Abstract
The intestinal microbiota is known to be a driving force in the development and maintenance of the immune system. While substantial shifts in the microbiota composition may influence immune functionality in a longer term, short occasional changes might also be sensed. The latter opens considerable perspectives for the use of nutritional interventions, intended to modulate immune functionality in a desired direction. Probiotics are discussed here as a possible way to achieve this goal. It seems that effects are not only strain specific, but will depend on many environmental factors that make the immune system receptive or not to the influences of a given probiotic strain. The interactions between probiotics on the one hand and enterocytes or immune cells on the other hand, are a complex interplay that is rarely mediated by a single mechanism. Immunomodulation through nutrition is therefore a complex phenomenon that needs careful consideration, understanding of immune functionality as well as insight into the mechanisms of probiotic activities. Only then can the proper clinical trials with proper readouts be set up to prove efficacy of each strain/mixture individually.

Introduction
Probiotic effects can be attributed to microbiological, metabolic or immunological activity. Although most of these effects can be investigated in vitro, they will be heavily influenced by the environmental conditions (mouth, gut, vagina),
lifestyle (drugs, diet, stress, hygiene) and host status (genetic background, newborn vs. adult or elderly; healthy or diseased). Consequently, in vitro screening for strains with desired probiotic properties will therefore require in vivo validation and final analysis in clinical trials targeted towards a well-defined population. As these clinical validations are expensive and time consuming, it is important to select the most suitable strains at the beginning of the strain selection pipeline.

The Importance of Immunomodulation

Inflammation, in many cases, is an immediate spontaneous response to acute body damage or infection. However, many different forms of chronic inflammation have been described which cause or are caused by different types of illness. Since initial symptoms are often subclinical, early disease remains mostly unnoticed, but in a longer term such low grade inflammation may result in severe metabolic disorders, immune-mediated diseases or even heart or mental problems. Over the last 50 years, we have seen a tremendous increase in this type of chronic diseases [1], an observation which has been linked to a lack of sufficient challenges for the immune system at the early stages of life (the hygiene hypothesis) [2].

In parallel, recent research projects like MetaHit [3, 4] or The Human Microbiome Project (http://commonfund.nih.gov/hmp/) have revealed the importance of the microbiota in many aspects of human health and disease. Research has focused on the quantity (10 times more cells and 150 times more genes than present in the human body) [5], quality and diversity or resilience of the microbiota [6] in IBD, type 1 diabetes or obesity. As a consequence, microbiota composition has now been extensively linked to similar immune-mediated disorders as previously considered by the hygiene hypothesis. The multiple possibilities to influence the quality of the microbiota through nutrition, medication or even stool transplantation opened many perspectives for further research focusing not only on the microbiota composition and diversity, but also on the immune modulation potential of the intervention considered. Therefore, the use of possible anti-inflammatory approaches, e.g. with respect to dietary interventions, has gained a lot of attention. One of these interventions comprises suitable probiotic strains. In this chapter, we will focus on some of the available screening pipelines useful to discriminate between strains with different immunomodulatory potential as well as to assist in providing mechanistic explanation of the observed differences.
The Mechanisms Involved

As mentioned above, the immune system of mammals involves a panoply of immune cells and signaling molecules which interact with microorganisms and antigens surrounding us. Because of the $10^{14}$ bacteria in our gut, the intestine has evolved to our main immune organ. No doubt that the intestine therefore is a receptive organ, suitable for nutritional interventions aiming at modifying or correcting immune responses of the host.

Below, we describe very briefly some of the currently known mechanisms involved in the interaction between the host and his or her microbiota. More extensive reviews of these processes can be found in Delcenserie et al. [7] and Lebeer et al. [8].

- The innate immunity allows to rapidly and radically react towards challenges caused by infectious agents. The responses are inflammatory in nature and include mainly phagocytic cells (macrophages, neutrophils, natural killer cells).

- The adaptive immune system, after activation by antigen-presenting cells, will mobilize specific T and B cells that will, through the production of specific signaling molecules (cytokines, chemokines), assist in the regulation of both the innate and adaptive immune responses. B cells secrete antibodies (providing humoral immunity), whereas T cells are subdivided into T helper cells (CD4+, also called Th) and T cytotoxic cells (CD8+). Since antigen-presenting cells in the intestinal mucosa continuously sample intraluminal intestinal antigens, the central challenge of the gut immune system is to continuously balance defense with tolerance: choosing to activate effector T cells that enhance defense against pathogens, or promote differentiation into various regulatory T cell subsets that induce tolerance. The latter mechanisms have to protect the host from excessive inflammation during the course of an infection or help to rebalance the immune system when disturbed. The innate immune cells will therefore continuously need to identify ‘self’ versus ‘non-self’, using specific surface markers. Table 1 lists the most common bacterial pathogen- or microorganism-associated molecular patterns, together with their respective pattern recognition receptors (PRRs) found in and on immune cells. The best known are the Toll-like receptors and the NOD (Nucleotide-binding Oligomerization Domain-containing protein)-like receptors, at the surface or the nucleus of immune cells, respectively.

Amongst the antigens sensed, lipopolysaccharides (LPS) of Gram-negative cell walls belong to the most powerful inducers of inflammatory responses. LPS is thought to be involved in chronic physiological inflammation, typically encountered in obese patients [9]. In contrast, peptidoglycans
Probiotic effects may be mediated through their interaction with PRRs in dendritic cells (DCs) or in intestinal epithelial cells. DCs, activated by probiotics, produce cytokines and express costimulatory molecules that will allow the polarization of naïve T cells towards the differentiation of regulatory T cells (Tregs), notably CD4+CD25+FoxP3+ Tregs [10, 11] or Th1 effector cells [12] that will produce proinflammatory cytokines like interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and interleukin-2 (IL-2) and IL-12. Th1 cells therefore stimulate phagocytosis and help fight microbial pathogens but can also be implicated in immune-mediated diseases such as arthritis or multiple sclerosis. Nonprobiotic antigens might polarize T cells towards the differentiation into Th2 cells, producing cytokines such as IL-4, IL-5, IL-6 and IL-13, which help to fight extracellular parasites, but are also seen in allergic reactions. Since both Th1 and Th2 cell types act antagonistically, the balance between Th1 and Th2 cytokine production will determine the direction of an immune response. In this process, Treg cells and type 3 T helper cells (Th3 cells) may interfere.

(PGNs) from Gram-positive bacteria (e.g. lactobacilli) can be involved in probiotic anti-inflammatory activity [10].
Treg cells have immunoregulatory properties mediated by IL-10 and TGF-β [13], while Th3 cells primarily secrete TGF-β [14]. The cytokines IL-10 and TGF-β, with assistance of CD4+ T cells from the GALT will promote the differentiation of B cells into IgA antibody-secreting plasma cells. The enterocytes will excrete the IgAs into the intestinal lumen, where they inactivate antigens and pathogens (viruses, bacteria, toxins) [15].

- Another subset of CD4+ Th cells, the Th17 cell, is characterized by the production of mainly IL-17 (besides IL-21 and IL-22) and by the differentiation by TGF-β and IL-6 cytokines [16], involved in the recruiting of neutrophils and macrophages to the infected tissue.

- Specialized epithelial cells, such as Paneth cells at the bottom of the crypts of the small intestine, can produce defensins, while goblet cells produce mucus that will protect the epithelium. Defensins are a major family of antimicrobial peptides that contribute to the protection of mucosal surfaces by binding to the microbial cell membrane where they form pore-like structures that cause membrane defects in the bacteria. IL-22 secreted by Th17 cells can not only induce the expression of β-defensin 2 and β-defensin 3 [17], assisting directly in the defense against bacterial infection, but through these cytokine signaling pathways it will also link to (and coordinate?) the different arms of the host defense system.

**The Probiotic Influence on the Immune System**

Over the last decades, hundreds of studies have looked at the potential of a wide range of (potential) probiotic strains to interact with a variety of immune cells using a diversity of in vitro, ex vivo or in vivo models. Table 2 lists a relevant selection of papers published between January and October 2012 which have studied or inventoried possible mechanisms of probiotic immune modulation. The variety of readouts, models and organisms clearly reflects the complexity of this type of studies. The immune functionality of any given strain in humans will depend on the environment as well as the surrounding microbiota (commensals, pathogens, bacteriophages, toxins). The final validation of a selected strain will therefore be through human clinical trials, confirming the effectiveness in certain target populations. Since clinical validations are expensive, the understanding of the possible mechanism may be a valuable factor in the definition of the clinical trial context and the end points to consider. Below is one example of a possible approach that starts with the screening of a (large) number of strains and ends with a purified molecule that is responsible for the observed anti-inflammatory effect of a selected strain.
Table 2. Selection of relevant papers published between January and October 2012 which have studied or inventoried possible mechanisms of probiotic immune modulation

<table>
<thead>
<tr>
<th>(Probiotic) organism</th>
<th>Target</th>
<th>Approach</th>
<th>Measure</th>
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<tbody>
<tr>
<td><em>Streptococcus thermophilus</em> and <em>Lactobacillus delbrueckii</em></td>
<td>human DC compared to PBMC</td>
<td>in vitro</td>
<td>surface markers CD86, HLA-DR and cytokine production</td>
<td>[34]</td>
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<tr>
<td>Living probiotics, probiotic DNA and CpG-ODN</td>
<td>allergic response</td>
<td>in vivo</td>
<td>↗CD4+CD25+high Treg cells, intestinal barrier, TLR-9 mRNA, NF-κB activity, p-IκB-α</td>
<td>[35]</td>
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<tr>
<td><strong>12 Lactobacillus strains</strong></td>
<td><em>E. coli</em> infection</td>
<td>in vitro</td>
<td>CXCL8 release from human urothelial cells</td>
<td>[36]</td>
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<tr>
<td>Probiotics</td>
<td>health and disease prevention</td>
<td>review</td>
<td>intestinal regulation of inflammatory processes</td>
<td>[37]</td>
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<tr>
<td><em>L. casei</em> Shirota, <em>L. rhamnosus</em> GG, <em>L. plantarum</em> NCIMB 8826 and <em>L. reuteri</em> NCIMB 11951, <em>B. longum</em> SP 07/3 <em>Bifidobacterium bifidum</em> MF 20/5</td>
<td>human PBMC natural killer (NK) cell activation</td>
<td>in vitro</td>
<td>↗proportion of CD69+ on lymphocytes, T cells, T cell subsets and NK cells, ↘the proportion of CD25+, mainly on lymphocytes and NK cells; ↘of IL-1β, IL-6, IL-10, TNF-α, granulocyte-macrophage colony-stimulating factor and macrophage inflammatory protein 1α; no effect on the production of IL-2, IL-4, IL-5 or TNF-β; strain-specific modulation of IL-10, INF-γ, TNF-α, IL-12p70, IL-6 and monocyte chemotactic protein-1</td>
<td>[38]</td>
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<tr>
<td><em>L. plantarum</em> NCIMB8826 and VSL#3</td>
<td>TNBS model</td>
<td>in vivo</td>
<td>↘colon morphology and less influx of CD11b+ and adaptive CD4+/CD8+ cells in the intestinal mucosa and ↗IFN-γ, IL-17, IL-1β, MCP-1; shift of gene expression profiles toward healthy controls (genes related to mast cells and antimicrobial peptides; suppression of chemokine genes)</td>
<td>[39]</td>
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<tr>
<td>Heat-inactivated <em>L. rhamnosus</em> GG and <em>L. delbrueckii</em> subsp. <em>bulgaricus</em></td>
<td>human DCs</td>
<td>in vitro</td>
<td>LGG ↘p38, L. delb ↘I-κB; modification of miRNAs expression: LGG ↘miR-146a expression (negative regulator of immune response targeting NF κB), ↗miR-155</td>
<td>[40]</td>
</tr>
<tr>
<td><em>L. acidophilus</em> NCFM</td>
<td>tumor mice model</td>
<td>in vivo</td>
<td>↘tumor volume growth by 50.3%, ↘severity of colonic carcinogenesis (level of colonic involvement and structural abnormality of epithelial/crypt damage), ↗apoptosis; ↘CXCR4 mRNA expressions in the colon, MLN and extraintestinal tissue, mean fluorescence index of MHC class I (H-2Dd, -Kd and -Ld) in flow cytometry analysis</td>
<td>[41]</td>
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<td><em>Bacillus coagulans</em> 30 metabolites at &lt;3, 3–30, and 30–200 kDa</td>
<td>maturation of mononuclear phagocytes</td>
<td>in vitro</td>
<td>↗CD14+ CD16+ proinflammatory cells (high molecular weight fraction); on CD-14dim cells: ↗CD80 and CD86 expression, in contrast to a selective ↗in CD86 expression on CD14bright cells (role in antigen presentation to T cells, support of T helper cell differentiation, generating T regulatory cells)</td>
<td>[42]</td>
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### Table 2. Continued

<table>
<thead>
<tr>
<th>(Probiotic) organism</th>
<th>Target</th>
<th>Approach</th>
<th>Measure</th>
<th>Reference</th>
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<tbody>
<tr>
<td><em>E. coli</em> Nissle 1917 versus <em>E. coli</em> W536 (intact cells versus lysates)</td>
<td>gene expression on human PBMCs measured by microarrays</td>
<td>in vitro</td>
<td>(\uparrow) IL-12p40 by both LPS molecules, dose dependent (\uparrow) IL-10 by both lysates; (\downarrow) CCL24 (eotaxin) by lysates (especially EcN compared to wild-type LPS)</td>
<td>[43]</td>
</tr>
<tr>
<td>Heat-killed <em>Bifidobacterium lactis</em> AD011, <em>B. bifidum</em> BGN4, <em>L. casei</em> IBS041, and <em>L. acidophilus</em> AD031</td>
<td>mouse DC (±IEC)</td>
<td>in vitro</td>
<td>BB and LC (single culture) (\uparrow) by DCs I-Ad, CD86 and CD40; all probiotics (\uparrow) IL-6, TNF-α; LC and LA (in coculture) (\downarrow) expression I-Ad; no probiotics (\uparrow) IL-6, TNF-α; LC (inverted coculture), (\downarrow) the expression CD40; all probiotics (\downarrow) IL-6; BL (\uparrow) IL-10; LC and LA (\uparrow) TGF-β</td>
<td>[44]</td>
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<tr>
<td><em>Bacillus cereus</em> var. Toyoi</td>
<td>adjuvant potential to improve BoNV-S vaccine (cattle pathogen)</td>
<td>in vivo</td>
<td>(\uparrow) IgG antibody response toward Th1, and (\uparrow) IFN-γ, IL-12 and IL-10 mRNA levels</td>
<td>[45]</td>
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<tr>
<td><em>L. reuteri</em> strains DSM 17938 and ATCC PTA 4659</td>
<td>NEC in newborn rats</td>
<td>in vivo ex vivo</td>
<td>(\uparrow) survival rate and (\downarrow) incidence and severity of NEC, IL-10; ex vivo and in vivo (\downarrow) intestinal LPS-induced pI-kB</td>
<td>[46]</td>
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<tr>
<td><em>Bifidobacterium animalis</em> ssp. <em>lactis</em> BB-12 and <em>Lactobacillus paracasei</em> ssp. <em>paracasei</em> 431</td>
<td>vaccination model in healthy subjects</td>
<td>human clinical trial</td>
<td>(\uparrow) vaccine-specific plasma IgG, IgG1 and IgG3; (\uparrow) number of subjects obtaining a substantial increase in specific IgG; (\uparrow) mean fold increases for vaccine-specific secretory IgA in saliva and total antibody concentrations; no differences for plasma cytokines or innate immune parameters</td>
<td>[47]</td>
</tr>
<tr>
<td><em>Bifidobacterium breve</em> UCC2003 (EPS+), UCC2003–EPSdel (EPS–), UCC2003:Bbr_0430 (EPS–)</td>
<td>murine treatment with EPS+ or EPS– strains</td>
<td>in vivo</td>
<td>surface EPS aids in long-term persistence, mediates immune evasion, avoiding B cell responses; colonization of mice with EPS+, but not EPS– strain, protects after <em>Citrobacter rodentium</em> infection</td>
<td>[48]</td>
</tr>
<tr>
<td>VSL#3 and conditioned culture medium</td>
<td>steatohepatitis and atherosclerosis mice model</td>
<td>in vivo</td>
<td>reversed insulin resistance, prevented development of histologic features of mesenteric adipose tissue inflammation, steatohepatitis and (\downarrow) the extent of aortic plaques; conditioned media: direct transactivation of PPAR-γ, Farnesoid X receptors and vitamin D receptor</td>
<td>[49]</td>
</tr>
<tr>
<td>Dietary probiotics</td>
<td>T cell (Treg) homeostasis</td>
<td>review</td>
<td>CD4+CD25+FoxP3+ Tregs could be valuable to control chronic inflammatory conditions directly affecting the gut (IBD, Crohn’s disease) or indirectly influencing other chronic tissue inflammatory conditions (T1D, MS and RA)</td>
<td>[50]</td>
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A Screening Approach for Better Probiotics

In 2007, Foligné et al. [18] described the homology between a simple in vitro screening test using human peripheral blood mononuclear cells (PBMCs) and an in vivo approach using a mice model of trinitrobenzene sulfonic acid (TNBS)-induced colitis [18]. While for most strains a high congruence was shown between both experimental approaches, considerable variation was found for most LAB genera and yeasts in their potential to induce IL-10 and IL-12, markers of anti-inflammation and proinflammation, respectively [18–22].

This variation is illustrated in figure 1a, where 8 different strains of LAB have been compared for their IL-10:IL-12 ratio. The strains of *Lactobacillus salivarius*, *Lactobacillus rhamnosus* and *Lactobacillus casei* have potential anti-inflammatory properties (cytokine ratio >15), while the two strains of *Lactobacillus plantarum* clearly do not have the same potential (cytokine ratio around 5). The strains investigated of *Lactobacillus acidophilus*, *Lactococcus lactis* and *Streptococcus gordonii* will probably have no anti-inflammatory effect at all, as their cytokine profile on human PBMCs tends to be very neutral to rather proinflammatory (cytokine ratio <5). Strains with a high IL-10:IL-12 ratio are generally considered anti-inflammatory, and are better suited for in vivo applications that require a reduction of inflammation, while strains which induce more...
Fig. 1. **a** Cytokine ratio IL-10:IL-12 obtained on human PBMCs. Data are averages of 4 different healthy blood donors. **b, c** Protective effect of lactic acid bacteria strains against TNBS-induced colitis in BALB/c mice. Bars represent the percentage protection (reduction in the mean Wallace macroscopic inflammation scores of bacteria-treated mice in relation to the mean score of a TNBS control group of mice, n = 10). Colitis index was assessed 48 h after TNBS administration. Each bar represents an independent experiment. The comparison between the TNBS control groups and the groups that received the corresponding untreated BMDCs (DC) was calculated using the Mann-Whitney U test (* p < 0.05, ** p < 0.01, *** p < 0.001). **b** Effects of oral administrations of live lactic acid bacteria on acute TNBS-induced colitis in BALB/c mice. **c** Effects of IP administrations. IP experiments are lacking for the strains of *L. casei* and *L. plantarum*. For further experimental details see Macho-Fernandez et al. [10].
pro-inflammatory cytokines can be interesting to activate the immune system e.g. in fighting certain types of infections [12].

In order to validate the anti-inflammatory nature of the strains, these 8 strains were further investigated in an in vivo animal model of colitis. The TNBS colitis model [18] was used. The administration for 5 consecutive days can result in a certain degree of protection against inflammation induced by TNBS. The protection is measured with a macroscopic score [23], histological score [24] or other immune parameters (intestinal myeloperoxidase, murine IL-6 or serum amyloid A protein levels). Myeloperoxidase is a major constituent of neutrophil cytoplasmic granules, and its activity therefore is a direct measure of neutrophil presence and an indirect indicator of tissue inflammation.

In figure 1b the results obtained in the TNBS colitis model are shown for the 8 strains previously investigated with the PBMC model. The macroscopic protection obtained, expressed as a percentage of the average Wallace score obtained for 10 nonprotected TNBS-treated mice, confirms the three different groups of strains defined using the human PBMC model [18]. The different bars in figure 1b represent repetitive experiments.

Although these models are interesting as a primary screening tool, they do not give any information about the possible mechanism(s) involved. The first question we tried to answer related to the nature of the immune cells involved in the signaling. Therefore, four of the bacteria from the previous models were administered intraperitoneally (IP) to TNBS-treated mice, rather than through gavage. The result of this experiment, shown in figure 1c, revealed that IP administration yielded virtually identical protection levels in mice, and this within a time frame of no more than 2 h. This raised the possibility that DCs were probably involved in this protection [25].

In order to test this hypothesis, the strains of *L. salivarius* (LS33) and *L. acidophilus* NCFM (indicated in bold in fig. 1) were selected as strains with opposite immunomodulation profiles to be compared in further mechanistic studies.

In the next experiment, naïve bone marrow DCs (BMDCs) were isolated from the bone marrow of healthy BALB/c mice and stimulated for 16 h in vitro with the respective LAB. The probiotic-conditioned DCs were then washed and readministered to the mice via the IP route. A treatment with TNBS followed, and protection levels were measured using the traditional readouts mentioned above. Results obtained confirmed earlier findings: BMDCs pulsed with strain LS33 could protect mice from colitis (58% protection) while BMDCs pulsed with strain NCFM did not [25]. Using Nod2−/− knockout mice, we could then show that protection needed Nod2 signaling as strain LS33 did not protect in these mice in contrast to the WT mice [10].
Based on this observation, we then hypothesized that the PGN at the bacterial surface might be responsible for the protection [10]. In collaboration with C. Hermann (Konstanz University, Germany), the PGN of both strains was isolated and purified. The use of the purified PGN injected via the IP way in the TNBS colitis model yielded again comparable results as obtained with the intact strains and moreover was dose dependent (fig. 2). A final comparison by HPLC of the chemical composition of PGN of strains LS33 and NCFM, performed in collaboration with Ivo Boneca (Institut Pasteur, Paris, France), yielded a significantly different peak, which was identified and chemically synthesized. Using the pure chemical structure of the monomer GlcN-MurNAc-L-Ala-γ-D-isoGln-L-Lys, released to about 10% of the total amount of the muropeptides in LS33, it was possible to mimic the probiotic effect of the live strain of *L. salivarius* LS33 in mice [26]. Moreover, further mechanistic research confirmed that the protective capacity of selected probiotic lactobacilli is NOD2 dependent and is correlated with local IL-10 production and induction of regulatory CD103+ DCs and regulatory T cells. CD103+ DCs have been shown to drive preferentially CD4+Foxp3+ T regulatory cells notably through the indoleamine 2,3-dioxygenase (IDO)-dependent pathway, and as such play a crucial role in intestinal homeostasis. IDO expression was indeed found to be upregulated in the colon and in the CD11c+ DCs purified from mesenteric lymph nodes in PGN-treated mice [10, 26].

While it is obvious that these results need to be followed by a proper clinical trial with either the probiotic strain or the purified molecule, the knowledge about the mechanism can have serious implications. The use of this approach might for example not be advised for patients with Crohn’s disease. As this probiotic mechanism is NOD2 dependent and mutations in the *Nod2* gene are as-
associated with susceptibility to Crohn’s disease, a positive outcome in clinical trials is not at all guaranteed. This finding seems to confirm the earlier observations that several well-characterized probiotic strains failed to fulfill the expected clinical outcome in patients suffering from Crohn’s disease.

**Other Mechanisms under Study**

Other mechanisms have been linked to anti-inflammatory activity. Sokol et al. [27] showed that *Faecalibacterium prausnitzii* was associated with a higher risk of postoperative recurrence of ileal Crohn’s disease. In Caco-2 cells transfected with a reporter gene for NF-κB activity, *F. prausnitzii* had no effect, whereas the supernatant abolished the NF-κB activity. PBMCs stimulation by *F. prausnitzii* induced significantly lower IL-12 and IFN-γ levels and more IL-10. The oral administration of either the strain or its supernatant protected mice from TNBS-induced colitis. Today, it is not clear what compound released in the supernatant is responsible for this effect.

Bifidobacteria have also been shown to have anti-inflammatory effects. Ivanov et al. [28] characterized a serpin from a strain of *Bifidobacterium longum*, an anaerobic Gram-positive bacterium that naturally colonizes the human gastrointestinal tract. The *B. longum* serpin was shown to efficiently inhibit eukaryotic elastase-like proteases. When inhibiting neutrophil elastase, involved in acute inflammation in the gut, this strain would be a very powerful anti-inflammatory agent [29]. A recombinant *L. lactis* strain overexpressing this bifidobacterial serpin showed substantial alleviation of inflammation in the TNBS model in mice [Arigoni et al., oral communication at the 8th Symposium on Lactic Acid Bacteria, August 28 to September 1, 2005, Egmond aan Zee, The Netherlands].

A strain of *Bifidobacterium breve* was shown to release soluble factors that alleviated the secretion of proinflammatory cytokines by immune cells [30]. TNF-α-induced chemokine (CXCL8) secretion, alteration of NF-κB and AP-1 signaling pathways all confirmed that the strain and its cell-free medium (CFM) induced a time- and dose-dependent inhibition of CXCL8 secretion by epithelial cells, involving AP-1 and NF-κB transcription pathways. Moreover, protection was shown in a model of TNBS-induced colitis in mice by the strain and its CFM. The same effect was obtained by DCs that were conditioned with the CFM. These results indicate that the *B. breve* strain was able to downregulate inflammation at the epithelial level by inhibiting phosphorylations involved in inflammatory processes and by protective conditioning of DCs. Again it was not the cells that seemed responsible for this effect, but secreted soluble factors.
These factors clearly contribute positively to intestinal homeostasis by attenuating chemokine production.

A last example of unidentified soluble factor with anti-inflammatory potential was described by Jones and Versalovic [31] for the strains of *Lactobacillus reuteri*. Supernatants from *L. reuteri* biofilms were added to human monocytic THP-1 cells in the presence and absence of LPS. LPS stimulates the production of pro-inflammatory TNF-α by THP-1 cells. *L. reuteri* strains ATCC PTA 6475 and ATCC PTA 5289, but not two other strains of the same species, suppressed TNF-α production when cultured as biofilms. The authors noted that the relative abilities to suppress human TNF-α in monocytoid cells were directly correlated with relative abilities to aggregate and form biofilms on polystyrene surfaces. Recently, it was suggested [32] that the histamine produced by strain ATCC PTA 6475 stimulated increased levels of cAMP, which inhibited downstream MEK/ERK MAPK signaling via protein kinase A, with subsequent suppression of TNF production by transcriptional regulation.

These observations once more indicate the complexity of the conditions that will promote or inhibit a possible anti-inflammatory effect and stress the importance of well-designed clinical trials in a well-controlled target population, with a well-controlled product.

**Conclusions**

The intestinal microbiota is largely involved in the development and maintenance of our immune system. A ‘balanced immune’ system probably does not exist, as the microbiota with all the variable intestinal content is continuously interacting with the immune system. Over a longer period, each immune system will show varying responses to a particular antigen or combinations of antigens. Healthy blood donors for example will react differently over time when their PBMCs are challenged with a set of bacteria (data not shown). Therefore, the constant anti-inflammatory and regulatory surveillance of the intestinal immune system is very important. External factors, including nutritional factors, can influence these immune-regulatory mechanisms. In this chapter, we focused on only a few of the possible mechanisms, involving regulatory cells, cytokines, chemokines, defensins and many other factors that manage immune responses, fight infections and toxins or cause immune-mediated anomalies. When these control mechanisms fail, external intervention is necessary. Probiotics have the potential to assist in this process. While some of the mechanisms by which they act are being elucidated, many more need to be clarified.
Although it is essential to understand these mechanisms in full, it is not very likely that probiotics, due to the complexity of all environmental factors, will bring solutions to all immune-mediated diseases. Likewise, it is not likely that probiotics alone will be able to restore ‘heavily damaged’ immune systems. The best results have been obtained with young children, probably because the immune system development still allows for modifications at the time oral tolerance and immunity are developing. This may become more difficult at later age.

Without any doubt, however, knowledge about the mechanism will allow to select better strains, design more effective clinical trials, with readouts that correspond to the expected effect in the targeted population. This probably means that other bacteria than the traditional lactobacilli and bifidobacteria are likely to become important, especially in a therapeutic context. The term pharmabiotics has been coined [33] and will probably be further elaborated to include heat-killed or irradiated strains as well as metabolically or genetically engineered bacteria. The latter will make optimal use of mechanistic studies providing insights into the molecular basis of the intended effects. The future of the *-biotics without any doubt is brilliant.

**Disclosure Statement**

The authors declare not to have any conflict of interest for this article.

**References**


