

## PROBIOTICS AND SMALL BOWEL MUCOSA: MOLECULAR ASPECTS OF THEIR INTERACTIONS

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**ABSTRACT:** *Probiotics are described as “friendly bacteria” that could improve the intestine defense by interacting with the resident microflora. There is a large body of evidence suggesting that consumption of functional food containing probiotics exerts positive effects on human health. Several clinical trials have highlighted the efficiency of probiotics in the prevention and treatment of different gastrointestinal disorders including the prevention of antibiotic associated diarrhea, the remission in patients with inflammatory bowel disease, beneficial effects against *Helicobacter pylori* infection, positive effects in patients affected by allergies and atopic diseases. The clinical benefits of probiotics use are mainly attributed to their antimicrobial substances production and their positive interactions with the enterocytes to reinforce the intestinal epithelial barrier. Moreover, there is evidence suggesting that probiotics stimulate both specific and non-specific host immune responses. Recently, have been published some experiments performed with the DNA microarray technology which provided a global gene screening of the complex bacteria-host interplay. Nevertheless, the molecular mechanisms by which probiotics enhance the intestinal host defense are still not completely elucidated. Here, we review the experiments and clinical studies to date on the complex mechanisms regulating the communication between probiotics and their hosts.*

**KEY WORDS:** Intestinal Barrier, Microarray, Mucosal immunity, Probiotics

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### INTRODUCTION

For some years there has been an increasing interest in the study of intestinal microflora and their relationships with human health. Indigenous microbiota, counting more than 500 species, not only support the digestive functions but act as well as a main actor of the immune system by preventing the colonization by

pathogenic microorganisms. The composition of the intestinal microflora is subject to constant variations by diet changes, emotional stress, age and use of antibiotics and immunosuppressive drugs. Furthermore, lifestyle in Western countries, increasing levels of hygiene has led to a poor intake of bacteria, detrimental for the microflora diversity. To counter this lack a number of food products have been developed to partially restore the intestinal ecosystem. Among these are probiotics, described as “friendly bacteria” which exert a beneficial influence on human health, which have been widely prescribed to positively influence the intestinal microflora balance. The most commonly used probiotics are lactic acid bacteria (LABs) *Lactobacilli* and *Bifidobacteria*. These are known to be safe to use even at an early age, able to survive passage through, and transiently colonize the intestinal tract. Other strains such as *Escherichia coli* spp, *Enterococci*, *Bacillus* species and yeasts have been shown, as well, to interact positively with the gastrointestinal microfloral ecosystem. There are various kinds of probiotic formulations. These could be added to yoghurt to produce the so called “functional food” or are prescribed as tablets with the main advantage of targeting their distribution to the correct area of the human intestinal tract.

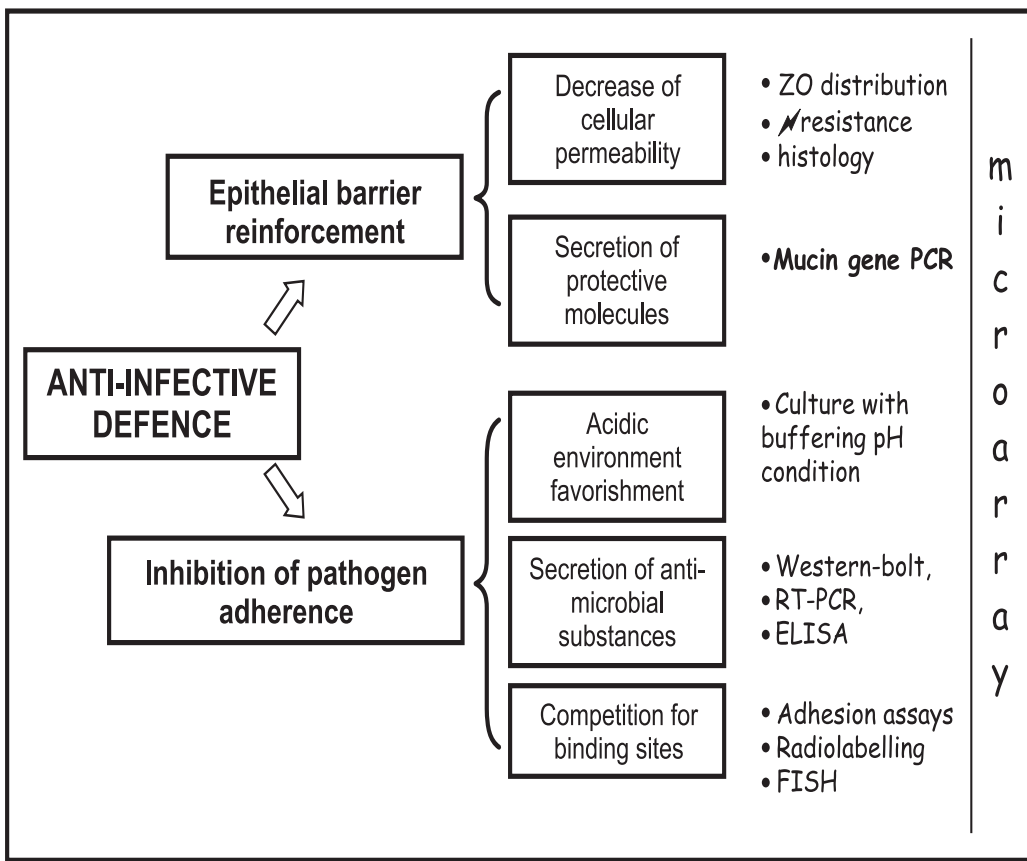
There is an increasing number of clinical trials showing positive effects of probiotic intake and their link to the prevention and treatment of gastrointestinal disorders. Several studies have shown positive effects for the reduction of diarrhea duration and frequency in patients affected by antibiotic associated diarrhea (AAD) (Surawicz et al., 1989; Cremonini et al., 2002a) and for the prevention of diarrhea in infants (Lin et al., 2005; Weizman et al., 2005). Probiotic use is associated with improvement of tolerance to the eradication treatment of *Helicobacter pylori* infection (Cremonini et al., 2002b; Myllyluoma et al., 2005). Moreover, probiotics ingestion has been associated with clinical improvements for patients suffering from inflammatory bowel disease by preventing pouchitis (Gionchetti et al., 2000; Mimura et al., 2004) and facilitating remission in ulcerative colitis (Bibiloni et al., 2005). Furthermore, probiotic administration exerts positive effects on people with allergic symptoms and dermatitis (Kalliomaki et al., 2001; Viljanen et al., 2005).

Probiotics benefits result from a specific communication between bacterial strains and the intestinal epithelial cells. Probiotics induce production of antimicrobial substances by the intestinal host cells, reinforce the epithelial barrier integrity, and compete with pathogenic microorganisms for enterocyte binding sites. Besides these anti-infective properties, probiotics are also main actors of the immune and inflammatory response, stimulating cytokines production. However, little is known regarding the molecular mechanisms of probiotics interactions with intestinal epithelial cells.

The scope of this review is to briefly summarize experimental data and clinical trials performed with treatments with probiotics to better understand their mode of action.

**Figure 1. Anti-infective processes exerted by probiotics onto epithelial cells and ex vivo methods commonly used to assess them**

Abbreviations: (M): electrical; FISH: Fluorescence In Situ Hybridization; PCR: Polymerase Chain Reaction; RT-PCR: Real-Time PCR; ZO: Zonula Occludens)



**PROBIOTICS AND THE ANTI-INFECTION DEFENCE**

The intestines fulfill an important function working as a barrier against the surrounding environment to permit only the selected substances to be absorbed by the organism and the dangerous or unnecessary compounds to be excreted via the feces. Probiotics ingestion has been shown to improve intestinal function by reducing the proliferation of pathogenic bacteria, reinforcing the epithelial barrier using many anti-infective processes such as competing with pathogenic bacteria for enterocyte receptors, regulating epithelial permeability and secreting antimicrobial substances (Figure 1).

**Probiotics inhibit the adhesion of pathogens**

The first aspect of probiotic benefits for the mucosa protection is the competition between favorable strains and pathogenic bacteria to colonize the gastrointestinal tract. Several studies using human intestinal cell models such as Caco-2 or HT-29 cells have shown adhesiveness of probiotics to intestinal cell receptors, preventing by this way the colonization by enteropathogenic bacteria (Chauviere et al., 1992; Bernet et al., 1994; Tuomola et al., 1998). These experiments have also shown that not all strains are able to adhere to enterocyte-like cells, underlining that this property is strain-dependent and cannot be extrapolated to all probiotics. Moreover, adherence

to intestinal cells seems to be achieved through different processes. Bernet et al. have reported that *Lactobacillus acidophilus 1* binds to the mucus secreted by the HT-29 cells whereas it adheres to Caco-2 cells via a proteinaceous adhesion promoting factor.

In addition to creating a barrier effect, probiotics may stimulate the intestinal defense through the induction of antimicrobial peptides by the host cells such as bacteriocins. *In vitro* experiments have provided several examples such as the release of human beta defensin 2 by Caco-2 cells cultured with *E. coli Nissle 1917* (Wehkamp et al., 2004). Defensins are a family of proteins playing a key role in the defense system of small intestine crypts against colonization by potential pathogens (O Neil et al., 2003). Recently, a study confirmed that 24 different strains of *Bifidobacteria* isolated from human feces were able to produce bacteriocin-like compounds that kill gram positive and negative bacteria

and yeast (Collado et al., 2005). *LGG* has been shown to induce production of nitric oxide (NO) by human T84 intestinal epithelial cells (Korhonen et al., 2002). NO is one of the major components of the immune response that acts as antimicrobial agent and plays a fundamental role in the regulation of gut mucosal immunity. Moreover, *Lactobacillus* bacteria, by producing metabolites such as acetic and lactic acids decrease the intestinal pH and thus inhibit the growth of some other bacterial pathogens such as *Staphylococcus aureus* (Fang et al., 1996).

**Table 1: Experiments showing the effects of probiotics on the intestinal barrier reinforcement**

PROBIOTIC STRAIN	EXPERIMENTAL MODEL	EFFECTS ASSESSMENT	RESULTS	REFERENCES
Lactobacillus GG	Rats fed with LGG supplemented milk	Immunospot assay of lactoglobulin antibody	Reinforce epithelial barrier	Isolauri et al. (1993)
VSL#3 mixture or fresh or heated VSL#3 conditioned medium	T84 monolayer	- cell permeability tested by mannitol flux - IL-8 secretion  - Pathogenic infection	↓ of mannitol flux, ↓ of permeability ↓ (modest) of IL-8 secretion ↑ monolayer resistance	Madsen et al. (2001)
VSL#3 mixture during 4 weeks	IL-10 deficient mice	- Histological score-  Colon bacterial content  - Cytokine secretion	↓ mucosal ulceration and epithelial hyperplasia ↑ nb of viable organisms in the colon ↓ TNF $\alpha$ and IFN $\gamma$	
Lactobacillus acidophilus strain LB heat-killed	HT-29 cells	- Zonula Occludens (ZO-1) marker immunofluorescence	↑ integrity of tight junctions	Montalto et al. (2004)
E. Coli Nissle 1917 or VSL#3	T84 and HT-29 intestinal cells	- IL-8 secretion - mucin gene expression - ZO-1 distribution - effects of pathogen	↓ IL-8 secretion ↑ MUC2, MUC3, MUC5A Cytoskeleton stabilization	Otte et al. (2004)

### Probiotics reinforce the epithelial barrier

Intestinal microflora plays a fundamental role in the control of cellular permeability. Several studies have shown beneficial effects of probiotics in reinforcing the epithelial barrier. Table 1 summarizes the different *in vitro* and *in vivo* experiments performed to study the positive effects of probiotics on epithelial cells. Briefly, probiotics reinforce the epithelial barrier (Isolauri et al., 1993), control cellular permeability (Madsen et al., 2001) and protect the tight junction integrity, key regulator of the paracellular permeability (Montalto et al., 2004; Otte et al., 2004).

Other studies have shown that some strains such as *Lactobacillus GG* (LGG) and *Lactobacillus plantarum 299v* induce an increase in transcription and secretion of protective molecules such as mucin MUC2 and MUC3 in human adenocarcinoma cell line HT-29 and the Caco-2 cells respectively (Matar et al., 2002; Mack et al., 2003). Mucin are glycoproteins secreted by the goblet cells that protect the intestinal surface by forming a gel which prevents the adherence of pathogens to the epithelial receptors and inhibits bacterial translocation.

However, the molecular mechanisms leading to mucin over-expression are not well-established. It is particularly unclear if probiotics express specific molecules activating the intestinal cells to produce the protein or if cells from the immune system are required to this end.

### PROBIOTICS AND REGULATION OF THE IMMUNE RESPONSE

Intestinal microflora is a key regulator of the immune system. The gastrointestinal tract (GIT) provides a protective interface between the internal environment and the food-derived antigens and microorganisms ingested. The GIT plays a duplicate role by eliminating pathogenic bacteria and tolerating harmless antigens. The correct execution of these functions is ensured by an important complex of immune cells consisting of lymphoid follicles (Peyer's patches) and lymphocytes distributed throughout the lamina propria. Any failure in the regulation of the immune response can result in a range of clinical disorders such as inflammation or allergies. Several clinical studies have reported beneficial effects of probiotics

in treatment and prevention of a number of intestinal disorders such as mentioned in the introduction and, on the other hand, probiotics immunomodulatory effects have been well documented by experiments demonstrating their action on innate and more specific humoral and cellular immune responses (Figure 2).

**Probiotics activate the innate defense**

Improvement of innate immune function in healthy subjects given specific strains has been reported in several clinical studies as assessed by the enhancement of phagocytosis and natural killer activity (Arunachalam et al., 2000; Gills et al., 2001a; Parra et al., 2004). To understand more precisely how probiotics act, *Lactobacillus rhamnosus HN001* has been mainly studied in a rodent model infected by pathogenic bacteria. The experiments revealed that probiotics ingestion induced a decrease of colonization of the GIT by pathogenic bacteria and an increase of the intestinal cell and leukocyte phagocytic activity (Gills et al., 2001b ; Shu et al., 2002). In their experiment, Gills et al. demonstrated that even heat-inactivated *L. rhamnosus HN001* induced a dose-response effect on the phagocytic defense system in mice. However, another study done with different LAB strains has shown that only live bacteria were able to enhance the phagocytic capacity of mouse peritoneal leukocytes (Lee Y. and Lee TS., 2005). These conflicting results underline the specificity of action of each strain.

To assess how probiotics attract immune cells, Kitazawa et al. (2002) measured the chemotactic activity of the culture supernatant of 14 strains of LAB for murine macrophages *in vitro*. They purified a secreted chemotactic factor designated as “Gasserokine” that could be the substance responsible for the recruitment of non-specific immune cells in the intestinal tract.

**Probiotics alter the humoral immune response**

Humoral immune response at the mucosal level is mainly constituted by secreted type A immunoglobulins (sIgA). sIgA play a fundamental role by creating a barrier against infection by pathogenic microorganisms. Numerous studies have reported an enhanced sIgA production during probiotics treatment in both rodents and humans. *Lactobacillus casei* and *Lactobacillus acidophilus* strains increased the number of IgA producing plasma cells in a dose-dependant manner (Perdigon et al., 1995). Germ-free mice colonized with *Saccharomyces boulardii* displayed an increase of anti-*S. boulardii* IgA expression compared to non-colonized mice (Rodrigues et al., 2000). *Bacillus lactis HN019* was shown to improve significantly the amount of sIgA in response to *Escherichia Coli O175:H7* infection (Shu et al., 2001). This elevated amount of sIgA was associated with lower morbidity rate than in untreated mice. In humans, *LGG* has been reported to promote sIgA production in children affected by Crohn’s disease (Malin et al., 1996). In a more limited number of studies, probiotics have been described to modulate the production of IgE. Mice preinjected with ovalbumin displayed a reduced expression of IgE in serum when orally treated with heat-inactivated *Lactobacillus casei* (Matsuzaki et al., 1998).

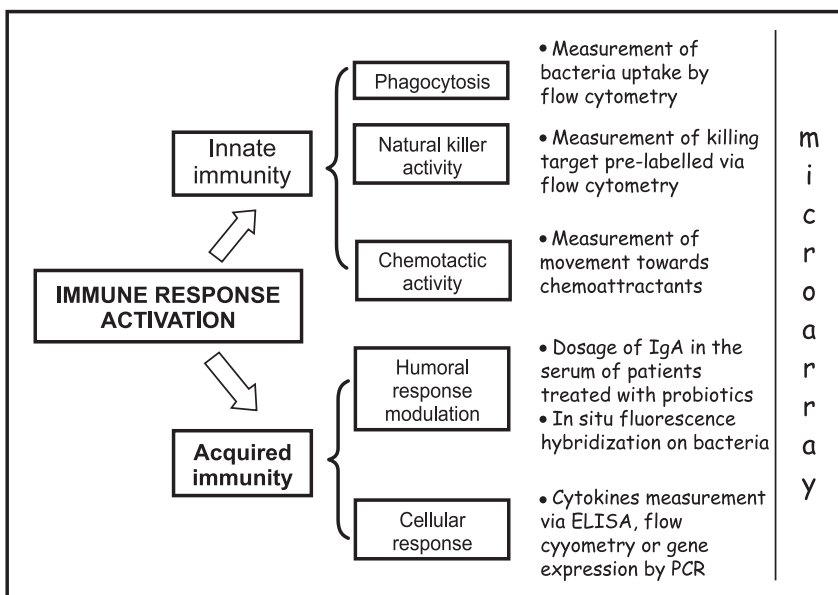
**Probiotics regulate the pro-inflammatory cytokines production**

Probiotics benefits for inflammatory diseases, such as preventing inflammation in chronic colitis and gastritis, Pouchitis and Crohn’s disease, have been clinically reported. These observations suggest that probiotics might down-regulate the enterocytes pro-inflammatory cytokines production. *LGG* has been largely used in clinical trials and experimental models, and shown to be able to

modulate the expression of many cytokines. *In vitro* studies with activated murine macrophages have indicated that *LGG* inhibited production of alpha tumor necrosis factor (TNF $\alpha$ ), one of the major pro-inflammatory cytokines implicated in chronic intestinal inflammation pathogenesis (Pena et Versalovic, 2003). Another experiment performed with Caco-2 cells confirmed the previous result and showed as well that heat-inactivated bacterial forms (and even antibiotic co-cultured probiotics) maintained their positive effects (Zhang et al., 2005). These results are supported by a clinical study with patients suffering from pouchitis. The study group treated with *LGG* showed regulation of the mucosal immune response and the reduction of pro-inflammatory-related interleukins IL-1 $\beta$ , IL-8 and interferon-gamma (IFN $\gamma$ ) (Lammers et al., 2005). Complementary studies suggest benefits of various other strains, such as *Escherichia coli Nissle 1917*, known to be efficient for the treatment of inflammatory bowel disease. It has been shown that this strain is able to down-regulate the diffusion of newly recruited T cells into the mucosa by inhibiting the expression of cytokines such as IL-2, IFN $\gamma$  or TNF $\alpha$  (Sturm et al., 2005).

**Figure 2. Summary of the experiments done to assess the probiotic effects onto the immune host cells**

The figure recapitulates the different experiments done *in vitro*, on animal models and from human samples to study the immune aspects of probiotic effects onto intestinal epithelial cells.



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Finally, it is interesting to highlight that specific strains have been shown able to exert their action by regulating the enzymes recruitment during inflammation. In this manner, *Bifidobacterium lactis* 420 has been shown to decrease cyclooxygenase 2 (COX2) expression in an *in vitro* model of enterocyte-like cells (Nurmi et al., 2005). COX2 is well-known to be involved in the inflammation cascade, chiefly by promoting prostaglandin synthesis. It has also been associated with chronic inflammatory and cancerous diseases.

All previous findings suggest that probiotics play an important role in cellular immune response regulation. However, the mechanisms leading to cytokines production and their associated beneficial effects on intestinal cell homeostasis have not been elucidated. To investigate the mode of action of *LGG*, Yan and Polk (2002) studied the survival of mouse and human colon cells in culture supplemented or not with *LGG* strain. They reported that *LGG* prevented cytokine-induced apoptosis by activating the anti-apoptotic Akt/protein kinase B and inhibiting the activation of the pro-apoptotic P38/mitogen-activated protein kinase (p38 MAPK). P38 MAPK is known to be critically involved in the pathogenesis of Crohn's disease and its inhibition could provide a therapeutic strategy for this pathology (Hommes et al., 2002). This set of results have proposed a new model of interaction between probiotics and epithelial cells allowing intestinal cell survival *in vitro* and confirming the interest of *LGG* therapy in the clinical setting.

Recently, an alternative model has been proposed to explain the protective effects of *LGG* on enterocytes (Braat et al., 2004). In this study, the authors investigated the capacity of dendritic cells (DCs) matured with *LGG* to instruct naïve T cells by measuring cytokine expression of the *ex vivo* stimulated T cells. The results have shown T cell hypo-responsiveness as assessed by cytometric analysis reporting a decrease of IL-2, IL-4 and IL-10 in stimulated T cells derived from *LGG* pre-incubated DCs. This model of probiotic-induced tolerance might lead to an explanation for the observed beneficial effects in the treatment of clinical diseases.

### Probiotics and allergies

The prevalence of allergies and atopic diseases has dramatically increased in the industrialized countries over the past decades. These affections are generally characterized by T-helper 2 (Th2) associated cytokines hyper-polarization. Probiotics, known to play immunomodulatory effects, could represent a therapy of choice to treat or prevent some allergies. *In vitro* models, such as the culture of macrophages with *LGG*, have shown that these bacteria were able to induce expression of chemokines including those that recruit Th1 cells to the site of inflammation (Veckman et al., 2003).

Probiotic immunomodulatory effects have been also tested in animal models such as the mouse (Kimoto et al., 2004). In this study, spleen cells from mice fed with the *G50 Lactobacillus* strain have been shown able to produce IFN $\gamma$ , a Th1 cytokine that plays an important role in the activation of antigen-presenting cells and T-cells. Thus in the phenomenon of tolerance. Moreover, the results in this study showed also that serum levels of IgE

antibodies in the treated group was significantly lower than in the control group. This observation confirmed the fact that the Th1 cytokines over-expression could inhibit IgE production (Powrie and Coffman, 1993).

Several clinical trials have reported beneficial effects of probiotic intake in allergic patients, especially in children. In work done by Pohjavuori & coworkers, it has been shown that *LGG* administration raised production levels of IFN $\gamma$  from the peripheral blood mononuclear cells in infants suffering from dairy products allergy or IgE associated dermatitis. These findings reflect some of the beneficial effects that *LGG* can provide. It is indeed well known that IFN $\gamma$  expression is deficient in atopic infants and contributes to the delayed maturation of cellular immune response. Generally, these children are characterized by the persistence of pronounced Th2 cytokines production after infancy, certainly due to a reduced exposure to microbes at an early age. A clinical trial was done, seeking to restore the normal shift from Th2 dominance to Th1 response (Rautava et al., 2002). Probiotics were administered to mothers before delivery and during breast-feeding period, and then to newborn. The study reported that prenatal administration of *LGG* to mothers 4 weeks before birth and 3 months during breast-feeding increased the expression of transforming growth factor  $\beta$ 2 (TGF- $\beta$ 2), a tolerance-induced growth factor in breast-milk. The newborn fed with that milk produced more IgA antibodies against dairy antigens. Other studies with perinatal *LGG* administration to infants for 6 months have shown a reduction in the incidence of atopic eczema in at-risk children until 4 years of age, suggesting that the protective effects exerted by *LGG* on atopic eczema could extend beyond infancy (Kalliomaki et al., 2003). Early probiotic administration could represent a safe and valuable method to induce immunoprotective effects in infants.

### GENERAL OVERVIEW OF THE COMPLEX PROBIOTICS-HOST INTERPLAY: A MICROARRAY ANALYSIS APPROACH

As previously shown, probiotics exert multiple beneficial effects on the host, playing a fundamental role to protect the epithelial barrier integrity. All the processes enumerated in the previous paragraphs resulted from clinical observations and experimental protocols studying one specific mode of action for each strain. To understand how probiotics act in a global context, a complete screening of probiotics-induced host genes would be necessary.

The DNA microarray technology, in allowing the study of the expression of thousands of genes simultaneously, could provide a valuable tool to characterize the complex probiotics-epithelia cross-talk. To date, two microarrays studies have been published reporting the global gene expression regulation of the small bowel mucosa of healthy patients treated with *LGG* or *Bacillus clausii* species (Di Caro et al., 2005a,b). Both studies revealed that probiotics affected the expression of genes involved in widely different functions, such as inflammation, immune response, cell adhesion, cell communication and apoptosis. These studies also confirmed the bacterial-mucosal interactions. Among the genes altered by probiotic intake, an interesting result concerned the immunomodulation-associated genes activated by each strain.

The first microarray analysis performed on small bowel mucosa samples harvested in healthy individuals treated with *LGG* for one month revealed numerous altered genes previously reported. Of particular interest are the results showing the modulation of Th1-type inflammatory response as assessed by the up-regulation of the allograft inflammatory factor 1 (AIF-1) and many members of the TNF family. The second study done with duodenal biopsies from healthy patients treated with *B. clausii* for one month, revealed the over-expression of cytokines stimulating a Th2-type response and proinflammatory genes such as members of nuclear factor kappa B (NF-KB) and mitogen activated protein kinases (MAPK). These findings seemed to contradict a clinical trial that showed immunomodulatory effects of *B. clausii* in nasal mucosa of allergic children (Ciprandi et al., 2004). However, it must be noted that the molecular analysis was done on a healthy population whereas the clinical trial was performed on allergic patients. It cannot be excluded that probiotics can have different effects according to the physiologic state of the individuals.

These preliminary results sketching the “molecular signature” of probiotic-enterocyte interactions could represent a base for a rational criteria to administer specific probiotics according to the patients’ immune deficiency.

Recently, a microarray study has been performed with a mouse colon epithelium cell line treated with *LGG*-conditioned media (Tao et al., 2005). The results have shown that the heat shock protein 72 (HSP-72) was one of the most up-regulated genes in treated cells. This over-expression has been shown to be MAP-kinase-dependent, suggesting that a specific signal transduction pathway was activated in epithelial cells. This data underlines a new molecular mechanism on how probiotics could preserve epithelial cells from oxidant stress by inducing the expression of cytoprotective HSP in the host cells.

These microarray analysis are useful to provide an insight in the molecular basis on the complex host-bacterium interplay. However, more studies are still required to analyze probiotic effects in different compartments of the GI tract, to specify the dose required for a maximal response, and to evaluate their effects on different pathologies.

## CONCLUSION

To date several clinical trials have suggested beneficial effects of probiotic intake to treat or prevent a wide range of diseases. *In vitro* experiments and animal models have shown some of the action mechanisms of probiotics in the gut, chiefly achieved by reinforcing the intestinal epithelial barrier and stimulating the immune response. However, as underlined in the different studies, the spectrum of probiotic effects is strain-dependent and may differ as well according to the target population. One important issue will be to determine the molecular and cellular mechanisms of interaction between each bacterial strain and the host cells both in healthy and diseased patients. To this end, DNA microarray technology could be an efficient tool allowing to determine sets of genes in the host that are affected by probiotics use. This “molecular signature” could help to determine which combination of strains with complementary effects could be the more efficient.

Some commercial probiotic mixtures are already available, such as the VSL#3. This “cocktail” of four strains of *Lactobacillus* (*L. casei*, *L. plantarum*, *L. acidophilus* and *L. debrueckii* subsp. *Bulgaricus*) has been reported as beneficial in several pilot studies (Bibiloni et al., 2005; Mimura et al., 2004; Kim et al., 2005).

A better characterization for the mode of action of probiotics would also allow for the isolation of their active principles eliminating the risks associated to the use of live bacteria.

Finally, the possibility of using genetically modified probiotics strains to secrete or deliver bioactive compounds to target sites in the GI tract has to be assessed with interest.

To give a glimpse on these future use of probiotics, a study on recombinant probiotics technology has shown its great potential for the treatment and prevention of *Escherichia coli*-induced travelers’ diarrhea (Paton et al., 2005). In this study, the authors achieved the expression of glycosyltransferase genes from pathogenic bacteria in a harmless *E. coli* strain, resulting in the production of a chimeric lipopolysaccharide capable of binding heat-labile enterotoxin with high affinity and reducing the pathogenic intestinal colonization.

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