCFA concentration²¹¹. Moreover, it had no effect on abundance of *L. acidophilus* and *B. lactis* but decreased *Eubacterium* count. However, no other bacterial taxa than those were analyzed.

Whole-grains have also been assessed for their potential to modulate the microbiome. Whole-grain wheat increased *Lactobacillus* and *Bifidobacterium* count compared to wheat bran (which is poorly fermented) but did not affect fecal SCFA concentration²¹². Similarly whole-grain maize increased *Bifidobacterium* levels compared to control without changing SCFA concentrations²¹³. In a newer study refined wheat was replaced with whole-grain wheat in obese subjects with a low intake of fruits and vegetables²¹⁴. The intervention had no

effect on overall microbiota community structure. This was the case both between control diet and whole-grain diet as well as between pre- and post-intervention in the whole-grain group. However, individuals eating whole-grains did increase their levels of *Prevotella* while reducing their levels of *Dialister*, *Blautia* (Firmicutes) and *Collinsella* (Actinobacteria)²¹⁴.

Prebiotic supplementation

As discussed earlier the prebiotic definition has its shortcomings and has also been modified numerous times since its creation¹⁸⁸. In most trials only a few MACs are considered to be prebiotics because of this. These typically include inulin, FOS and galacto-oligosaccharides (GOS) and they have been studied in human trials. Inulin has been shown to induce *Bifidobacterium* in multiple studies²¹⁵⁻²¹⁹ with the specific species being *B. longum*, *B. adolescentis* and *B. bifidum*. One study also found that inulin induced *Lactobacillus*²¹⁷. Furthermore inulin decreases the prevalence of *Bacteroides*, *Prevotella*^{215,217} and *Desulfovibrio*²¹⁹. However, inulin does not seem to increase SCFA concentration^{215,217}, unless total dietary fiber intake is included in the statistics²¹⁹. GOS has also been shown to induce proliferation of *Bifidobacterium*^{220,221} while inhibiting *Bacteroides*²²¹. Likewise, FOS-enriched inulin was found to increase *Bifidobacterium spp.*²¹⁸.

The health benefits of these fermentable prebiotics have been studied in relation to metabolic function. A meta-analysis including 831 subjects found that prebiotics (FOS, GOS and inulin) generally increase satiety and lower blood glucose and insulin response after eating²²².

Resistant starch supplementation

Resistant starch is another type of dietary fiber that comes from many different sources²²³. As the name implies RS is starch that is not broken down and absorbed in the small intestine but is instead fermented in the large bowel. RS is classified into three types, namely: RS1, a RS physically inaccessible to digestion because of other components of the food such as cell walls, and is typically found in grains, seeds and legumes; RS2, a type of starch with conformations that prevents digestion, which can be found in raw potatoes, green bananas, legumes and corn; RS3, a so-called retrograded starch generated from first cooked then cooled foods such as potatoes and bread; RS4, a group of chemically modified starches; and lastly RS5, a type of RS formed during food processing and other procedures²²³.

The examination of human consumption of RS has been shown to have several effects on the microbiome. The ability of RS to increase butyrate concentration was demonstrated early on²²⁴. Here RS2 and RS3 were given to 24 healthy subjects in a crossover trial. The analysis showed that the ratio of butyrate to total SCFAs rose significantly upon RS consumption. Another group replicated these results²²⁵. They confirmed that RS3 raised fecal butyrate but not total SCFA concentrations. In a more recent study with 20 healthy participants, researchers found that RS3 only had an insignificant tendency to increase the butyrate ratio²²⁶. However, when supplementing with the polysaccharide pullulan along with RS3 the butyrate ratio was significantly higher²²⁶.

Besides SCFA alteration RS has also been shown to modulate the composition of the microbiota. In one crossover trial participants receiving non-starch polysaccharides increased their relative proportions of *Ruminococcus bromii* only when also taken with HI-Maize (an RS product)²²⁷. In another double-blind crossover trial RS4 increased *Bifidobacterium adolescentis* and *Parabacteroides distasonis*, while RS2 increased *R. bromii* and *E. rectale*²²⁸. Similarly Walker et al. showed that

R. bromii, *E. rectale* and *Oscillibacter* bacteria increased in amount when consuming RS3¹⁸⁴. Furthermore, they found that there was great variation in people regarding the amount of RS that was fermented in their colon. This seemed to be influenced by baseline *R. bromii* numbers¹⁸⁴. In line with this, a culturing study found that *R. bromii* and *B. adolescentis* were very efficient at breaking down RS type 2 and 3²²⁹. In contrast *E. rectale* and *Bacteroides thetaiotaomicron* showed low efficiency in breaking down RS. Surprisingly, however, when co-culturing the four species *R. bromii* could induce an increased RS utilization in the other species²²⁹ showing the key role of *R. bromii* in RS breakdown. A study from 2014 found an increase in several bacterial taxa in subjects on an RS3 diet²³⁰. These were *Oscillospira guillermondii*, *R. bromii*, *Sporobacter termitis*, *Clostridium leptum* and *C. cellulosi*. However, the RS diet decreased the overall microbiota diversity. In contrast to previous findings a study in rural Malawi children showed that RS2 intake did not cause an increase in butyrate ratio²³¹. Instead the SCFA propionate was increased. Furthermore, the diversity of the microbiota declined as a consequence of RS intake and an increase in the fecal inflammatory marker calprotectin (associated with colitis) was seen²³¹.

Regarding health benefits of RS supplementation, mostly metabolic ones have been reported. A recent review concluded that RS seems to be beneficial in overweight individuals and obesity prevention²³². They found that numerous studies showed that RS decreases weight gain and improves insulin, glucose as well as cholesterol homeostasis. As shown by these studies dietary MACs of many kinds can have an effect on the microbiome.

Together the studies in this part of the review suggest that antibiotics, infant feeding strategy and delivery mode have significant impacts on the microbiome. This is both in regard to host health and microbe composition and metabolism in the gut. Antibiotics can shift the stable state configuration long- and short-term or affect the resilience of the gut to future antibiotic disturbances. Likewise, it can affect the metabolic function of the microbiome and increase disease risk for years to come. The many components found in breast-milk shape the infant gut. Among these components are live bacteria and bacteriogenic non-digestible oligosaccharides. Formula-fed and breast-fed infants show significantly different microbiota compositions, which may lead to long-term detrimental health effects. Delivery mode also plays an important role in development of the infant microbiome. Infants born via cesarean section inhabit microbes resembling the skin whereas infants delivered through the birth canal display a microbiome resembling that of the vagina. Like other alterations delivery mode also has long-lasting effects on both the microbiota and possibly the disease risk. Probiotics have been shown to be beneficial in alleviating symptoms in some diseases. Also, it has been found to improve baseline immunity and preventing occurrence of certain diseases. Other types of supplementary microbes not considered probiotic have valuable use as well. Supplementing with commensal microbes could be a strategy to improve health by inhibiting low-diversity associated diseases and this has been explored in some FMT trials. Another non-probiotic approach is fermented foods. These could potentially have positive impacts on disease risk that are not observed in non-fermented foods due to the beneficial bacterial proliferation. Furthermore, dietary MACs can have an effect on the microbiome both long- and short-term through its fermentation in the colon (Figure 5). These effects are mainly alterations in metabolite output of the gut as well as microbiota composition. These effects are seen both when viewing

entire populations and when performing intervention trials in humans. The interventions evaluated here include restriction as well as enrichment of various types of dietary MACs. They show that not one single type of MAC is responsible for the effects seen. Overall MAC supplementation seems to be beneficial for the microbiome and the health state of the host even though a few studies suggest otherwise.

Concluding remarks

This review has demonstrated that the microbiome is able to modulate the immune response of the host. Furthermore, it has illustrated that different lifestyle interventions such as antibiotics and fiber can modulate the microbiome. The things considered in this review suggest that people can shape the environment of the microbiome with their lifestyle choices as new knowledge is acquired. In this way the host could possibly manipulate its own susceptibility to certain diseases of autoinflammatory nature indirectly such as rheumatoid arthritis. This could be a missing link in considering the health benefits of a “healthy” diet and lifestyle.

Perspective

Besides those interventions mentioned here, new environmental factors that drive the microbiome health are continuously being discovered. In mice and/or rats exercise has proven to be another valuable tool. Exercise can restore intestinal integrity²³³ and change gut microbes observed^{233–236}. Furthermore composition of the microbiota depends on whether it is voluntary or forced exercise²³⁷ and human athletes exhibit a different composition from non-athletes²³⁸. Likewise, circadian rhythms also appear to be of importance for the microbiome^{239–241} implying the consequence of factors such as less than optimal sleep patterns and light exposure cycles. As discussed earlier, the mouse model has its limitations even though it has helped the field to evolve much further than it otherwise would have. New model systems are being developed and recently HuMiX was presented²⁴². HuMiX is a “modular microfluidics-based human-microbial co-culture model”. It represents an approach with which interactions between actual human intestinal cells and microbes are observed outside a living system. With time systems like these and the further developed models to come may replace the mouse model and possibly decrease the limitations of modeling the human microbiome.

In addition to the major influence the microbiome can have on intestinal and systemic immunity, another new area of interest is the impact on behavior. This connection is referred to as the gut brain axis²⁴³ and show that the microbiota can directly influence stress-related behaviors like depression and anxiety. The microbiome is a field in rapid exponential growth and thus far only the sky is the limit.

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