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**Author:** Mariman, Rob  
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Chapter 8

Summary & General Discussion
The microbiota present in the gastrointestinal (GI)-tract has a fundamental role in the maintenance of host homeostasis [1]. Host-microbiota interactions are of critical importance for tissue formation, metabolism, and the development and function of innate and adaptive immunity [2]. Shifts in the composition of the microbiota induced by improved hygiene, antibiotic treatment, dietary changes or (invasive) pathogens can perturb immune-regulatory networks [3]. Due to these environmental changes many individuals in industrialized countries are no longer exposed to the microbiota of our evolutionary past [4]. The rapid increase in prevalence of immune mediated disorders - autoimmune diseases, inflammatory bowel disease (IBD) [5,6] and allergies [7,8]- in western societies is thought to be mediated in part by disturbances in host-microbiota interactions [9]. Restoring immune homeostasis by modulating host-microbiota interactions with probiotic bacteria has been demonstrated to be effective in chronic intestinal diseases and allergies [10,11]. Therefore, a better understanding of the mode of action of probiotics may help to advance the development of nutritional and pharmaceutical intervention strategies that may help to reverse the current rise of immune-related disorders. This thesis aimed to provide insight into the role of probiotic bacteria in the regulation of mucosal and systemic immune responses and contribution to suppression of inflammatory diseases. This thesis shows that probiotic bacteria have favorable effects in a model of IBD and provides evidence regarding the underlying mechanism.

**Immune regulation in Inflammatory Bowel Disease**

IBD patients are currently treated with anti-inflammatory and immunosuppressive drugs, antibiotics, surgery and/or biologicals such as anti-TNF [12]. Monoclonal antibodies directed against TNFα have substantially improved therapy, although a considerable proportion of the patients is non-responder, develops neutralizing antibodies after prolonged use, or experiences serious side effects [13-16]. Classical immunosuppressive drugs and antibiotics have rather limited efficacy, and show adverse side effects. So there is a clear medical need to develop novel anti-inflammatory treatments for IBD patients; this may include the use of probiotic bacteria. Probiotics with potential health promoting effects could most probably be validated through the use of proper animal models.

One of the key issues with respect to animal models is their translational value for patients. Chapter 2 describes a model based on the previous published recurrent TNBS-induced colitis model [17,18]. We characterized the processes associated with the early stages of colitis by genome-wide transcriptome analysis of colon tissue. TNBS-induced colitis has originally been positioned as a model for Crohn’s disease (CD), based on histopathological features like transmural cellular infiltrations and local production of $T_h^{1/17}$ cytokines [19,20]. When
compared with recently published gene expression data from inflamed biopsies from ulcerative colitis (UC) [21] and CD [22] patients, the recurrent TNBS colitis model overlapped respectively 18 and 16 percent. This overlap is comparable with other genome-wide transcription studies aimed to identify genes and biological processes affected in experimental colitis, i.e. acute TNBS colitis model in rats [23], murine DSS colitis [24], IL-10^{-} colitis [25], and CD4CD45Rb^{hi} T-cell transfer colitis [26]. Yet, pathway analysis identified major processes important for IBD i.e. tissue morphogenesis, wound healing, immune/inflammatory response, cell adhesion and angiogenesis. The model therefore represents several aspects of mucosal inflammation in UC and CD patients. Indeed, expression of α-defensins and chemokines as well as the influx of macrophages, T-lymphocytes and mast cells in the intestinal mucosa represents aspects of mucosal infiltration in UC and CD. Moreover, local budesonide treatment, a corticosteroid used to treat IBD patients[27,28], partly reduced colitis features in this model. A hallmark of chronic inflammatory intestinal disorders is the rapid recruitment of leucocytes, in particular T cells, to the site of inflammation [29]. Mediators that facilitate these processes include adhesion molecules and chemokines [30], and these were induced in the model particularly 2 days after each TNBS challenge. Recent studies indicate that targeting these molecules may be promising for the treatment of IBD [31-33]. Novel compounds evaluated in this respect include Natalizumab(anti-α_{4}) [31,34,35], Vedolizumab (anti-β_{7}) [36,37], ISIS2302 (anti-ICAM1) [38], and CCX282-B (anti-CCR9). However, targeting these leucocyte migration markers, which are not gut specific, may also raise safety issues [39]. Since the microbiota is involved in shaping the intestinal immune system, i.e. by targeting leucocytes and their migration mediators, modulating the microbiota might be a new treatment approach/strategy in individuals susceptible for intestinal inflammation. The study described in chapter 3 shows that recurrent TNBS colitis model is sensitive to probiotic bacteria. Possible mechanisms underlying these beneficial effects will be discussed in the next paragraph.

Modulating mucosal immune responses by probiotic bacteria

To gain insight into the anti-inflammatory potential of probiotic bacteria the probiotic mixture VSL#3 versus a single strain-\textit{L. plantarum}-, were evaluated by studying their effect on gene expression profiles in the colon. In contrast to studies with these probiotic bacteria in immune compromised mice such as the IL-10^{-} [25]and T cell transferred Rag2^{-} colitis model [40], our results in the recurrent TNBS colitis model reflect immune modulation in mice with a fully functional immune system. Probiotic treatment reduced colitis associated influx of T-lymphocytes, mast cells and macrophages. This was accompanied by suppression of chemokines like MCP-1, eotaxin-1, and eotaxin-2. These chemo-attractants have been implicated in the recruitment of a variety of innate and adaptive immune cells [30,41] and
can be produced by intestinal epithelial cells or innate immune cells in the colonic mucosa. Therefore, it is tempting to speculate that probiotics ameliorate experimental colitis in this model by interfering with these chemokine-secreting cells which consequently results in reduced influx of inflammatory cells.

The induction of regulatory T cells (Treg) represents another potential mechanism by which oral administration of VSL#3 may render mice resistant to TNBS induced colitis. As described in chapter 7, prolonged administration of VSL#3 drives the induction of these FoxP3+Treg locally in the MLN of healthy BALB/c mice. Such cells have the capacity to migrate from a secondary lymphoid organ like the MLN to the inflamed mucosa and suppress T effector cell responses [42].

In IBD, dysregulated T cell responses perpetuate the disease and the vicious cycle of chronic inflammation [43]. It is unknown whether overabundance of T effector cell responses in IBD are the result of reduced numbers of Treg, dysfunction of Treg or resistance of T effector cells for suppression. Apart from a small sub-population of IBD patients with reduced Treg activity caused by a mutation in FoxP3 [44,45], most IBD patients have normal numbers of these cells in the inflamed mucosa [46]. Some reports even show an increase in numbers of Treg cells during inflammation [47-49] possible as a compensation mechanism in an attempt to control inflammation. Since the induction of Treg by probiotic bacteria is considered as a mechanism by which they induce anti-inflammatory effects, prophylactic and therapeutic treatment may have different outcomes. Treg can prevent disease development in spontaneous and chemical induced colitis [50,51]. In immune-compromised mice, adoptive transfer of Treg has also been shown to be efficient in amelioration of an established inflammation [52]. In contrast to chemical-induced colitis models, were Treg lost their potency to suppress colitis when these cells were transferred to mice with established colitis [51]. This might be related to environmental factors, since Treg cells have reduced suppressive capacity if pro-inflammatory cytokines or co-stimulatory signals are present in the inflamed site as shown in various experimental models for autoimmune diseases. [53-55].This implicates that induction of Treg by probiotics could be effective in a prophylactic setting but would fail in IBD patients during periods of active inflammation. Although this is beyond the scope of this thesis, it would be interesting to evaluate the therapeutic potential of probiotics in the recurrent TNBS colitis model.

**Modulation of cytokine and chemokines release by probiotics**

In chapters 3 and 7 it is shown that probiotic bacteria modulate expression patterns of inflammatory mediators like cytokines and chemokines *in vivo* under conditions of inflammation and homeostasis. Probiotic bacteria have been shown to interact with innate
immune cells and intestinal epithelial cells via binding to pattern recognition receptors [56], thereby modulating inflammatory signaling pathways[57]. In chapter 5, *in vitro* studies with bone marrow derived dendritic cells (BM-DC) were employed to evaluate the effects on probiotics on the expression and release of these inflammatory mediators. Studies described in this chapter showed that immune modulating capacities of probiotics on DC depend on host genetics and the presence of other stimulatory signals. DC derived from the bone marrow of two polarized mouse strains i.e. C57BL/6 and BALB/c mice responded differently to ultrapure Toll-like receptor ligands that mimic microbe-associated molecular patterns(MAPS) on bacteria, fungi, and viruses. Host specific responses of BMDC stimulated with these ultrapure ligands that trigger one single receptor, underline the impact of genetic factors on MAMP responses in the gut, but underestimate the complex environment of the gut. DC in the lamina propria(LP) integrate signals simultaneously received from the commensal microbiota and (potential) pathogenic microorganisms. Moreover, during active intestinal inflammation that is often accompanied by impaired barrier function, intestinal DC are exposed to an enormous load of Gram-negative bacteria that reside in the lumen [58]. In chapter 5 it is shown that probiotic bacteria synergistically enhance the production of IL-12p70 and IL-23 in DC from C57BL/6 mice induced by LPS (as a representative component of Gram-negative bacteria). Previous studies have shown that simultaneous activation of TRIF and MyD88 coupled TLR (by bacteria and/or pure ligands) induces a robust induction of IL-12p70 [59]. It is therefore tempting to speculate that Gram-positive probiotic bacteria may induce robust T₈₁ and T₈₁₇ responses in individuals with established inflammation. This implies a risk of probiotics use under these conditions, because steering the immune system towards a T₈₁/T₈₁₇ inflammatory response could be harmful to patients suffering from T₈₁/T₈₁₇-mediated (autoimmune) disease such as CD.

Apart from this pro-inflammatory effect, probiotic bacteria induced anti-inflammatory effects with respect to chemokine expression and release. Irrespective of the mouse strain, VSL#3 and *L. plantarum* suppressed LPS induced up-regulation of CXCL-9 and CXCL-10. These chemokines target the CXCR3 chemokine receptor that is expressed on T₈₁ cells, CD8+ T cells and DC [60]. Both chemokines have been shown to play a role in a variety of autoimmune diseases and infectious diseases by recruitment of inflammatory cells to the site of inflammation [61-65]. Moreover, therapy with anti-CXCL-10 antibody reduced experimental colitis in IL-10⁻/⁻ mice by interfering with recruitment of T effector cells [66,67]. Chapter 6 describes comparable *in vitro* studies with human monocyte derived DC. With the use of dedicated PCR arrays for cytokines and chemokines, the gene expression signature of human DC in response of LPS and VSL#3 was identified. This unbiased approach, not focusing on one single inflammatory mediator, identified a cluster of LPS-induced genes that was suppressed by VSL#3. Chemokines present in this cluster did not target a single receptor, but a diversity of chemokine receptors including CCR8, CCR1, CCR3, CCR7 and CXCR3.
Consequently, this reduced chemokine expression may potentially dampen attraction of a broad panel of immune cells including T cells, NK-cells, DC, B cells, eosinophils, basophils and mast cells [68]. In silico analysis of transcription factors appeared a powerful approach to identify a common regulator of LPS-induced genes that were blocked by probiotics. Transcription factor analysis could be used because a large panel of chemokines and cytokines was under investigation in this study. The majority of these inflammatory mediators was not affected by probiotic treatment and could therefore be used as a reference set. STAT-1 was predicted as a dominant driver of LPS-induced genes that were suppressed by VSL#3, which was confirmed by demonstrating that phosphorylation of this transcription factor was inhibited by probiotic bacteria. This suggests that VSL#3 can reduce production of specific chemo-attractants in vitro by selective inhibiting of STAT-1 induced transcription regulation. STAT-1 is relevant in intestinal disease, since STAT-1 deficiency reduced the intensity of spontaneous and chemical induced colitis [69,70]. Furthermore, a recent report showed that selective regulation of STAT-1 signaling in T-cells by fusaraside resulted in reduced production of pro-inflammatory cytokines and improved TNBS induced colitis [71]. Understanding how MAMPs present on probiotic bacteria modulate intracellular signaling induced by pathogenic bacteria could be helpful to identify mechanisms by which probiotics promote health in vivo. Accordingly, a better knowledge of the molecular mechanisms of induced signaling pathways could allow selection of probiotic strains with distinct properties, leading to more specific and efficacious therapeutic strategies in the prevention or treatment of allergic and autoimmune diseases.

**Probiotics modulate immune responses outside the gut**

Commensal bacteria influence local immune responses in the gastrointestinal mucosa under inflammatory and homeostatic conditions[10]. However, immune modulation by the GI-microbiota is not restricted to the intestine and may influence immune responses in peripheral tissues as well [8]. Epidemiological studies found strong correlations between altered fecal microbiota and atopic diseases. Atopic diseases, such as asthma and AD are the result of an inflammatory reaction triggered by a T\(_2\) cell mediated immune response. In chapter 4, it is shown that there is an association between gut inflammation and atopic dermatitis (AD) in human ApoC1+/+ transgenic mice. Interestingly, transcriptome profiling of the colon further indicated that these mice have reduced intestinal chemokines and cytokines that are associated with T\(_h\)\(_1\) immune responses along with increased allergic markers like IgE\(^+\) and FcεRI\(^+\) cells, suggesting a T\(_h\)\(_2\) driven inflammation in the intestine. Recent evidence shows that the commensal microbiota programs many aspects of the adaptive immune system, including T cell responses [75]. Environmental changes such as
antibiotics, diet and infant feeding regimes have been shown to influence the composition of the microbiota [76-79]. It is hypothesized that these changes in the gastrointestinal microbiome composition contribute to the increase in prevalence of immune mediated disorders. Studies in experimental models for rheumatic arthritis [80], experimental autoimmune encephalomyelitis [81] and various animal colitis models demonstrate that the commensal microbiota contributes to autoimmune and allergic disorders at sites distal to the intestinal mucosa[82]. Exacerbation of arthritis and EAE is likely the consequence of pathogenic T_h17 cells that traffic out the LP to their target tissue [80,81,83]. Other studies also indicate that T-cell activation in the intestinal mucosa by microorganisms influences systemic immunity[75]. For example B. fragilis PSA affects the nature of systemic T cell responses evident from increased circulating T_h1 cells in mice colonized with this bacterial strain[84]. Likewise, it has been shown that Clostridium IV and XIV in the GI-tract not only induce expansion of Treg in the LP, but also in the periphery[85,86]. Interestingly, oral administration of L. plantarum result in amelioration of skin pathology in the human ApoC1+ transgenic mice model for atopic dermatitis (AD) as shown in chapter 4. This might be due to the induction of local inflammatory responses by this specific L. plantarum strain as observed in an ex vivo study employing human colon explants [87]. Moreover, oral administration of this lactobacillus strain to healthy mice increased the frequency of IFN-γ positive T cells locally in the mesenteric lymph nodes, as well as in peripheral immune tissue like the spleen [88]. The beneficial effect of probiotics intervention in this mouse model was not limited to the skin. L. plantarum also reduced the number of mast cells in the intestinal mucosa. Therefore, it is tempting to speculate that L. plantarum exerts the beneficial effect in this mouse model for AD by balancing T_h1/ Th2 responses. Failure of the probiotic mixture VSL#3 in preventing skin pathology suggests that different strains may act differently in diverse pathological conditions. This implicates a careful selection of probiotic strains for therapeutic use is required, based on the type of inflammation.

Concluding remarks

A better understanding of host-microbiota interactions in the pathogenesis of (auto)-immune and infectious diseases may contribute to new treatments of inflammatory diseases. Manipulating the enteric microbiota with fecal microbiota transplantation and probiotics might have great potential for treating IBD[89-91]. However, to fully benefit from the potential of such strategies deeper understanding of the immune-modulating mechanisms of these interventions is required.

We and others have shown that oral administration of specific probiotic strains reduce the severity of intestinal inflammation and atopic dermatitis in experimental animal models.
However, to improve the application of probiotics in healthy and diseased individuals it is important to define the proper probiotic bacterial strains for a specific therapeutic application. In addition, we showed in chapter 4 and 7 that effects of probiotics on immune related gene expression in the intestine largely depend on host genetics. This is in line with clinical studies, where human biopsies from healthy volunteers taken before and after probiotic intervention also showed large intra-individual differences in their response to probiotics. Therefore, it seems doubtful that all individuals experience similar physiological benefits of a certain probiotic strain. Current probiotics have not been selected for specific treatment purposes but they rather are “all purpose” probiotics. The development of a new generation of probiotic strains for disease specific application therefore may benefit from detailed knowledge of their immune modulating properties. Design of probiotic therapies should be based on the abnormalities in the microbial composition, the underlying genetic defect and desired biological response.

Due to their long history of use in food formation, the FDA has designated most probiotics to be generally recognized as safe (GRAS-status). However, it should be noted that a concern regarding the safety of using probiotics has recently been raised due to increased risk of mortality in patients with severe acute pancreatitis [92]. We showed that the involvement of other immune activating signals could also affect the innate immune responses towards probiotics. This implicates that patients with an established intestinal inflammation may respond differentially to probiotic intervention than healthy individuals. Consequently, it will be necessary to monitor safety issues when probiotics are used to treat IBD patients.

In conclusion, it was shown that immune-modulating properties of probiotic bacteria are strain specific and depend on host genetics and environmental factors. This illustrates the likelihood that personalized probiotic therapies for individuals should be designed, that are based on host genetic polymorphism and the desired biological response rather than non-discriminating heterogeneous patients with a single approach. Improved knowledge about the working mechanisms of probiotics will contribute to proper strain selection for a specific prophylactic or therapeutic application, ultimately leading to more personalized medicine.
References


