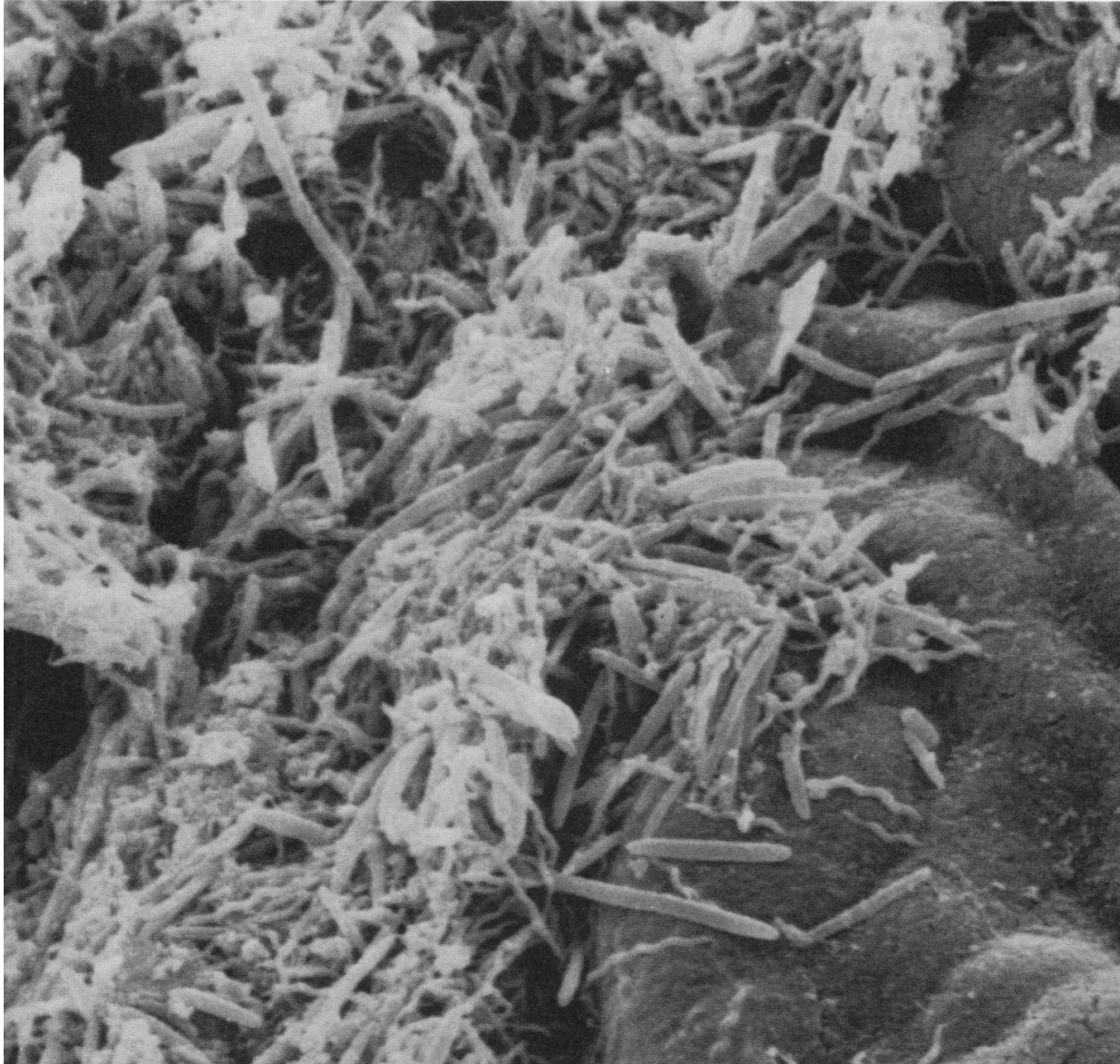


# Interactions between the host and intestinal microflora that maintain homeostasis in the gut



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**About the cover image**

Members of the microbiota associate themselves with the mucosa. This scanning electron microscopy image displays the level of colonization of the mouse cecum, with a variety of rod- and spiral shaped organisms predominating on the mucosal layer. Image adapted from Lee A. *et al*, *Infect. Immun* 51: 536-546, 1986.<sup>51</sup>

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## ABSTRACT

The intestinal flora is increasingly recognized as an important determinant of human health. Commensal bacteria drive the maturation of the intestinal barrier and modulate the development and function of the mucosal immune system, and awareness is growing that changes in the microbiota also influence diseases such as diabetes and atherosclerosis. However, many of the mechanisms that drive the interactions between the microbiota and the host remain to be elucidated. The aim of this thesis is to review the interplay between the gut microbiota and the host immune system, with emphasis on the events following birth and early bacterial colonization. A number of experiments will be performed as well, to demonstrate the ability of pathogens to penetrate the epithelium and invade epithelial cells.

## Introduction

Humans are known to have a complex relationship with the microbial world. Although some of the relationships with microbes are pathogenic, the majority of the interactions between humans and microbes do not cause disease. In fact, many of the interactions between mammals and microbes are of symbiotic nature. The microflora in the gut for example makes key contributions to host health, including increased digestive and metabolic efficiency, promoting the development of the host immune system and providing resistance against intestinal pathogens.

To maintain the symbiotic nature of the microbiota, immunological homeostasis in the gut is essential. Indeed, the intestinal microflora and host immune system appear to interact extensively to maintain the environment that is favorable for both the host and the microflora. The mechanisms that drive the interactions between the host and the microflora in these events remain to be elucidated, as our knowledge of the microbiota is still limited. The aim of this thesis is to review the molecular interactions between the intestinal microflora and the host immune system, with emphasis on the interactions occurring during the initial colonization of the gut and the first months of life.

## The adult gut

### The microbiota in the adult gut

The adult human gut is populated by an enormous number of bacteria. Its number – approximately  $10^{14}$  (100 trillion) – even exceeds the total number of cells of a human body by an order of magnitude. These bacteria form a very diverse population, which is not completely mapped up until today due to difficulties culturing many of the species resident in the gut.<sup>1</sup> The majority of the bacteria in the gut establish symbiotic relationships with their hosts, aiding significantly in the digestion and metabolism of the host diet. As mammals do not carry the genes for a range of enzymes capable of digesting all the plant fibers present in their diet, adopting a population of bacteria capable of digesting this otherwise indigestible part of the diet is highly beneficial. The importance of the microbiota for digestion is demonstrated in sterile animals lacking an intestinal microflora, known as germ-free animals. These animals take up approximately 30% more calories in order to maintain their body weights.<sup>2</sup>

As a result of the very high density of bacteria, the intestine is a very competitive microenvironment. In order to persist in this environment, bacteria have developed mechanisms that allow them to shape the host environment. An example of this principle is the ability to change the amount of fucosylation of host proteins: *Bacteroides thetaiotaomicron* carries a cluster of genes that form a feedback loop, allowing it to coordinate its energy need with the availability of a host-derived energy source. Disruption of these genes decreases the ability of this bacterium to persistently colonize the intestine.<sup>3</sup>

Commensal microbiota are also responsible for increasing resistance against intestinal pathogens and inflammatory disease. Since most pathogens lack the repertoire of digestive enzymes carried by the symbiotic microbiota, they are poorly equipped to compete for nutrients of the host diet, limiting their ability to colonize the intestinal lumen. Commensal bacteria also stimulate host immunity by activation of pattern receptors like toll-like receptors, increasing the production of antimicrobial peptides by cells in the gut epithelium and the proliferation of immune cells, as discussed later in this thesis. Research has demonstrated this potential in different models using germ-free mice, showing that germ-free mice have reduced resistance against intestinal pathogens like *Shigella flexneri* and *Listeria monocytogenes*.

## The intestinal immune system

Although a large part of the microbiota in the intestine is harmless to its host, many food-borne pathogens will target the intestine as a primary route of entry, necessitating an extensive immune system protecting the gut and the cells surrounding it, preventing contact with the epithelium and dissemination of pathogens to other host tissues.

A significant part of limiting bacterial penetration is facilitated by the mucus lining the epithelium. The mucus consists mainly of the heavily glycosylated protein mucin, which assembles into a thick protective gel-like substance. The mucus can be divided into two distinct layers, with a loose outer layer that can be colonized by bacteria, and a dense inner layer. This inner layer is resistant to bacterial penetration, aiding in the prevention of contact between bacteria and the host epithelium lying underneath this layer of mucus. The protective effects of the mucus are demonstrated in mice lacking one of the major constituents of the mucus, MUC2. These mice are not able to prevent contact between bacteria and the epithelium, leading to the development of colitis several weeks after birth.<sup>4</sup>

Another major mechanism employed by the immune system to prevent bacterial penetration is the secretion of bactericidal proteins. The majority of these antimicrobial proteins kill bacteria by disrupting the bacterial inner membrane, or by enzymatically attacking the bacterial cell wall.<sup>5</sup> Others starve bacteria of essential components, like lipocalin 2, which deprives bacteria of iron.<sup>6</sup> Virtually all cell types present in the intestine express antimicrobial proteins, although not all proteins are regulated in the same way. Some are expressed constitutively, meaning they are expressed independently of bacterial signals. Others require bacterial stimulation, like  $\alpha$ -defensins and cryptidins secreted from Paneth cells. This stimulation usually occurs through activation of pattern recognition receptors, which recognize conserved characteristics shared by different bacteria. Toll-like receptors or nucleotide-binding oligomerization domain-containing protein (NOD) 2 are part of this group.<sup>7</sup>

A third mechanism employed to prevent bacterial spread is the secretion of IgA by B cells in the intestine. The resulting IgA<sup>+</sup> B cells home to the gut epithelium where they produce IgA, which is then transcytosed into the gut lumen. The secreted IgA protects the gut lumen. How this system works is not fully known, but it may involve trapping bacteria in the mucus, or opsonizing them for phagocytosis if bacteria do manage to penetrate the epithelium.

In the event that bacteria manage to breach the protective measures described above, there is another layer of defense, mainly consisting of phagocytic cells. Macrophages and neutrophils beneath the epithelium very efficiently phagocytize and kill invading bacteria, thereby preventing dissemination further into the host.

## Colonization of the newborn gut

Most mammals are born germ-free, naïve to the environmental bacterial presence. Over the course of a very short timeframe however, the epithelial surfaces of the newborn are colonized by countless microorganisms, occupying their own distinct niches. The first colonization occurs during birth, as a result of contact with the microbes present in the vagina and cervix. These initial colonists are typically facultative anaerobes like *Escheria coli*, staphylococci and streptococci, which can be detected in the neonatal feces within hours. This process is different in children delivered by caesarian section: since these children are directly delivered from the sterile womb to the outside environment, their sources of primary inoculation are different. This means the newborn is initially colonized with different bacteria when compared to a conventionally delivered neonate.<sup>8</sup>

During the first months after birth, the microbiota develops towards a more stable pattern resembling that of an adult, which consists mainly of obligate anaerobic species such as *Bifidobacterium*, *Bacteroides* and *Clostridium*. One of the factors that may direct this transition of bacterial flora is breast milk. Breast milk contains large amounts of glycans that stimulate the growth of bacterial species that

can utilize these glycans as an energy source, such as bifidobacteria and lactobacillae.<sup>9, 10</sup> Breast milk also contains antibodies, which may act as a selective force on the initial microflora by eliminating possibly harmful species. Other compounds in breast milk may also have shaping effects, either by facilitating or restricting the growth of a certain part of the microflora.

Another large event in the development of the microbiota is weaning, the transition from breast milk to a diet containing solid food. During weaning a large influx of obligate anaerobic species can be seen, shifting the microflora towards its obligate anaerobic climax state. In this period bacteria also start manipulating host processes that provide them with a competitive edge. An extensively studied example is the change of host glycans during weaning, caused by an obligate anaerobic member of the microbiota, *Bacteroides thetaiotaomicron*. Before weaning, glycans on the epithelium are usually terminated with a sialic acid residue. Shortly after weaning however, these glycans are mainly terminated with a fucose residue instead. In germ-free mice, this switch is not seen, while in mice monocolonized with *B. thetaiotaomicron* this switch is readily observed.<sup>11</sup> This switch is the result of a cluster of genes encoding a feedback loop system, allowing the metabolism of fucose as an energy source. The system allows the bacterium to 'ask' the host for more fucose as well, stimulating the fucosylation of host proteins. The upregulation of fucosylation is one of the mechanisms employed by the bacterium to facilitate its own persistence in the intestine. The importance of this system has been demonstrated using a strain of *B. thetaiotaomicron* lacking the fucose sensing system: the strain was unable to persistently colonize the exposed animals.<sup>3</sup> Other commensal bacteria may also employ mechanisms that increase their ability to persistently colonize the intestine, although further research would be needed in order to elucidate the other various mechanisms.

## **Interplay of bacteria with the host immune system**

The immune system is a formidable barrier against many pathogens, capable of defending against the wide variety of pathogens that try to invade the body via the gut. Its development is strongly dependent on interaction with the symbiotic bacteria present in the intestinal lumen that promote both the correct development of immune reactions, as well as the induction of immune homeostasis with the high bacterial load in the intestine.

### **Development of lymphoid tissue**

A very important part of the immune system protecting the gut is the lymphoid tissue in the gut. The lymphoid tissues generate B-cells producing secretory IgA direct against the intestinal flora, but also include lymphoid follicles, Peyer's patches and mesenteric lymph nodes, all containing cells that constantly monitor the status of the luminal content and protect against pathogens. To generate this lymphoid tissue, bacterial stimulation is required. Bouskra *et al* demonstrated this in mice using germ-free mice and mice with a limited microflora. Germ-free mice, as well as mice deficient in the innate pattern recognition receptor NOD1 displayed fewer lymphoid follicles, demonstrating the need for innate sensing of the increasing bacterial load during the early stages of life.<sup>12</sup> Other parts of the immune system may be affected by lack of bacterial stimulation as well, such as the spleen and thymus.<sup>12-14</sup> Bacterial stimulation also affects the secretion of innate antimicrobial peptides by Paneth cells. For example, expression of the microbicidal protein Angiogenin-4 increases rapidly during the weaning period, in response to the influx of bacteria, and its expression is maintained during life. In germ-free animals, the expression level of Angiogenin-4 remained low after weaning. Colonization of these mice however restored the expression of Angiogenin-4 to wildtype levels.<sup>15, 16</sup> Angiogenin-4 appears to be species-specific, meaning that bacterial induction of Angiogenin-4 may help shape the intestinal environment to favor certain populations in the gut. Other antimicrobial proteins induced by bacterial stimulation include  $\alpha$ - and  $\beta$ -defensins.<sup>15, 17, 1718</sup>

### Induction of regulatory T cells

The commensal microflora also modulates the adaptive immune system in the gut to maintain homeostasis. In order to achieve this, the microbiota is able to induce regulatory T (Treg) cells. Treg cells are essential for tolerance to both self and foreign proteins by preventing inflammatory reactions. This effect is mainly achieved by secreting IL-10, a cytokine with anti-inflammatory properties. By inhibiting the secretion of pro-inflammatory cytokines such as IL-1, IL-12 and tumor necrosis factor (TNF)- $\alpha$  it is able to prevent excessive or unnecessary inflammation.<sup>19</sup> The correct development of regulatory T cells in the gut is dependent of several species of the intestinal microbiota. One of these species is *Bacteroides fragilis*, a gram-negative anaerobic commensal of the gut. *B. fragilis* has been shown to have a number of immunomodulatory properties, one of them the induction of Treg cells. This effect appears to be the result of a single carbohydrate synthesized by the bacterium, Polysaccharide A (PSA). PSA achieves this effect by activation of TLR2 on host CD4<sup>+</sup> T cells. Bacteria deficient in PSA are unable to induce regulatory T cells and fail to persistently colonize the host.<sup>20-22</sup> Other bacterial species are able to induce regulatory T cells as well: the inoculation of mice with a mixture of *Clostridium* species resulted in a marked increase of CD4<sup>+</sup> Foxp3<sup>+</sup> T cells in the intestine. This research also showed that the detected Treg cells in the gut are induced Treg (iTreg) cells, possibly meaning that the cells are generated in the gut as a result of contact with the intestinal microbiota.<sup>23</sup> However, It is not known how *Clostridium* is able to induce regulatory T cells. While it has been shown that the effects of *B. fragilis* PSA are mediated via TLR activation, *MyD88*, *Rip2* or *Card9* deficient mice colonized with *Clostridium* species still showed similar numbers of regulatory T cells in the mucosa, demonstrating that the bacterial induction of Tregs is not solely dependent of TLR activation.<sup>23</sup>

Other bacterial species may induce regulatory T cells as well, although these have not been fully elucidated. Other mechanisms of Treg induction may involve the activation of other pattern recognition receptors like those from the NOD family, the induction of an environment rich in the cytokine TGF- $\beta$ , which is a major stimulant of Treg formation<sup>19</sup>, or by production of specific metabolites, like butyrate or other short chain fatty acids (SCFA)<sup>24</sup>.

### Modulation of the T helper 17 response

Homeostasis in the gut is not solely dependent on the induction of regulatory T cells. The microbiota also modulates other immune cell populations in order to further promote homeostasis. One of these are T helper 17 (Th17) cells, which are essential in the defense against bacterial and fungal pathogens at mucosal barriers. When stimulated with TGF- $\beta$  and IL-6, antigen-activated CD4<sup>+</sup> T cells upregulate the transcription factor ROR $\gamma$ t and express Th17 specific cytokines like IL-17 and IL-22.<sup>25</sup> These cytokines contribute to the barrier function of the intestinal epithelium, by increasing tight junction formation and stimulating the secretion of microbicidal proteins. The Th17 response also stimulates the expansion of neutrophils, which are required for the clearance of invading bacteria.<sup>26, 27</sup> The Th17 response is essential for protection against mucosal pathogens like *Klebsiella pneumoniae* and *Salmonella typhimurium*. Mice deficient in Th17 cytokines display severe pathology during infection with pathogens like *Salmonella* or *C. rodentium*, with increased translocation of bacteria into lymph nodes.<sup>28</sup> Since Th17 cells possess significant proinflammatory potential, tight regulation of these cells is required to prevent potentially disastrous inflammatory responses against the microbiota.

Bacterial species are able to modulate the Th17 response under experimental conditions. One group of bacteria able to do so are the segmented filamentous bacteria (SFB), a non-culturable *Clostridia*-related species. These bacteria adhere tightly to the host epithelial barrier, where they interact with the epithelial cells. In animals monocolonized with SFB, robust Th17 responses were detected. The main mediator in this process appeared to be Serum Amyloid A (SAA) produced by dendritic cells, which was able to transform CD4<sup>+</sup> T cells to Th17 cells.<sup>22, 25, 29, 30</sup>

As mentioned before, *Bacteroides* species have been shown to have immunomodulatory effects. Colonization of mice with *B. fragilis* resulted in the suppression of pro-inflammatory Th17 reaction. This modulation could be attributed solely to the carbohydrate PSA, demonstrating the wide range of effects this carbohydrate can have on the immune system. *B. fragilis* lacking PSA was unable to suppress Th17 response, and also failed to persistently colonize the intestine.<sup>21</sup>

What is important to note about the Th17 response is its proinflammatory nature, making the Th17 response an interesting target for research on its role in other diseases as well. Indeed, disruption of Th17 responses is observed in autoimmune diseases<sup>25</sup>, and mice deficient in Th17 cells are protected from several experimental autoimmune diseases.<sup>31</sup> Since there is evidence for the influence of the microbiota on the status of Th17 cells, there may be a link between a disturbed microbiota and the status of autoimmune disease, making the microbiota an interesting target for research in relation to autoimmune disease.

### **Secretion of IgA**

As mentioned before, an important effector of the mucosal immune system is the secretion of IgA. The secreted IgA helps to protect the intestine against pathogens, and prevents overgrowth of the intestinal microbiota. Dendritic cells beneath the epithelium of Peyer's patches support the generation of IgA in the intestine. They are able to sample both invading bacteria as well as bacteria that are still in the gut lumen by means of extended dendrites. These dendritic cells, carrying live bacteria, traverse to nearby mesenteric lymph nodes, where they are capable of inducing class switching to IgA of B cells. The class-switched B cells then traverse back to the gut, where they start producing IgA, which is transcytosed into the gut lumen.<sup>32</sup>

The homing of B cells towards the lymphoid tissue in the gut is mediated through chemokine receptors on the B cells. The expression of these chemokine receptors varies throughout infancy, displaying marked changes in the first months after birth, possibly in response to colonization by the gut microbiota.<sup>33</sup> Another observation suggesting the involvement of the microbiota in the development of the intestinal B cell population is the effect of TLR4 activation in the gut epithelium on the secretion of IgA. Mice expressing a constitutively active form of TLR4 expressed in the epithelium displayed increased B cell recruitment to the gut and a higher sIgA content in their feces. Although these results are based on genetic manipulation, they give an indication that interaction with the microbiota through TLR4 activation benefits the development of the B cell population and the secretion of IgA in the gut.

### **Protection of the premature gut**

A large portion of the microbiota has beneficial effects on the gut, both for the host metabolism and host health. While many of these bacteria are harmless when they are residing in the gut, they can pose a serious threat to the host health when they do manage to get below the gut epithelium. This can lead to exaggerated immune responses in the gut, with loss of electrolytes and fluids as a result. This is especially true for the newborn intestine, which has not fully matured yet. The intestinal epithelium is still leaky, allowing bacteria easier access to underlying tissues. The adaptive immune system has not fully developed either, adding difficulty to mounting an effective immune response to these invading bacteria. Considering that the neonate is still relatively unable to effectively manage the high bacterial load of the microbiota, one would expect to see disease and inflammation shortly after birth, as a result of the primary colonization. The opposite is true however, as most neonates seem to be protected from disease and inflammation during the first weeks after birth suggesting that external factors and immune adaptations are essential for maintaining homeostasis in the immature gut.



## Breast feeding

Breast feeding appears to contribute significantly to preventing harmful effects of the bacterial colonization. Breast milk contains large amounts of IgG and IgA antibodies as well as glycans and triglycerides, each having protective effects on the infant's intestine. The antibodies in the breast milk have a neutralizing effect on many of the possibly pathogenic microbes in the gut. Since the infant's own production of antibodies is delayed, the breast milk provides a temporary source of protection. In neonatal mice, the production of antibodies is delayed to the point of weaning, after which their own production of antibodies starts.<sup>9</sup> Another major component of the breast milk is the diverse repertoire of glycans and oligosaccharides. Many of these glycans help protect the infant gut from several toxins and viruses. Glycans and oligosaccharides from the milk inhibit bacterial toxins, and help prevent invasion of *Helicobacter pylori*, which normally binds to host cell glycans. It even offers limited protection from viruses.<sup>9</sup> Breast milk also contains several growth factors, one of them being TGF- $\beta$ . TGF- $\beta$  is one of the cytokines involved in the development of Treg cells, so although it may not directly protect from intestinal infections, the TGF- $\beta$  received from the mother may regulate the immune status of the neonatal epithelium, dampening excessive reactions from the developing neonatal gut.<sup>34</sup> Another growth factor useful for the gut may be secreted epidermal growth factor (EGF). Research has demonstrated its protective effect in studies observing necrotizing enteric colitis (NEC). One of the beneficial effects is the downregulation of pro-inflammatory cytokines and the upregulation of anti-inflammatory cytokines like IL-10.<sup>35</sup>

## Intestinal macrophages

Breast milk is not the only factor protecting the infant gut from excessive inflammation. A good example of the coevolution of the microbiota and the intestinal immune system is the way certain cell populations have evolved. One of these populations would be the intestinal macrophages. Normally, macrophages display a wide array of innate response receptors, including receptors for LPS, and receptors for growth factors like IL-2 and IL-3. They also secrete proinflammatory cytokines including TNF- $\alpha$ , IL-1, and IL-6. The intestinal macrophages are different however: they do not secrete pro-inflammatory cytokines in response to phagocytosis and lack innate response receptors. Interestingly, these macrophages do have full scavenger and bactericidal abilities, displaying full killing potential against *S. typhimurium* and *E. coli*. *In vitro*, the intestinal macrophages displayed downregulated responses against several bacterial components as well as whole bacteria.<sup>36</sup> Therefore, intestinal macrophages are able to protect the gut from invading bacteria, while preventing the release of proinflammatory cytokines that might disturb intestinal homeostasis.

Intestinal macrophages contribute to intestinal homeostasis in other ways as well. Closely associated with the epithelium, the intestinal macrophages are capable of stimulating growth of the epithelial cells in response to injury. Activation of the intestinal macrophages occurs through toll-like receptors, as mice deficient in the TLR signaling adaptor molecule MyD88 show marked irregularities in response to intestinal injury by dextran sodium sulphate, indicating the need for TLR activation for successful repair of damaged epithelium. Interestingly enough, germ-free mice displayed the same irregularities as *MyD88*<sup>-/-</sup> mice, meaning that the required TLR activation results from contact with the microbiota.<sup>37</sup> As the epithelial barriers are still leaky shortly after birth, contact with the microbiota on incompletely developed surfaces may help speeding up the development of these surfaces through growth factors released by the intestinal macrophages.

Much of the information on intestinal macrophages is based on *in vitro* experiments, or experiments performed in adult animals using experimental models. Therefore, it is hard to fully translate much of the results to the state of the neonatal human intestine, where many parts of the immune system have not yet fully developed and the gut microbiota is different from that found in the intestines of lab animals. However, since innate immune cells may be present earlier during the

development of the neonate, it is tempting to speculate that these macrophages contribute to the prevention of any detrimental effects that colonization may have on the still developing gut while speeding up the development of the epithelial barrier.

### **Regulation of toll-like receptor signaling**

Toll-like receptors are one of the most prevalent innate immune receptors expressed by the intestinal epithelium, capable of recognizing conserved parts of bacteria. However, since they recognize widely conserved patterns, there is a real risk that these PRRs are activated in response to harmless commensals. As a result, mammals have evolved a variety of strategies to prevent TLR activation against the commensal microflora.<sup>38-40</sup>

For example, the localization of TLRs is limited to the basolateral side of the epithelium, preventing recognition of commensals in the intestinal lumen. This restriction still allows the sensing of pathogens which penetrate the epithelium to spread into the host or commensals that accidentally cross the epithelial barrier after damage. Other adaptations in the host epithelium also help restrict TLR induced signaling, to further reinforce the homeostasis of the gut. Cells of the intestinal epithelium are able to inhibit signaling as a result of TLR activation on multiple levels using a number of different mechanisms. One of the key targets for inhibition is the transcription factor NF- $\kappa$ B, a key effector of several TLR pathways. For example, the peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) inhibits the activation of NF- $\kappa$ B and the following response, attenuating inflammatory response.<sup>41</sup>

The importance of regulated TLR signaling for homeostasis in the gut is further accentuated by the observation that some members of the microbiota induce inhibitory reactions in the intestine. For example, *B. thetaiotaomicron* may inhibit the function of NF- $\kappa$ B by exporting the p65 subunit in a PPAR- $\gamma$  dependent manner, although it is not known how the bacterium is able to induce the upregulation of PPAR- $\gamma$ .<sup>41</sup> Other research demonstrates the effect of the microbiota as well, as germ-free mice express PPAR- $\gamma$  at significantly lower levels than conventionally colonized mice.<sup>42</sup>

Although it is known that regulation of TLR signaling is critical for maintaining homeostasis, many aspects remains to be elucidated. It is known that the microbiota helps maintaining homeostasis, but exactly which species are able to do so, or what mechanisms they employ remain unknown. Future research may also identify new targets for regulation by the microbiota and the effect of other defects in disease.

## **Disease in the intestine**

### **Disruption of intestinal homeostasis**

The healthy gut poses a strong barrier to pathogens. Due to the dense microbiota populating the intestine, pathogens first need to overcome this barrier in order to successfully colonize and infect an individual. For many pathogens it is still unknown how they are able to do so, although it is in many cases achieved by disturbing the homeostasis between the host and the microbiota. One of the mechanisms pathogens employ to disturb intestinal homeostasis is the induction of inflammation in the host. Inflammation triggers antibacterial responses<sup>26</sup>, such as increased secretion of antibacterial peptides. Although these peptides are primarily secreted in response to pathogens, they also affect the microbiota, altering its composition and lowering its resistance against colonization. Other effects of the inflammation may include changes in the mucus layer, or the disruption of tight junctions between the epithelial cells, allowing penetrant bacteria easier access to underlying tissues.

Homeostasis in the intestine can also be disturbed by processes other than those stimulated by pathogens. Suppression of the host immune system by treatment with steroids or other immunosuppressive conditions decrease the ability of the host to resist colonization. This is displayed in SIV infected macaques. Under experimental conditions, *Salmonella* can be seen disseminating far into

the tissue beneath the epithelium in these macaques, while in SIV-free monkeys the infection is relatively well contained.<sup>28</sup> Genetic defects that increase the risk on inflammation in the gut also allow pathogens to gain a competitive edge over the microbiota, which can be seen in IL-10 deficient mice.<sup>43</sup>

Changes in host immunity are not the only disturbing factor. Alterations in the microbiota itself, by use of antibiotics for example, also influence the colonization resistance of the intestine. The effect of antibiotics can be displayed using animal models, in which intestinal pathogens only display their full pathologic physiology after treatment with antibiotics. Alterations in the microbiota are mostly reversible, but during this recovery period the colonization resistance of the gut is significantly lower, offering pathogens a competitive edge.<sup>43</sup>

### ***Campylobacter jejuni***

One example of a bacterium that benefits from disturbed intestinal homeostasis is *Campylobacter jejuni*. Normally a commensal in poultry, it is capable of causing infection in humans, causing symptoms ranging from watery diarrhea to dysentery. In rare cases however, *C. jejuni* infection causes the development of Guillain-Barré syndrome (GBS), an autoimmune disease affecting peripheral neurons.<sup>44</sup>

When compared to the microbiota, *C. jejuni* is poorly adapted to compete with the microbiota for nutrients. Instead, *C. jejuni* mostly relies on amino acids like serine and aspartate and Krebs cycle intermediates as a carbon source<sup>45, 46</sup>, forcing it to escape the gut lumen to localize to a more favorable nutrient environment. *C. jejuni* utilizes a number of taxis systems that aid in this process. One system includes the chemotaxis genes *CheY*, *CheA*, *CheV*, *CheW* and *CheR*.<sup>47</sup> This system senses nutrients and controls the rotation of *C. jejuni*'s flagella accordingly via *CheY*, directing movement in response to nutrients. The system is also required to cause disease, as mutants were unable to cause disease in a ferret model. Another taxis system, dependent on energy taxis revolves around the proteins *CetA* and *CetB*<sup>48</sup>, which also determine flagellar motility. These taxis systems are major determinants for virulence, as they allow *C. jejuni* to find the environment in which it can optimally replicate.

It is known that *C. jejuni* penetrates the mucus layer in contrast to most commensal bacteria. This penetration of the mucus precedes the invasion of the underlying epithelium. How *Campylobacter* manages to penetrate the mucus remains to be elucidated<sup>49, 44, 45, 49</sup>. One possible factor influencing this ability is the viscosity of the mucus. Therefore, experiments were performed to test the hypothesis that *C. jejuni* is able to penetrate media with different viscosities. Experiments demonstrating the importance of the taxis systems of *C. jejuni* in the infection of epithelial cells were performed as well.

## **Experiments: invasive behavior of *Campylobacter jejuni***

### **Materials and methods**

*Growth of Chang cells under methylcellulose.* Cells from a Chang cell line were grown in sterile culture flasks at 37°C and 5% CO<sub>2</sub>. These cells were treated with trypsin in order to break adhesion and then transferred to a 24-well plate. The wells in this plate contained RPMI-1640 medium supplemented with 5% fetal calf serum (FCS) different concentrations of methylcellulose. These cells were then grown at 37°C and 5% CO<sub>2</sub> for 72 hours and analyzed by phase-contrast microscopy.

Subvasion by *Campylobacter jejuni* 108. Cells from a Chang cell line were grown for 48 hours on coverslips in 24-well plates in RPMI-1640 medium containing 5% (FCS), at 37°C and 5% CO<sub>2</sub> for 48 hours. The cells were then checked for adhesion, density and possible infections. Prior to the experiments, the cells were washed and the medium was changed to either fresh RPMI-1640 or HEPES buffer, both containing varying concentrations of methylcellulose. Bacteria were grown in liquid medium under anaerobic conditions and spun down at 3,000 rpm for 5 minutes. The supernatant was removed after which the bacteria were resuspended in phosphate buffered saline (PBS). The optical density was

determined using a spectrophotometer set to 550 nm.  $5.0 \times 10^7$  bacteria (m.o.i of 200) were added to the cells in each well, which were then incubated at 37°C and 5% CO<sub>2</sub> for 2 hours. After incubation, the cells were washed twice with Dulbecco's PBS to remove any bacteria present in the supernatant. The cells were then fixed for 2 hours using Dulbecco's PBS supplemented with 1% PFA and 0.1% glutaraldehyde, and stained overnight with crystal violet. Using this protocol, bacteria stained purple, while the Chang cells assumed a light pink color. The stained coverslips were then transferred from the 24-well plates to microscope slides and intracellular bacteria were counted by means of light microscopy.

## Results

### *Growth of Chang epithelial cells under methylcellulose.*

After 72 hours of growth, cells grown in medium supplemented with 0.5% 400 centipoise or 0.5% 4000 centipoise methylcellulose showed unaltered morphologically compared to cells grown in normal medium. The cell number appeared to be slightly less in the presence of methylcellulose. Cells grown in 1% 400 centipoise or 1% 4000 centipoise methylcellulose were more round compared to the more extended cell morphology of cells grown under normal conditions. They also appear significantly less numerous, suggesting a reduced growth rate. Since most experiments only require the layer of methylcellulose during the assays themselves, it should be possible to supplement the medium with methylcellulose shortly before the start of the assay, ruling out any harmful effects prolonged exposure to the methylcellulose might have.

### *Subvasion by Campylobacter.*

In order to test the ability of *C. jejuni* to penetrate a mucus-like layer, we needed to create a model that resembled the situation in the gut. To achieve this, we supplemented the medium in which the cells were grown with low concentrations of methylcellulose, creating a viscous layer on top of the adherent cells in the wells. By changing the concentration of methylcellulose in the wells, we were able to change the viscosity of the medium.

*C. jejuni* penetrated the slightly viscous medium (0.5% 400cP methylcellulose) (Fig. 1), both when RPMI or HEPES buffer was used as medium. At higher viscosities (0.5% 4000cP and 1% 400cP) bacterial invasion of the cell layer diminished (fig. 1 and 2).

*Taxis-driven invasion.* We also wanted to test the effect of mutations in genes related to taxis on the ability of *C. jejuni* to invade cells in different nutrient conditions. To achieve this, we used a knockout strain defective in several genes required for serine and aspartate chemotaxis ( $\Delta$ CheVAY) and a strain lacking energy taxis ( $\Delta$ CetA). We also used a strain unable to synthesize a capsule ( $\Delta$ Cps). Each strain displayed some marked differences compared to the wildtype strain. The  $\Delta$ CheVAY mutant always invaded the cell layer, both in the rich RPMI medium and in the nutrient-lacking HEPES buffer used to simulate nutrient starvation (Fig. 1 and 2). The  $\Delta$ CetA knockout displayed very aberrant invasive behavior, regardless of the nutrient environment, in some cases it did not invade at all (fig. 1 and 2). The capsule mutant displayed higher invasion both in rich medium and nutrient-lacking medium.

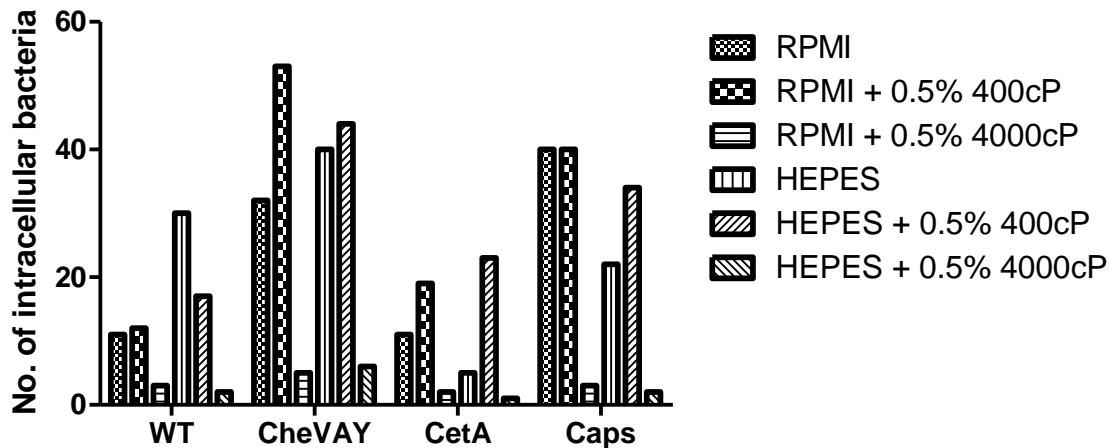


Figure 1: Invasion of *C. jejuni* mutants is reduced in highly viscous media. In a highly viscous media (0.5% 4000 centipoise methylcellulose) both wildtype and mutant *Campylobacter* strains are unable to invade the cell layer. The different 'taxis' mutants of *C. jejuni* display large differences in invasion.  $\Delta$ CheVAY, a chemotaxis mutant invades the cell layer, regardless of the nutrient situation.  $\Delta$ CetA, an 'energy taxis' mutant displays a disturbed phenotype, with aberrant numbers of invading bacteria.  $\Delta$ Cps, a capsule mutant also displayed a disturbed phenotype, with higher numbers of invading bacteria regardless of the nutrient situation.

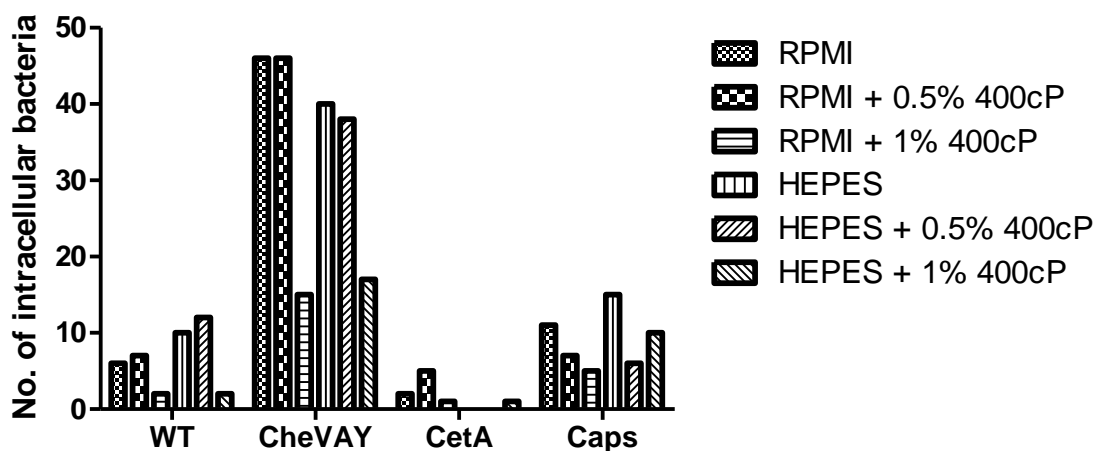


Figure 2: The invasive behavior of *C. jejuni* is disturbed by mutations in taxis-related genes. The  $\Delta$ CheVAY mutant invades a cell layer, regardless of its nutrient situation.  $\Delta$ CetA shows very little invasion under any medium condition. The  $\Delta$ Cps mutant behaves almost the same as wildtype *C. jejuni*.

## Discussion

Although there was not enough time to confirm all the results, the experiments performed suggest that a high viscosity of the medium hinders the invasion of cells by *C. jejuni*. This observation is consistent with previous research in which the mucosal layer was examined. The different concentrations of methylcellulose used may have some correlation with the viscosity of the two layers of the mucus that exist *in vivo*, at least in terms of viscosity. However, *In vivo*, mucus may also serve different purposes such as retaining antimicrobial peptides or IgA, which help restricting motility of microbes.

In order to successfully persist, *Campylobacter jejuni* needs to be able to migrate to the environment with the most nutrients. Chemotaxis is a very important process to help achieve this, allowing the microbe to move to the location with the highest availability of nutrients. In the event of nutrient starvation, *C. jejuni* will invade cells, as they present a rich nutrient environment. Mutations in genes required for taxis will therefore modify the invasive behavior of *C. jejuni* in response to different nutrient conditions. The performed experiments suggest that the invasive behavior of *C. jejuni* is partially determined by chemotaxis.

The *CheVAY* strain lacks chemotaxis for serine and aspartate. Since these two amino acids are a major energy source for *C. jejuni*, the inability to sense if these resources are abundant in the environment will force the bacteria to migrate to other locations, in this case inside the cell layer under the medium. In the *CetA* knockout strain, one of the energy taxis pathways is disrupted. Since energy taxis is required to find the most favorable environment for replication, bacteria lacking this system will be unable to determine where they need to migrate to. The performed experiments appear to confirm this hypothesis, displaying aberrant behavior of *CetA* mutants.

Experimental studies on the taxis systems of *Campylobacter* may provide insight in its ability to persist in the gut, even though it is not adapted to competing with the intestinal microbiota. These experiments may also provide insight on the invasive ability of *C. jejuni*, and the involved pathways.

## Conclusion

Recently, the microbiota has been recognized as a major influence in many processes of mammalian health. Not only do they provide the host with increased digestive efficiency and limited protection from intestinal pathogens, they also drive the correct development of the host intestinal epithelium and the immune system, as well as develop several parts of the host metabolism. Yet, we are only just beginning to appreciate the full range of effects caused by the microflora.

The current understanding of the influence of the microbiota on these host processes mainly rests on experiments performed with defined bacterial microflora, such as specific pathogen-free inoculates, or monocolonization assays, usually performed in adult mice. To fully understand the influence of the colonization of the gut by the microbiota shortly after birth, other animal models or assays would have to be designed. It would also require deeper knowledge on the entirety of the microbiota, as we are still unable to fully determine the composition of the microflora due to its complexity and the inability to isolate the non-cultivable portion of the microbiota.

The current knowledge about the microbiota does allow speculation: with basic knowledge about the processes modulated by the microbiota it is possible to predict the effects of altered composition of the microflora on a variety of diseases, such as the development of metabolic syndrome<sup>11</sup>, or inflammatory bowel disease. This also opens up the future for treatment: by targeting the microbiota it may be possible to treat diseases, or increase the colonization resistance of the gut. Indeed, early work in these fields display the possibility of treatment of murine colitis with probiotics that induce Treg cells<sup>50</sup>, suggesting that treatment of diseases by targeting the microbiota may be a valid strategy for the future.

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