SUMMARY AND DISCUSSION

The human gastrointestinal (GI) tract is colonized by microbiota, an assemblage of microorganisms that is approximately 10 times higher in numbers compared to its host’s cells\(^1-^3\). This vast amount of microorganisms mainly includes Bacteria, but also contains the two other domains of life: Archaea and Eukarya. The composition of this complex microbial ecosystem is under influence of several elements including environmental factors, such as diet and smoking, as well as age, host’s health state and phylogeny\(^4-^7\).

The relationship between the microbiota and human body as a host can be defined in several ways. Intestinal microbiota has a large impact on gut health, since it maintains intestinal homeostasis, stimulates the maturation of the immune system and provides nutrients for the host by metabolizing food ingredients thereby providing energy for the host\(^8,^9\). Intestinal microbiota, their metabolites and cell components play a major role in development of a functional immune system, both innate and adaptive. Moreover, they participate in the development of the immune system by initiating tolerance towards specific bacterial strains that is necessary for maintenance of intestinal homeostasis. Lack of this tolerance or malfunctioning of the intestinal immune system may be followed by serious consequences, such as inflammatory bowel disease (IBD). The interaction with the immune system can be direct, in which mucosal microbiota, their components and metabolites are in direct contact with intestinal epithelial cells and related immune pathways. Such interaction can also be indirect, especially for the luminal microbiota that are not in close contact with the epithelial cells or the associated mucosal layer, where only their metabolites modulate immune responses. Therefore, the localization of different bacteria towards the host epithelial cells plays a crucial role in the development of the (mucosal) immune system and host’s health and disease.

The research described in this thesis was aimed at revealing the interaction between the intestinal microbiota and the host cells. The first main focus point of the thesis was to study the ecology of the abundant intestinal commensal *Faecalibacterium prausnitzii* in the human gut environment, investigate its potentially beneficial anti-inflammatory properties on the host cells and the possible ways that the host might influence its ecology. The second main focus was to investigate the host immune response towards the symbiotic intestinal microbiota and pathosymbionts in IBD patients.

In Chapter 2 of the thesis, the localization of the different bacterial groups in human feces, so-called “bio-structure” was analyzed. We visualized the bio-structure of some of the populations of the dominant gut bacterial species, in particular *F. prausnitzii* and *Roseburia*. *F. prausnitzii* is a major member of the firmicutes that may represent up to 25% of the total gut microbiome in healthy individuals. This major butyrate producer is found predominantly close to the epithelial cells, much more than other butyrate producers, such as *Roseburia*\(^10\).
Summary, Discussion and Future perspectives

In this chapter, we propose that there are at least three phylogroups of *F. prausnitzii*, of which only 2 have been cultured so far. Our observations suggest that the expansion of *F. prausnitzii* close to the mucus layer and close to the epithelial cells is mainly due to the outgrowth of phylogroup 2 rather than phylogroup 1, while both of them are distributed evenly in the luminal section of the feces. Swidsinskii *et al.* categorized microbiota to three different groups, bacteria that mainly reside in the luminal section of the feces, bacteria that colonize closer to the intestinal epithelial cells and inhabit the mucus layer and the third group, namely feco-mucus bacteria, which reside in both locations. They categorized *F. prausnitzii* in the latter group. Our observations confirm the findings of Swidsinskii *et al.* and shows the faecalibacteria localize throughout the fecal sections with a dominant presence of phylogroup 2 in the fecal-mucosal interface of the human gut. The ability of *F. prausnitzii* A2-165, a representative of phylogroup 2, to grow to visible colonies in the oxygenated area of agar cultures in gas tubes has previously been shown by Khan *et al.*

They hypothesized that faecalibacteria are capable of growing in the oxygenated zone by using free thiols as electron acceptors and flavins as the redox mediators and extra cellular electron transferors. Our findings in this chapter regarding the localization of phylogroup 2 of *F. prausnitzii* close to the mucus layer may indicate the capability of these cells in employing flavins, such as riboflavin, to localize in this area that may contain oxygen from the blood circulation.

Another important aspect in microbiota-host interactions is that the different bacterial groups of microbiota are able to digest food particles, specifically polysaccharides and non-digestible fibers by the host that aids to the host’s energy balance. The higher activity of those bacterial strains could lead to the enhanced production of specific substances, such as short chain fatty acids (SCFAs).

*F. prausnitzii*, *Clostridium* group XIVa members and *Roseburia* are among the most important and abundant butyrate producers in the human gut. Our findings in Chapter 2 demonstrate the formation of biofilms of these bacterial species around specific food particles. In all cases, *Roseburia* is part of biofilms, which were formed by *Clostridium* group XIVa. In contrast, biofilm formation by *F. prausnitzii* is observed on food particles that are different in shape and structure compared to those surrounded by *Roseburia/Clostridium*. Bacteria in these biofilms stained by the universal *F. prausnitzii* probe did not completely overlap with the various phylogroup-specific probes, suggesting that there is a third phylogroup of *F. prausnitzii* that is particularly able to form biofilms on non-digestible fibers and carbohydrates.

Riboflavin, which is found in a variety of foods, such as dairy products, meat and vegetables, might act as a redox mediator for faecalibacteria and stimulate their growth at oxic-anoxic interphases. In Chapter 3, we performed a proof-of-concept study to determine whether riboflavin supplementation enhances the presence of *F. prausnitzii* in human feces.
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The results show an increase in abundance of *F. prausnitzii* in fecal samples of healthy volunteers that took a daily oral dose of 100 mg riboflavin for 14 days. The observed increase in abundance of faecalibacteria was observed for both phylogroups and occurred in 8 out of 11 volunteers. These findings confirm the findings of Khan et al. In addition, we demonstrate that *Roseburia* takes advantage of riboflavin supplementation as their numbers increase as well. Since it has not been shown that *Roseburia* can employ riboflavin as an electron transfer mediator in *in vitro* experiments, the mechanism behind this increase and whether they directly benefit from riboflavin or not, remains speculative.

Previous studies have suggested that low abundance of *F. prausnitzii* and enhanced numbers of adherent invasive *E. coli* (AIEC) are associated with Crohn’s disease (CD). In *Chapter 2*, we found that enhanced numbers of *F. prausnitzii* close to the gut epithelium in some volunteers are accompanied by the absence of *Enterobacteriaceae*, like *E. coli*. Conversely, fecal samples of volunteers that have micro-colonies of *E. coli* in the mucus layer show lower numbers of *F. prausnitzii* at that location. In addition, in *Chapter 3*, we demonstrate that an increase in the abundance of butyrate-producing bacteria like *F. prausnitzii* is counterbalanced by decreased numbers of *Enterobacteriaceae* upon riboflavin supplementation, which supports the observed counterbalance between the two bacteria in *Chapter 2*. Understanding the mechanisms how riboflavin decreases the abundance of *Enterobacteriaceae* might provide clues about how colonization of such pathosymbionts could be controlled. This may be beneficial for IBD patients as well as for infectious outbreaks of pathosymbionts. The fact that *Enterobacteriaceae* preferably grow close to the epithelial cells and the role of AIEC in the pathogenesis of the CD, the unique specificity of *F. prausnitzii* to colonize the fecal-mucosal interphase supports our hypothesis that promoting the *F. prausnitzii* population is important for a healthy gut.

Sokol *et al.*, have described anti-inflammatory properties of *F. prausnitzii*, in particular related to the inflamed gut. Reduction of the oxidative stress in the fecal-mucosal interphase of the gut as a result of those anti-inflammatory properties could limit the growth of pathosymbionts, such as *E. coli* that benefits from the oxygen stress. In contrast, inflammation of the intestinal epithelial layer caused by colonization of *E. coli* could lead to an increased oxygen stress and subsequently to suppression of *F. prausnitzii*. This counterbalance is in line with the findings of Harmsen *et al.* and Willings *et al.*, who described the reduction of *F. prausnitzii* and increase of *E. coli* in CD patients compared to healthy volunteers. Though this study was performed with non-IBD volunteers, our findings could indicate that *F. prausnitzii* controls the growth of *E. coli*, which may lead to a healthier gut homeostasis. Considering the important role of *E. coli* in the pathogenesis of CD and the potential anti-inflammatory properties of *F. prausnitzii*, the use of riboflavin to stimulate *F. prausnitzii* growth may be of benefit for these patients. Future studies should...
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be performed with IBD patients to determine the potential therapeutic effects of riboflavin for this patient group.

Our findings may indicate that the anti-inflammatory properties of *F. prausnitzii* are due to the suppressive effect on bacterial groups like *E. coli*. In addition or alternatively, the anti-inflammatory effect may arise from the enhanced production of butyrate by the enhanced numbers of *F. prausnitzii* and *Roseburia*, as butyrate has been shown to suppress chemical-induced colitis in mice\(^\text{15}\). It is therefore crucial to further study the interaction between epithelial cells and gut microbes, in particular *F. prausnitzii*, and how this and its products modulates inflammatory signaling in the gut.

Various experimental models have been developed to study the interaction between gut bacteria and intestinal cells, including *in vitro* approaches where bacteria and gut epithelial cells are cocultured, and in animal models\(^\text{14,16,17}\). In most of these systems, animal tissue or cultured cell lines are exposed to (an)aerobic bacteria. The requirement of oxygen to maintain the vitality of epithelial cell (lines) strongly limits the use of living anaerobic bacteria in such systems. The main focus in these kind systems is to analyze the effect of bacteria or their secretory products on inflammatory markers in the gut epithelial cells, such as cytokine expression and/or production. However, such systems do not allow the analysis in the opposite direction, e.g. the effect of epithelial cells on microbiota. In Chapter 4, we developed a coculture system for oxygen-requiring human gut epithelial (Caco-2) cells and extremely oxygen sensitive (EOS) *F. prausnitzii*. This “Human oxygen-Bacteria anaerobic” (HoxBan) coculture system truly allows coculturing of both cell types, e.g. they simultaneously proliferate, for up to 36 h. Specifically, the HoxBan model supports the growth of the *F. prausnitzii* in the low oxygen zone of the system and therefore makes it possible to study the aerobic-anaerobic interactions, which resemble the interaction between those cells within the intestinal tract. Findings described in Chapter 4 show the anti-oxidative stress properties of growing *F. prausnitzii* on Caco-2 cells, as well as its anti-inflammatory effects that were demonstrated using *F. prausnitzii* cell extracts in previous studies\(^\text{14}\). Our findings show the same rim forming phenomenon for growth of *F. prausnitzii* as described by Khan *et al.*\(^\text{18}\). However, this phenomenon is much more pronounced in the presence of Caco-2 cells and occurs outside the anaerobic chamber.

The explicit colony formation by *F. prausnitzii* in cocultures could be an indication for the beneficial interaction between Caco-2 cells and possibly the secreted substances of the bacteria. In addition, the oxygen consumption of Caco-2 cells might decrease the local oxygen concentrations, which is favorable for faecalibacteria.

Given the anti-inflammatory and anti-oxidative stress properties of *F. prausnitzii*, this phenomenon highlights the importance of the location of *F. prausnitzii* close to the epithelium to maintain a healthy gut, as discussed in Chapter 2. Furthermore, the substantial decrease in *F. prausnitzii*, specifically in the oxic-anoxic zone of the gut where
they are in close intact with epithelial cells, may play a role in the pathogenesis of CD\textsuperscript{12,13}. The anti-inflammatory properties of \textit{F. prausnitzii} were shown in Chapter 4 by down-regulation of IL1\(\beta\) and iNOS, which is in line with findings of Sokol \textit{et al.} who showed the suppression of IL1\(\beta\)-induced NF\(\kappa\)B activity by \textit{F. prausnitzii}-conditioned media on Caco-2 cells\textsuperscript{14}. Such effect could not be established in the same level with \textit{F. prausnitzii} cells, which in that experimental setting were dead because of exposure to oxygen. The HoxBan coculture system allows simultaneous growth of Caco-2 cells and \textit{F. prausnitzii} and revealed a clear suppression of IL1\(\beta\) and iNOS expression in the Caco-2 cells when cocultured with faecalibacteria. Moreover, the expression of reactive oxygen species (ROS)‐sensitive HO-1 was also suppressed in cocultures, supporting both anti-oxidative stress and anti-inflammatory properties of \textit{F. prausnitzii}. Future studies using the HoxBan coculture system will be aimed at determining the anti-inflammatory and anti-oxidative stress properties of \textit{F. prausnitzii} in combination with inflammatory signals like LPS or pathosymbionts.

Exo-metabolome analysis of different (co)culture conditions in Chapter 4 showed that there are differences between Caco-2/\textit{F. prausnitzii} cocultures, the two monocultures and the original culture medium. For instance, formate production is a known feature of \textit{F. prausnitzii} phylogroup 2\textsuperscript{11}. Formate levels increased significantly in media of HoxBan cocultures with \textit{F. prausnitzii} A2-165 and Caco-2 cells compared to media from \textit{F. prausnitzii} monocultures. This is most probably a consequence of increased bacterial biomass and/or bacterial activity in the growth rim. However, the enhanced biomass did not result in increased levels of butyrate, while it has been shown to be produced in equimolar amounts with formate under these conditions by this \textit{F. prausnitzii} strain. Given the fact that butyrate is a primary energy source for intestinal epithelial cells\textsuperscript{19}, we assume that part of the butyrate produced by \textit{F. prausnitzii} is consumed by Caco-2 cells. The expression of the butyrate transport proteins H(+)-coupled monocarboxylate transporter (MCT1) and Na(+)-coupled monocarboxylate cotransporter 1 (SMCT1), levels of which are controlled by butyrate, may be indicative for the induced butyrate consumption by Caco-2 cells\textsuperscript{20,21}.

Increased levels of formate coincided with a decrease in concentrations of compounds of purine metabolism, such as adenine and inosine, in cocultures compared to monocultures. This may be indicative of accelerated growth of \textit{F. prausnitzii}, as these metabolites are required for the synthesis of DNA (cell proliferation) and ATP (energy).

The HoxBan system is readily adaptable to coculture any other anaerobic gut bacterium in combination with different cell lines. Moreover, it provides a simple but practical method to study the effect of environmental factors such as smoking and dietary compounds on the relation between the intestinal microbiota and intestinal cells. The HoxBan system provides a platform to analyze infection and/or inflammatory models with triggers, such as pathosymbiotic bacteria, reactive oxygen species (ROS) and inflammatory cytokines and
study the anti-inflammatory/anti-stress properties of potentially beneficial microbiota in such a model.

Crohn’s disease (CD) and ulcerative colitis (UC) are the two main forms of IBD, which are inflammatory disorders that develop as a result of a complex interplay between genetic susceptibility, environmental factors, intestinal microbiota and the immune system. The intestinal immune system of healthy individuals effectively prevents penetration of pathogens and pathosymbionts into the mucosa, while at the same time it maintains tolerance to resident commensal microbes. An impaired balance in the host immune system’s activity and the intestinal microbiota may lead to inflammation of the gut wall.

Recent genome wide association studies have identified over 163 loci in the human genome that are associated with IBD. Many genes residing in these loci play a role in the immune response against bacteria invading the epithelial layer. Still, our knowledge about the way how individual susceptibility variants may affect the gut microbiome composition, specifically the mucosal microbiota, is limited.

\textit{ATG16L1} is an important factor involved in innate immunity, macro-autophagy and phagocytosis of microbes from the gut. Macro-autophagy is a starvation-induced cellular pathway for the lysosomal degradation of cellular proteins and organelles that aids to cell survival during conditions of nutritional limitation. In addition, it shares part of its machinery with the intracellular degradation of pathogens after they have been taken up by phagocytosis. Uptake of bacteria activates autophagy, a process also called xenophagy, in which it assists in the immune system to clear invading pathogenic or pathosymbiotic bacteria. A specific variant of \textit{ATG16L1}, e.g. \textit{ATG16L1-T300A}, disturbs the elimination of specific bacteria after internalization through phagocytosis, linking disturbed autophagy to the pathogenesis of CD. In Chapter 5, we studied the interrelationship between the \textit{ATG16L1} genotype of CD patients and the bacterial composition of the inflamed and non-inflamed ileal mucosa of these patients. Our findings show that the microbial composition of mucosal biopsies of the inflamed terminal ileum of CD patients that are homozygous for the \textit{ATG16L1} protective allele (e.g. \textit{ATG16L1-T300}) is different from patients that are homozygous for the risk allele. Remarkably, such difference was not detected for the non-inflamed intestinal mucosa of the same patients. CD patients who are homozygous for the \textit{ATG16L1} risk allele are unable to adequately clear pathosymbionts, such as \textit{Enterobacteriaceae}, \textit{Bacteroidaceae} and \textit{Fusobacteriaceae}, upon inflammation in the ileum. In contrast, CD patients who are homozygous for the \textit{ATG16L1} protective allele are capable to clear these bacterial species during inflammation and this is accompanied by a relative increase in \textit{Lachnospiraceae} when compared to CD patients homozygous for the risk allele. Despite our knowledge about the crucial role of microbiota in the progression of inflammation in CD, it remains elusive whether pathosymbionts actually trigger the inflammation or take advantage of the inflammation to outgrow in the inflamed tissue. Our
findings in Chapter 5 are in line with findings of Neut et al. who reported that the increased abundance of Bacteroides, fusobacteria and E. coli is associated with an earlier relapse of CD in patients after ileocolonectomy. The suggestion that the ATG16L1 risk allele impairs the autophagy process of pathosymbionts is in line with a previous study by Raju et al. who showed that the ATG16L1 risk allele enhances the susceptibility to H. pylori infection. Moreover, in vitro studies have shown that human epithelial cells of individuals with the ATG16L1 risk allele are unable to complete the autophagy process to clear intracellular pathogens. Similarly, Lapaquette et al. showed that siRNA knockdown of ATG16L1 enhances the survival of internalized AIEC in HeLa cells. In Chapter 5, we also studied the effect of inflammation on the survival rate of AIEC in monocytes isolated from healthy volunteers homozygous for the ATG16L1 risk or protective allele. Primary monocytes were activated by phorbol 12-myristate 13-acetate (PMA) or exposed to inflammatory cytokines, such as IL1β and TNFα, and were analyzed for the processing and killing of AIEC. Results of the in vitro experiments confirm the in vivo data that AIEC survival is enhanced in monocytes homozygous for the ATG16L1 risk allele when exposed to inflammatory conditions. Interestingly, no such differences were detected in untreated (control) monocytes. Fujita et al. did not observe a difference in autophagic processing of S. typhimurium and group A Streptococci in mouse embryonic fibroblast carrying the ATG16L1 risk allele, which is in agreement with our findings as the observed differences only occurred under inflammatory conditions.

Intestinal microbiota play a crucial role in development of the immune system and establishing a specific tolerance towards certain bacterial strains. Impairment in this tolerance, especially in CD, strongly contributes to the development of inflammation. In normal conditions, immune reactions are specifically targeted against pathosymbionts like E. coli to eliminate them from the epithelial environment. This is particularly important in conditions when beneficial commensal bacteria are incapable of suppressing expansion of these pathosymbionts and colonize the mucus layer of the gut epithelium. The suppressive effect of F. prausnitzii on E. coli growth is an example of how “beneficial” bacteria restrict expansion of pathosymbionts in the gut, as discussed in Chapter 2 and 3. Increased presence of pathosymbionts in the mucus layer may lead to alterations in the immune response. Ineffective suppression of those bacterial groups may lead to inflammation that breaks the epithelial barrier of the intestinal wall. Loss of intestinal epithelial integrity may progress to a leaky gut. Pathosymbionts take advantage of the broken barrier and invade the submucosa which will initiate a variety of immunological responses and induce inflammation. Presence of bacteria beyond the mucus layer triggers the innate immune response in which specific immunoglobulins (IgGs) are produced against the invading bacteria.
Summary, Discussion and Future perspectives

Given the fact that inflammation and a leaky gut will allow blood to leak from the circulation into the intestinal lumen, gut bacteria may be recognized by specific IgGs\textsuperscript{36}. In Chapter 6, we aimed to determine to which bacteria such humoral immune response is targeted in IBD patients. We sorted the IgG-binding bacteria after incubation of fecal samples with autologous serum by magnetic-activated cell sorting after suspension in streptavidin-coated magnetic beads and differentiate the selection by flow cytometry. The bacterial compositions of both the IgG-sorted samples and the original fecal samples were determined by 16S rRNA-gene analysis using Illumina sequencing. The results reveal that the IgGs from IBD patients bind to specific bacteria. Interestingly, the IgG immune response is preferentially directed against typical small intestinal bacteria, like Streptococcus, Lactobacillus, Enterococcus and Veillonella.

Moreover, our results show that the IgG immune response is also directed towards inflammation-associated bacteria, such as R. gnarus and Enterobacteriaceae. In contrast, there is a very limited IgG immune response directed towards the beneficial and anti-inflammatory F. prausnitizii and Roseburia. No significant differences were observed in the enrichment comparing the two types of IBD patients, CD and UC. From those IgG sorted bacteria some are pathosymbionts and therefore have the ability to trigger the immune system. However, the non-pathosymbiotic bacteria that were enriched are of interest, since they are not known to be involved in the pathogenesis of IBD. Characterization of the antigens involved in the IgG immune response towards these specific bacteria may contribute in unravelling the pathogenesis of IBD.

FUTURE PERSPECTIVES

The results in this thesis highlight that there is a complex interaction between the gut microbiota and the human host. This heavily intertwined relation and the different ways that the gut microbiota influence human health and disease, might allow us to consider the microbial cohort as an “organ”. Similar to the other organs like liver, lungs and kidneys, different bacterial cells of the microbiota are in specific relations with each other, which organize the collective functions of the gut microbiome, including fermentation of food that escaped digestion by the host and training and development of the immune system.

This thesis focused on the role of gut bacteria, specifically the extreme oxygen-sensitive F. prausnitizii, in inflammatory bowel disease, especially Crohn’s disease. As a result of most scientific activities, the research described in this thesis answered a few scientific questions, but generated even more to be addressed in future work.

More in-depth studies to address the phylogeny of F. prausnitizii need to be performed. A significant number of F. prausnitizii cells that form a biofilm on food particles were not identified by one of the two phylogroup-specific probes, suggesting the presence of at least one additional phylogroup of this bacterium that is responsible for biofilm formation.
Chapter 7

Localization of the third and maybe more phylogroups of *F. prausnitzii* in feces needs to be determined in future experiments. The next logical step is to characterize the food particles that are utilized by *F. prausnitzii*. Including such foods that support the growth of *F. prausnitzii* in the diet of CD patients may aid to increase the abundance of *F. prausnitzii* in those patients.

The effect of riboflavin supplementation on the composition of the microbiota in IBD patients and how inflammatory markers change by this intervention during relapse and remission needs to be explored. Providing specific support for *F. prausnitzii* by food and vitamins, such as riboflavin, may also control the expansion of pathosymbionts in CD patients and therefore prolong the period of remission. Moreover, the possible effects of riboflavin supplementation on other bacterial groups than *F. prausnitzii* and *Roseburia* have to be studied as well, since those may have beneficial effects on the disease status also. This asks for a systems biology approach, in which the metabolome of the host should be included.

It is evident from the work presented in this thesis, as well as from many contributions from researchers in the field, that IBD is a result of genetic susceptibility, impaired host gut barrier and immune response to specific bacteria in the gut. *In vitro* models to study this are very limited and suboptimal at best. The HoxBan coculture method described in this thesis has great potential to be further optimized to also study the interaction between epithelial, immune cells (of individuals with different genotypes) and intestinal microbiota, either with pure cultures, as we did with *F. prausnitzii*, or as complex mixtures. This could include the reaction of monocytes and/or neutrophils isolated from IBD patients homozygous for *ATG16L1* and *NOD2* risk and protective alleles towards *F. prausnitzii* and other gut bacteria.

In addition, the HoxBan system allows a detailed investigating of the anti-inflammatory properties of *F. prausnitzii* in a “competition” model with pathosymbionts like adherent invasive *E. coli* (AIEC) in combination with epithelial cells and/or immune cells, like we did with the killing-survival assay in this thesis. Furthermore, this model may provide the opportunity to study the effects of drugs used for IBD treatment on the physiology and metabolism of the intestinal microbiota.

The differences between mucosal microbiota of CD patients homozygous for the *ATG16L1* risk or protective allele was shown in this thesis. In addition, many more susceptibility loci play an important role in the development of CD, including the autophagy-related genes *NOD2* and *IRGM*. Analyzing larger patients cohorts for the potential effect of specific IBD associated SNPs on the mucosal bacterial profile will further our understanding of the molecular pathways that control microbiome homeostasis in the gut.
Summary, Discussion and Future perspectives

Research on the effect of environmental factors, such as diet and hygiene, will provide new insights into the mechanisms that underlie the development and propagation of IBD. The results presented in this thesis may help to develop dietary or pharmacological strategies to induce and/or maintain remission in the treatment of IBD.

REFERENCES:
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