Application of molecular biology and genomics of probiotics for enteric cytoprotection

Moslehi Jenabian, Saloomeh; Nielsen, Dennis Sandris; Jespersen, Lene

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J.J. Malago
J.F.J.G. Koninkx
R. Marinsek-Logar
Editors

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Editors:
Dr. Joshua J. Malago
Department of Veterinary Pathology
Faculty of Veterinary Medicine
Sokoine University of Agriculture
P.O. Box 3203, Chuo Kikuu, Morogoro
Tanzania
malagojj@yahoo.com

Dr. R. Marinsek-Logar
Zootechnical Department
Biotechnical Faculty
University of Ljubljana
Domžale
Slovenia
romana.marinsek@bf.uni-lj.si

Dr. Jos F. J. G. Koninkx
Division Pathology
Department of Pathobiology
Faculty of Veterinary Medicine
Utrecht University
Yalelaan 1, 3508 TD, Utrecht
Netherlands
j.f.j.g.koninkx@uu.nl

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Contents

Contributors

Hans Christian Beck The Pr
Holbergsvej 10, 6000 Kolding
e-mail: hcb@teknologisk.dk

Giuseppe Caramia Hemeritu
Specialized Maternal-Infantil I
e-mail: caramiagm@libero.it

Christophe Chassard Labora
Science and Nutrition, ETH Z
Zurich, Switzerland
e-mail: christoph.chassard@il

J. Kevin Collins Department
Ireland
Cork Cancer Research Centre,
e-mail: microbiology@ucc.ie

Sebastiano Collino BioAnaly
Research Center, P.O. Box 44,
e-mail: Sebastiano.Collino@r

E. P. Culligan Alimentary Ph
University College Cork, Cork
e-mail: eamonnculligan@gma

Rossana D'Arienzo Institute
Avellino, Italy
e-mail: rdarienzo@isa.cnr.it

Véronique Delcenserie Cana
of Guelph, 43, McGilvray Stre
e-mail: vdelcens@uoguelph.c

Anne Donnet-Hughes Nestlé
Blanc, 1000 Lausanne, Switze
e-mail: anne.donnet@rdls.nest

Chapter 6
Application of Molecular Biology and Genomics of Probiotics for Enteric Cytoprotection

Saloomeh Moslehi-Jenabian, Dennis Sandris Nielsen and Lene Jespersen

6.1 Introduction

The intestinal microbiota plays an essential role in host nutrition, intestinal cell proliferation and differentiation, development of the immune system and acquired responses to pathogens. Alterations in the composition of the intestinal microbiota have recently been linked to various diseases, including inflammatory bowel disease, allergy and diabetes type II (Guarner and Malagelada 2003; Larsen et al. 2010; Lomax and Calder 2009). Probiotics are among the variable indigenous constituents of the gut mictobiota. There are various evidences for different beneficial functions of probiotics and the mechanisms underlying these health effects include both microbe-microbe and microbe-host interactions. Nevertheless, the molecular basis of these mechanisms is still largely unknown. However, recent modern molecular biology based –omics technologies (genomics, proteomics and metabolomics), allowing simultaneous analysis of huge numbers of genes, proteins or metabolites, have revealed insights into understanding the molecular basis for these health promoting activities and increased our knowledge concerning the roles of probiotics in microbe–microbe and host–microbe interactions. The microbial genomic content reflects metabolism, physiology, biosynthetic capabilities of the microorganism, and its ability to adapt to varying conditions and environments. Hence, genome analysis of probiotics will help us to understand their metabolic processes and functionality in human health and well-being. Beside the scientific importance, it will provide a way to improve functional foods, which attracts the interest of the industry and consumers. Consequently, it is of significant concern to exploit the recent studies on the molecular details of the interaction of probiotics with the human host and other microbes. This chapter provides an overview of current progresses in molecular and genomic technologies of probiotics to elucidate the role of these microorganisms in human health and well being. Emphasis will be on the model probiotic

S. Moslehi-Jenabian (✉)
Department of Food Science, Food Microbiology, Faculty of Life Sciences, University of Copenhagen, Rolighedsvej 30, 1958 Frederiksberg, Denmark

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bacteria *Lactobacillus* spp. and *Bifidobacterium* spp., which are phylogenetically distant relatives with different features. When relevant, references will be made to the probiotic yeast *Saccharomyces cerevisiae* var. *boulardii* (van der Aa Kühle and Jespersen 2003), which is widely used as a therapeutic agent.

6.2 Functional Genomics

Functional genomic analyses including whole genome sequencing, genome data mining and comparative genomics have been useful in understanding the influence of genetic content, organization, function and regulation on gut and probiotic functionality as well as to identify the differences and similarities between probiotics since many of the probiotic features are species and even strain dependent. Functional genomic analysis is therefore essential to understand the cellular physiology, metabolic pathways, sensing and signalling in order to clarify mechanisms underlying the probiotic functions of these microorganisms (Klaenhammer et al. 2002). In addition, genomic tools to investigate the gene regulatory networks are important in order to analyse the response of microorganisms to different environmental conditions, especially, the gut-related environmental stresses.

Various studies have investigated the molecular response of probiotics using *in vitro* models mimicking the gut and intestinal environment, for instance acid and bile stress response and tolerance. In many cases, the genes and proteins identified encompass the general stress proteins like GroEL, GroES and DnaK (Frees et al. 2003; Lim et al. 2000; Weiss and Jespersen 2010), and functions related to maintenance of the cell-envelope integrity due to the destructive effect of bile on the cell wall (Bron et al. 2006). It has been shown that these responses are controlled by different regulators that are involved in control of the general stress response (Ferreira et al. 2001, 2003). *In vitro* models are useful for investigating the response of the microorganism to a specific intestinal stress. However, investigation of the full response of a given microorganism will only be achieved using *in vivo* approaches. Therefore, some functional genomic approaches have focused on the study of genetic responses of microorganisms *in vivo* with the goal of identifying bacterial genes that are important during residence in the gut.

Three main strategies have been developed for the identification of genes that are highly expressed *in vivo*, as compared with laboratory conditions: (1) (recombination-based) *in vivo* expression technology ((R-)IVET), (2) signature-tagged mutagenesis (STM), and (3) selective capture of transcribed sequences (SCOTS). These *in vivo* gene identification strategies have been applied for investigation of important genes in bacterial pathogenesis (Mahan et al. 2000). In addition, IVET has recently been employed to identify genes potentially influencing the probiotic functionality in both *Lactobacillus reuteri* 100-23 (Walter et al. 2003) and *Lactobacillus plantarum* WCFS1 (Bron et al. 2004). This approach allows identification of promoter elements that are expressed during *in vivo* transit of probiotic cultures, and reveals the corresponding genes driven by these promoters.

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The DNA microarray technique is a functional genomic approach enabling monitoring the global transcriptional response at the time of sampling and can be used to elucidate the genomic expression of gut-related bacteria in the intestinal tract (Azcarate-Peril et al. 2004; Denou et al. 2007). This approach together with real-time PCR, can be used for quantitative analysis of the transcriptional response of the cells under conditions of interest, e.g., cells that are located at specific intestinal sites (Tao et al. 2006).

Targeted insertional mutagenesis is another alternative to study the gene regions that are presumed to be involved in probiotic traits, and thereby a number of gene regions have been characterized and functionally correlated to important phenotypes (Azcarate-Peril et al. 2004; Velez et al. 2007).

Thus far, functional genomic analyses have revealed a number of interesting features that are generally considered to be important for the roles of probiotics in enteric cytoprotection and health.

6.3 Genes and Molecules Involved in Adaptation of Probiotics to the Gut Niche

Tolerance of probiotics to the stress conditions of the intestinal environment and their adaptation to the gut niche play significant roles in the functionality of probiotics. Different genomic studies have demonstrated the genetic adaptation and metabolic activity of *Lactobacillus* spp. or *Bifidobacterium* spp. in the intestinal environment, which will be discussed in detail in the following sections.

6.3.1 Genes and Molecules Involved in Stress Adaptation

Genes encoding acid resistance responses are essential in tolerance of probiotics to intestinal stress. As an example induction of putative heat shock proteins, i.e., DnaK, DnaJ, GrpE, GroES and GroEL, in acid adapted cells (exposure of cells to sub-lethal adaptive acid conditions) has been shown in *Lactobacillus acidophilus* CRL 639 (Lorca et al. 2002). Recently, a transcriptomic study has shown the expression of stress related genes GroEL, DnaK and ClpP in *L. acidophilus* NCFM after exposure to gastric juice following passage through an *in vitro* gastrointestinal tract model (Weiss and Jespersen 2010). In *L. acidophilus*, the *atp* operon is an acid inducible operon containing 8 genes encoding the various subunits of the F_1F_0-ATPase, a multimeric enzyme either synthesizing ATP using protons or conversely expulse protons out of the cell with the energy provided by ATP hydrolysis. Acidic stress induces expression of the *atp* operon accompanied by an increase in the activity of the membrane-bound enzyme, which results in active expulsion of protons out of the cell and maintenance of cytoplasmic pH under acidic environmental conditions (Kullen and Klaenhammer 1999). Further studies have shown the presence of

four loci contributing to acid resistance in the *L. acidophilus* NCFM genome. The role of the four loci in acid tolerance was proved by insertional mutagenesis in these regions, which resulted in acid sensitive derivatives (Azcarate-Peril et al. 2004). A two-component regulatory system has been found in *L. acidophilus* NCFM playing a role in acid resistance (Azcarate-Peril et al. 2005). Insertional mutagenesis of this two-component regulatory system resulted in an acid sensitive mutant. Whole-genome microarray analysis of the mutant showed that expression of 80 genes including two oligopeptide-transport systems, other components of the proteolytic enzyme system, and a *luxS* homolog was affected by the mutation. The gene *luxS* is involved in AI-2 mediated interspecies quorum sensing (cell-to-cell communication) among bacteria (Federle and Bassler 2003). A transcriptomic study has shown that the *luxS* gene is induced by acidic stress in *L. acidophilus* NCFM and *Lactobacillus rhamnosus* GG and plays a role in the acid stress response in these probiotics. It was observed that in both species, the *luxS* gene was transiently up-regulated after acidic shock (pH 4.0). Acid adaptation of cells attenuated the transcription of the *luxS* gene. Thus, this gene might be important in not only the survival of *Lactobacillus* spp. during the passage through the gastrointestinal tract, but also in the cell-to-cell communication among bacteria in the intestinal microbiota (Moslehi-Jenabian et al. 2009). Genome wide expression analysis experiments using microarrays have revealed that in *L. reuteri* ATCC 55730, the *clpL* chaperone gene (encoding an ATPase with chaperone activity) was involved in the early response to severe acidic shock. This was validated by mutation in *clpL* and the mutant was significantly more sensitive to acidic stress compared to the wild type (Wall et al. 2007).

Genes involved in the tolerance to bile salts are also important for survival of probiotics after passage through the gastrointestinal tract. DNA micro-array analysis of the global transcriptional response of *L. plantarum* WCFS1 against bile revealed 12 bile-responsive gene clusters. Seven of the identified bile-responsive genes and gene clusters encoded typical stress-related functions, including glutathione reductase and glutamate decarboxylase, involved in oxidative and acid stress defence, respectively. Besides, 14 bile-responsive genes and gene clusters were detected that encoded proteins located in the cell envelope, including the *dlt* operon and the F_1F_0 ATPase. The induction of a high number of genes encoding cell envelope functions show the significant effect of bile salts on the integrity and/or functionality of the cytoplasmic membrane and cell wall (Bron et al. 2006). Genes encoding bile salt hydrolases (*bsh*) have been identified in intestinal *Lactobacillus* spp., i.e., *L. acidophilus* NCFM (McAuliffe et al. 2005), *Lactobacillus johnsonii* 100-100 (Elkins et al. 2001) and *L. plantarum* WCFS1 (Lambert et al. 2008a), which shows the ecological adaptation of these species to the intestine and the importance of this trait for *Lactobacillus* spp. in order to colonize the lower gastrointestinal tract.

As for *Lactobacillus* spp., *Bifidobacterium* spp. have developed a system that attempt to maintain their cytoplasmic pH near neutral under acidic stress. In this respect, the proton-translocating ATPase (F_1F_0-ATPase) plays an important role and is encoded by the *atp* operon including nine genes. This multi-subunit enzyme is essential for growth of *Bifidobacterium* spp. under acidic conditions (Ventura et al. 2004). It has been shown that bile induces expression of the F_1F_0-ATPase

and increases the membrane-bound H^+-ATPase activity in *Bifidobacterium animalis*. Comparison of *B. animalis* IPLA 4549 and a mutant with acquired resistance to bile (*B. animalis* 4549dOx) has shown that the bile-resistance mutant was able to tolerate bile by increasing the intracellular ATP reserve and by inducing proton pumping by the F_1F_0-ATPase (Sanchez et al. 2006). Genes encoding bile salt hydrolases (*bsh*) have been detected in *Bifidobacterium longum* BB536 (Shuhaimi et al. 2001), *Bifidobacterium bifidum* ATCC 11863 (Kim et al. 2004), *Bifidobacterium adolescentis* ATCC 15705 (Kim et al. 2005) and a bile tolerant strain of *B. animalis* subsp. *lactis* KL612 (Kim and Lee 2008). In a recent study, two putative multidrug resistance (MDR) transporter genes, i.e. the BL0920 gene from *B. longum* subsp. *longum* NCC2705 and its homolog, Bbr0838 gene, from *Bifidobacterium breve* UCC2003, were induced after exposure to sub-inhibitory concentrations of bile. The expression of the BL0920 gene in *Escherichia coli* conferred resistance to bile, which was probably mediated by active efflux from the cells. This study represents the first identified bifidobacterial bile efflux pump (Gueimonde et al. 2009). Molecular analysis of *B. longum* NCC2705 cells grown in the intestinal tract of mice revealed that different genes and proteins are expressed in the cells for adaptation of *B. longum* to intestinal stress. Among these, EF-Tu (related to the retention or attachment), bile salt hydrolase and stress proteins which protect *B. longum* against the action of bile salts and other destructive components of the gastrointestinal tract have been identified. In addition, it has been found that intestinal growth triggered phosphorylation of LuxS protein (the active form of LuxS) that possibly play a key role in the regulation of quorum sensing between microorganisms of intestinal microbiota (Yuan et al. 2008).

6.3.2 Genes and Molecules Involved in Nutritional Adaptation

The complete sequencing of several *Lactobacillus* spp. genomes has revealed a considerable degree of auxotrophy for amino acids and other cellular components. To compensate for these auxotrophies, *Lactobacillus* spp. have been shown to encode multiple genes for transport and uptake of macromolecules and metabolism of complex carbohydrates (Pfeiler and Klaenhammer 2007). Due to their auxotrophy, *Lactobacillus* spp. will predominantly be present in the ileum, which is a nutritional richer environment than e.g. the colon. Comparing the genome sequence of intestinal isolates of *Lactobacillus* spp. with food isolates indicates a strong degree of niche adaptation. As an example, *Lactobacillus helveticus* DPC 4571, a cheese starter culture, has additional genes for fatty acid biosynthesis and specific amino-acid metabolism, but remarkably fewer cell-surface proteins and phosphoenolpyruvate phosphotransferase systems for sugar utilization compared to *L. acidophilus* NCFM, which is a closely related species well adapted to the intestine. In addition, no functional mucus-binding proteins or transporters for complex carbohydrates are encoded by the *L. helveticus* DPC 4571 genome, indicating adaptation to the milk environment. Whereas *L. acidophilus* that is adapted to the gut ecological niche,

contains functional gene sets such as mucus-binding and cell surface proteins and enzyme complexes that are absent from *L. helveticus* DPC 4571 (Altermann et al. 2005; Callanan et al. 2008), emphasizing the importance of these gene sets for gut adaptation and probiotic functionality. The genes encoding the mucus-binding or cell surface proteins found in the genome of intestinal *Lactobacillus* spp. are predicted to produce secreted proteins such as the S-layer proteins, which are maintained at the cell envelope via either covalent interactions affected by the sortase enzyme or electrostatic interactions, and interact with human intestinal compounds such as extracellular matrix proteins and mucus (Åvall-Jääskeläinen and Palva 2005). These extracellular proteins are essential not only in the interaction of probiotics with host cells or tissues, but also in degradation of complex extracellular carbon sources and have a prominent role in the adaptation to environmental changes and intestinal persistence (Boekhorst et al. 2006; Buck et al. 2005). Analysis of the predicted extracellular proteins of *L. plantarum* WCFS1 has revealed that at least 12 proteins are predicted to be directly involved in adherence to host components like collagen and mucin, and about 30 extracellular enzymes, mainly hydrolases and transglycosylases, predicted to be involved in substrate degradation by *L. plantarum* WCFS1 to maintain the growth in different environmental niches (Boekhorst et al. 2006).

In vivo studies using an IVET strategy based on the *in vivo* selection of an antibiotic-resistant phenotype have shown induction of 3 *in vivo* induced genes that are highly expressed in *L. reuteri* 100-23 during intestinal colonisation in *Lactobacillus*-free mice (Walter et al. 2003). In another study using a recombinase-based-IVET approach in *L. plantarum* WCFS1, 72 different genes were induced during passage through the gastrointestinal tract of conventional mice. Most of these genes were related to carbon and amino-acid metabolism and stress response (Bron et al. 2004). The homologues of many of these genes have been found in intestinal pathogens and associated with survival and adaptation to the gut environment. Whole genome transcriptional profiling of *L. plantarum* during colonization in the cecum of germ-free mice showed up-regulation of genes involved in carbohydrate transport and metabolism, compared with *in vitro* growth conditions. Indeed, the mouse diet had an essential impact on the *in situ* transcriptome of *L. plantarum* WCFS1 (Marco et al. 2009). Similar studies have shown transcription of metabolic genes in *Lactobacillus casei* DN-114 001 (Oozeer et al. 2005) and in *L. johnsonii* NCC533 (Denou et al. 2007, 2008) as adaptation to the environmental conditions in the murine intestine. In the latter species the expression of different sets of genes was observed to depend on its location in the mouse intestine (Denou et al. 2007, 2008). Some *Lactobacillus* spp. can utilize fructo-oligosaccharides which are known as prebiotics (non-digestible oligosaccharides which stimulate growth and/or metabolic activity of probiotics in the host intestine) and thereby interact metabolically with host and other microbes. *L. acidophilus* NCFM metabolise fructo-oligosaccharides by inducing the transcription of a specific transport and degradation system (Barrangou et al. 2003). Similarly, *L. plantarum* WCFS1 have a specific gene expression pattern when exposed to fructo-oligosaccharides, even though it is only able to degrade the short chains of these compounds (Saulnier et al. 2007).

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Contrary to *Lactobacillus* spp., *Bifidobacterium* spp. are autotrophic and are therefore well adapted to growth in an environment with low concentrations of some growth substrates such as the human colon (Ventura et al. 2007a). This property gives them an ecological advantage in the intestinal ecosystem. *Bifidobacterium* genomics has demonstrated their relative broad autotrophy for amino acids, nucleotides, vitamins, and cofactors and has verified their ability to degrade and utilize complex carbohydrates (Schell et al. 2002). Gene clusters coding for complex sugar degradation pathways are abundant in bifidobacterial genomes (Ventura et al. 2007b) and preliminary intestinal transcriptomic studies have shown expression of bifidobacterial genes including oligosaccharide metabolism and vitamin production in the human infant gut (Klaassens et al. 2009). *Bifidobacterium* spp. are also able to hydrolyse different types of fructo-oligosaccharides (prebiotics) and the operons for fructo-oligosaccharide metabolism, specific transporters and hydrolases for oligosaccharides have been identified in the bifidobacterial genome (Gonzalez et al. 2008; Ryan et al. 2005).

6.4 Genes and Molecules Involved in Interaction of Probiotics with Enteropathogens and Gut Microbial Symbionts

Interaction of probiotics with enteropathogens in the intestinal tract involves different mechanisms, including nutrient-based interactions, competition for specific adhesion sites (competitive exclusion) and production of antimicrobial compounds.

6.4.1 Genes and Molecules Involved in Nutrient-Based Interactions

Nutrient-based interactions between probiotic bacteria and other members of the gut microbiota has been proved using germ-free mice models colonized by *Bacteroides thetaiotaomicron* ATCC 29148 (a prominent component of the adult human gut microbiota), *B. longum* NCC2705 and *L. casei* DN-114 001 or combinations of these microorganisms. Whole genome transcriptional profiling of all bacterial species as well as the intestinal epithelium showed that presence of *B. longum* triggered an expansion in the diversity of polysaccharides targeted for degradation by *B. thetaiotaomicron* (e.g., mannose- and xylose-containing glycans), and induced host genes involved in innate immunity. Presence of *L. casei* in this model resulted in an expanded capacity of *B. thetaiotaomicron* to metabolize polysaccharides and increased expression of genes for inorganic ion transport and metabolism, the same results as those observed by *B. longum*. This model showed how a resident symbiont and a probiotic species adapt their substrate utilization in response to each other (Sonnenburg et al. 2006). Indeed, it has been proposed that depletion of iron by *Bifidobacterium* spp. which is an essential nutrient for many intestinal pathogens

(but not for *Lactobacillus* spp.) could be an important factor in the protective effect of *Bifidobacterium* spp. against pathogens in the gut (Kot and Bezkorovainy 1993).

6.4.2 Genes and Molecules Involved in Competitive Exclusion

One of the beneficial roles of probiotics is competition with enteropathogens to adhere to intestinal mucus or competitive exclusion. Therefore, the capacity of probiotic bacteria to adhere to the intestinal mucosa is an important factor for competitive exclusion. Different molecular methods including comparative genomics have revealed a number of genes involved in the adhesion of probiotic *Lactobacillus* spp. to the intestinal tract, such as genes encoding mucus-binding proteins (Altermann et al. 2005; Buck et al. 2005), surface layer proteins (Buck et al. 2005; van Pijkeren et al. 2006), fibronectin-binding proteins (Altermann et al. 2005; Buck et al. 2005), fimbrae (Pridmore et al. 2004), EPS clusters (Altermann et al. 2005; Pridmore et al. 2004), mucus-binding pilli (Kankainen et al. 2009) and mannose-specific adhesion proteins (Pretzer et al. 2005).

Multiple copies of genes encoding mucus-binding proteins have been found in different *Lactobacillus* spp. The predicted mucus-binding proteins are unusually large proteins representing the largest open reading frames (ORFs) in the genome, with relatively low amino acid identity offering considerable sequence variability within surface proteins which are supposed to have important roles in mucus binding (Altermann et al. 2005; Pridmore et al. 2004). Inactivation of genes encoding a mucus-binding protein, a fibronectin-binding protein and a surface layer protein in *L. acidophilus* had a great impact on adherence to intestinal Caco-2 epithelial cells. The adhesion ability was reduced significantly in the mucus-binding protein mutant (65%), the fibronectin-binding protein mutant (76%), and the surface layer protein mutant (84%). However, the decreased adhesion ability in the latter mutant was due to the loss of multiple surface proteins that may be embedded in the S-layer. This study showed that in *L. acidophilus* NCFM multiple cell surface proteins individually have a role in the ability of organism to attach to intestinal cells (Buck et al. 2005). Recently, a transcriptomic study using an *in vitro* gastrointestinal tract model has shown up-regulation of the genes encoding mucin binding protein and fibronectin-binding protein in *L. acidophilus* NCFM after exposure to duodenal juice and bile (Weiss and Jespersen 2010). The important role of mucus-binding pilli in the adhesion ability has been proved in *L. rhamnosus* GG. Comparative genomics of this probiotic bacterium with a starter culture strain *L. rhamnosus* LC705 (exhibiting reduced binding to mucus) revealed one genomic island in *L. rhamnosus* GG which was not present in the other strain and contained 3 pilli encoding genes (spaCBA). Molecular analysis showed that the spaC gene is involved in the adherence of strain *L. rhamnosus* GG to human intestinal mucus and presence of this gene is crucial for the interaction between *Lactobacillus* spp. and host tissues offering a likely explanation of the longer persistence of *L. rhamnosus* GG in the intestinal tract compared to other *L. rhamnosus* strains (Kankainen et al. 2009). Furthermore,

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a mannose-specific adhesin gene (*msa*) which encodes a sortase-dependent cell surface protein has been found in *L. plantarum* WCFS1 (Pretzer et al. 2005). In a pig model, a *msa* knock-out mutant of *L. plantarum* 299v exhibited decreased association with intestinal epithelia and increased jejunal fluid absorption. The wild-type *L. plantarum* 299v induced expression of the gene encoding pancreatitis-associated protein, a protein with proposed bactericidal properties but this feature was not observed in the *msa* mutant that suggests a role for the *msa* gene in the induction of host responses in the pig intestine (Gross et al. 2008). Adhesion to mannose residues is a likely mechanism behind various bacterium-host interactions. Presence of mannose specific adhesin genes and mannose-specific binding properties has been observed in different pathogens such as *E. coli* and *Salmonella enterica* serovar Typhimurium and is the basis for competitive exclusion by the potent probiotic yeast *S. cerevisiae* var. *boulardii* that have mannose containing polysaccharides in the cell wall (Moslehi-Jenabian et al. 2010). *S. cerevisiae* var. *boulardii* prevents bacterial adherence and translocation in the intestinal epithelial cells, due to the capacity of cell wall to bind enteropathogens. The *S. cerevisiae* var. *boulardii* cell wall has been shown to bind enterohaemorrhagic *E. coli* and *S. enterica* serovar Typhimurium (Gedek 1999).

The genome sequence of *B. longum* and other *Bifidobacterium* spp. contain predicted glycoprotein-binding fimbriae and mucus and fibronectin-binding proteins that could be involved in the bacterial adhesion to the intestinal tract (Klaassens et al. 2009; Schell et al. 2002). *B. adolescentis* BB-119 binds to type V collagen at galactose chains as target site via its two cell surface proteins with molecular masses of 36 kDa and 52 kDa and lectin-like activity (Mukai et al. 1997). It has been shown that several species of *Bifidobacterium* produce a compound in the growth media which inhibits binding of enterotoxic *E. coli*-expressing colonization factor antigen II to gangliotetraosylceramice (asialo GMT1 or GA1), a common bacterium-binding structure (Fujiwara et al. 1997).

6.4.3 *Genes and Molecules Involved in Production of Antimicrobial Compounds*

Probiotics are able to interact with enteropathogens by production of bacteriocins (antimicrobial peptides). Bacteriocins are a heterogeneous family of small, heat stable peptides with antimicrobial activity against closely related bacteria (Cotter et al. 2005). Numerous studies have shown the production of various bacteriocins by probiotics with antimicrobial effect against enteropathogens (Corr et al. 2007; Todorov and Dicks 2004; Zamfir et al. 2007). However, in most of these studies, it was not proved that the bacteriocin production was the main reason for inhibitory effect against pathogens by the probiotics. Nevertheless, bacteriocin-based interaction of probiotics and enteropathogens have been proved for *L. salivarius* UCC118 which has the ability to eliminate *Listeria monocytogenes* EGDe and LO28 from a mouse model due to the production of the broad spectrum bacteriocin Abp118 (also

known as salivaricin) (Corr et al. 2007). It was observed that a bacteriocin-negative derivative of *L. salivarius* UCC118 was not able to protect mice against listerial infection. On the other hand, *L. salivarius* UCC118 could not protect the mice against infection with a *L. monocytogenes* derivative expressing the bacteriocin-immunity protein (Corr et al. 2007). This study demonstrates precisely the importance of bacteriocin production by probiotics for the protection against enteropathogens. In addition to bacteriocins, production of lactic acid and H_2O_2 has also been shown to be important measures used by *Lactobacillus* spp. against enteropathogens (De Keersmaecker et al. 2006; Pridmore et al. 2008).

In addition to inhibiting enteropathogens by production of antimicrobial compounds, it has been shown that the probiotic yeast *S. cerevisiae* var. *boulardii* produces two proteins of 54 and 120 kDa being responsible for degradation or neutralisation of bacterial toxins. The 54 kDa protein is a serine protease that decreases the enterotoxic and cytotoxic activities of *Clostridium difficile* by proteolysis of *C. difficile* toxin A and inhibits binding of the toxin to its brush border membrane receptor. *In vivo* studies have shown that oral administration of *S. cerevisiae* var. *boulardii* or its supernatant decreases toxin A-induced intestinal secretion and permeability due to activity of this enzyme (Castagliuolo et al. 1996, 1999; Pothoulakis et al. 1993). The 120 kDa protein has no proteolytic activity but competes specifically with the chloride secretion stimulated by the toxins of *Vibrio cholera* by reducing the cyclic adenosine monophosphate (cAMP) in the intestinal cells (Czerucka et al. 1994; Czerucka and Rampal 1999). Both *S. cerevisiae* var. *boulardii* and *S. cerevisiae* W303 have the ability to protect Fisher rats against cholera toxin (Brandão et al. 1998). *S. cerevisiae* var. *boulardii* also synthesizes a protein phosphatase that dephosphorylates endotoxins such as lipopolysaccharides of *E. coli* 055B5 and inactivates its cytotoxic effects (Buts et al. 2006).

6.5 Genes and Molecules Involved in Interaction of Probiotics with Host

Probiotic-host interactions that benefit the host can be investigated by genome mining and molecular analysis of the bacterial proteins or macromolecules, which might be involved. Probiotics interact with host and confer beneficial effects by means of different mechanisms including metabolic interactions, modulation of mucosal barrier function and modulation of the innate and adaptive immune system.

6.5.1 Genes and Molecules Involved in Metabolic Interactions

Probiotics interact metabolically with the host by modifying the nutritive function of the epithelium. For example, expression of the *ldh* gene encoding lactate hydrogenase by *Lactobacillus* spp. after entrance to the gastrointestinal tract and

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production of lactate by these bacteria, that later could be converted to butyric acid by the activity of some of the colon bacteria such as *Eubacterium hallii* (Duncan et al. 2004; Marco et al. 2007; Oozeer et al. 2005). The production of butyrate is important due to its beneficial effect on the gut epithelium. Butyrate is an important source of energy for the colonic mucosal cells, and it has been suggested to be essential for the maintenance of the colonic epithelium (Hamer et al. 2008).

Bile salt hydrolase activity and metabolism of bile salts is another positive effect of probiotics on the host positively influencing host physiology due to its role in biological processes in the host, i.e. in serum cholesterol lowering. Bile salts have antimicrobial and emulsification properties and probiotics by metabolizing these compounds might affect the amount of fat absorbed by the body. Bile salt deconjugation is the obligatory first reaction in further oxidation and dehydroxylation steps of bile salts by intestinal bacteria, and it includes the production of secondary bile salts, which negatively affects the host by being involved in formation of gallstones and colon cancer. On the other hand, bile salt deconjugation plays a role in mucin production and excretion in the intestinal lumen, and this could affect the nutritional environment encountered by the intestinal microbiota (Lambert et al. 2008b). Besides, bile acids act as local signalling molecules that regulate innate immunity (Inagaki et al. 2006), while re-absorbed bile salts act as signalling molecules involved in regulation of systemic endocrine functions (Watanabe et al. 2006).

6.5.2 *Genes and Molecules Involved in Modulation of Mucosal Barrier Function*

Probiotics preserve the barrier function by different mechanisms such as induction of mucin secretion (Mack et al. 2003), up-regulation of cytoprotective heat shock proteins (Petrof et al. 2004; Tao et al. 2006), enhancement of tight-junction functions (Klingberg et al. 2005; Seth et al. 2008) and modulation of epithelial cell apoptosis (Yan et al. 2007). Some of the signalling pathways involved in these mechanisms have been identified; however, the probiotic effector molecules and the genes encoding them are mostly unidentified.

Induction of mucin secretion is one of the mechanisms by which probiotics strengthen the intestinal barrier functionality. This mechanism is dependent on direct adhesion of probiotics to the epithelial cells as it has been shown by losses in the ability of mucin induction followed by spontaneous mutation in the *adh* gene (involved in adhesion) in *L. plantarum* 299v (Mack et al. 2003).

Increase in the level of inflammatory cytokines and apoptosis of intestinal epithelial cell lead to disruption of epithelial integrity. It has been indicated that *L. rhamnosus* GG prevents cytokine-induced apoptosis in human and mouse intestinal epithelial cells by regulating signalling pathways, i.e., by activation of the anti-apoptotic Akt/protein kinase B and inhibition of activation of the pro-apoptotic p38/mitogen-activated protein kinase by tumor necrosis factor-alpha (TNF-α), interleukin-1 alpha (IL-1 α), or gamma-interferon (IFN-γ) (Yan and Polk 2002). Two

secreted proteins (p75 and p40) have been found in the spent culture of this probiotic bacterium, which activate Akt that has inhibitory effects on cytokine-induced apoptosis and loss of intestinal epithelial cells. Thereby these two proteins promote cell growth in human and mouse colon epithelial cells and cultured mouse colon explants (Yan et al. 2007). Intestinal epithelial tight junction is structured by distribution of different specific proteins such as occludin, zonula occludens (ZO-1, ZO-2, and ZO-3), claudins, E-cadherin, beta-catenin and junctional adhesion molecules (Anderson and VanItallie 1995). Hydrogen peroxide induces the re-distribution of these proteins and cause disruption of tight junctions. Secretory proteins of *L. rhamnosus* GG have been shown to protect intestinal epithelial tight junctions and the barrier function from hydrogen peroxide-induced damages by preserving the distribution of occludin, zonula occludens (ZO-1), E-cadherin, and beta-catenin in the intercellular junctions by a protein kinase C (PKC)- and mitogen-activated protein (MAP) kinase-dependent mechanism (Seth et al. 2008). In addition, an acid and heat stable low-molecular-weight peptide has been found in the spent culture of *L. rhamnosus* GG that induce expression of heat shock proteins (Hsp25 and Hsp72) in intestinal epithelial cells in a time- and concentration-dependent manner (Tao et al. 2006). DNA microarray experiments showed that Hsp72 is one of the genes most highly up-regulated in response to exposure to *L. rhamnosus* GG spent culture. Real-time PCR and electrophoretic mobility shift assays indicated that the *L. rhamnosus* GG spent culture modulates the activity of certain signalling pathways in intestinal epithelial cells by activating MAP kinases. In addition, functional studies suggested that treatment of gut epithelial cells with *L. rhamnosus* GG spent culture protects them from oxidative stress, possibly by preserving cytoskeletal integrity. Inhibition of nuclear factor-kappaB (NF-κB) and induction of heat shock proteins in colonic epithelial cells through proteasome inhibition has also been observed after exposure of the epithelial cells to spent culture of the probiotic mixture VSL#3 (*L. casei, L. plantarum, L. acidophilus, L. delbrueckii* subsp. *bulgaricus, B. longum, Bifidobacterium infantis, B. breve* and *Streptococcus salivarius* subsp. *thermophilus*) (Petrof et al. 2004). Investigation of individual strains of VSL#3 showed that spent culture of *B. infantis* had the highest effect on increasing the TER compared with spent cultures of other probiotic strains in the mixture. *B. infantis* spent culture decreased claudin-2, and increased ZO-1 and occludin expression in T84 cells, which was mediated by changes in MAP kinases. Besides, *B. infantis* spent culture inhibited reduction of TER induced by TNF-α and IFN-γ and re-distribution of tight junction proteins. In addition, oral administration of spent culture reduced colonic permeability in mice (Ewaschuk et al. 2008). These results may account for the anti-inflammatory and cytoprotective effects reported for probiotics and the mechanism of microbial-epithelial interaction. However, more research is needed to identify the unknown factor(s) in spent culture of various probiotics, which exert the protective effects on intestinal epithelial cells mediated by multiple signalling pathways.

Anti-inflammatory effects and lowering the proinflammatory response has also been shown for *S. cerevisiae* var. *boulardii* upon exposure to enteropathogens (Chen et al. 2006; van der Aa Kühle et al. 2005). Production of products with anti-inflammatory effect has also been shown by *S. cerevisiae* var. *boulardii*. This yeast pro-

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6.5.3 *Genes and Molecules Involved in Modulation of the Innate and Adaptive Immune System*

Modulation of the innate and adaptive immune system is another beneficial effect of probiotics on human health. Modulation of the immune system by *Lactobacillus* spp. is mostly ascribed to priming immunoregulatory responses via modulation of dendritic cell (DC) function and induction of regulatory T cells (Rook and Brunet 2005). Numerous studies have shown the immunomodulating effects of probiotics. However, only a few probiotic effector molecules have been identified in this context. One of the probiotic effector molecules which has been identified based on genomics of *B. longum* NCC2705 is a gene encoding a homologue of the eukaryotic-type serine protease inhibitor (serpin) (Schell et al. 2002). Some of the members of the serpin family have the capacity to suppress inflammatory responses through inhibition of human neutrophil elastase (Ivanov et al. 2006).

The ability of probiotic *Lactobacillus* spp. to prime DCs to drive development of regulatory T-cell varies among different species and depends on their recognition of the C-type lectin receptor DC-SIGN (DC-specific intercellular adhesion molecule 3-grabbing nonintegrin), which has an important role in regulatory T-cell stimulation (Smits et al. 2005). It has been shown that mutation in a surface layer protein (SlpA) in *L. acidophilus* NCFM resulted in significant reduction in binding capacity to DC-SIGN. The protein SlpA is the first identified probiotic bacterial DC-SIGN ligand that is functionally involved in the modulation of DC and T cell functions (Konstantinov et al. 2008).

Teichoic acids, and especially lipoteichoic acids (LTA), are one of the major immunostimulatory components of pathogenic Gram-positive bacteria (Morath et al. 2001). Transcriptional profiling of *L. plantarum* WCFS1 during colonization of the cecum of germ-free mice have shown that a set of bacterial genes encoding cell surface-related functions were differentially regulated compared with *in vitro* growth conditions. Genes encoding the biosynthetic pathway responsible for addition of D-alanyl substituent to LTA were down regulated, while the expression levels of teichoic acid biosynthetic genes remained virtually unchanged and it has been proposed that *L. plantarum* WCFS1 modifies its gene expression *in vivo* and minimize the levels of D-alanylated LTA present on the cell surface (Marco et al. 2009). The importance of teichoic acid composition on the cell wall of probiotic *Lactobacillus* spp. in their immunomodulatory effect has been studied using a mu-

tant of *L. plantarum* NCIMB8826 (*dlt*) which was modified in the teichoic acid biosynthesis pathway and presented much less D-Ala in its teichoic acids compared to the wild type strain. This mutation positively influenced the Toll-like receptor 2 (TLR-2)-dependent immunomodulatory properties of *L. plantarum* NCIMB8826 (Grangette et al. 2005). A considerably lower secretion of proinflammatory cytokines by peripheral blood mononuclear cells and monocytes in parallel with a significant increase in IL-10 production was observed after stimulation with the mutant as compared to the parental strain. In addition, the mutant was significantly more protective in a murine colitis model compared to its wild type counterpart. These studies demonstrated that composition of teichoic acids has a great impact on the immunomodulatory effect of *L. plantarum* and this probiotic modifies its gene expression *in vivo* in a way that will increase its immunomodulatory effect. However, it should be kept in mind that *dlt* mutants of other strains of *Lactobacillus* spp. have shown other behaviour. As an example *dlt* mutations in *L. rhamnosus* GG resulted in unaltered immunomodulation and the mutant showed lower survival under intestinal conditions (Velez et al. 2007). Similar results were found for a *L. reuteri* 100-23 *dlt* mutant (Walter et al. 2007). These studies confirm that the immunomodulatory effects of probiotic *Lactobacillus* spp. are strongly species and even strain dependent.

Investigation of the immunomodulatory effect of probiotic preparation VSL#3 has shown that VSL#3 is a strong inducer of IL-10 by DCs from blood and intestinal tissue, and prevents generation of Th1 cells. However, individual strains within VSL#3 presented different immunomodulatory effects on DCs and bifidobacteria strains (*B. longum, B. infantis* and *B. breve*) offered the highest anti-inflammatory effects. Interaction of cell wall components of these *Bifidobacterium* spp. with human intestinal lamina propria mononuclear cells, whole blood, or an enriched blood dendritic cell population showed that *Bifidobacterium* spp. up-regulate IL-10 production by DCs and decrease IFN-γ production by T cells (Hart et al. 2004). In a similar study using a murine macrophage-like cell line, *B. adolescentis* and *B. longum* induced higher secretion of a proinflammatory cytokine IL-12 and TNF-α, compared to *B. bifidum, B. breve,* and *B. infantis*, whereas *B. adolescentis* did not stimulate the production of anti-inflammatory IL-10 as the other tested bacteria, showing that this bacterium is less capable of down-regulating the inflammatory response in macrophage-like cell line (He et al. 2002). However, the signalling pathways and genes involved in these immunomodulatory effects have not yet been identified.

6.6 Conclusion and Future Perspectives

Recent functional genomic analyses and molecular studies have identified some of the genes and molecules offering the health benefits of probiotics. These studies have proved the role of both stress response genes and genes involved in adaptation to new ecological environments being important for the functionality of the probiot-

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ics. In addition, different genes have been found to be involved in adhesion of probiotics to intestinal epithelial cells and production of antimicrobial products, which might be crucial for competitive exclusion and interaction with enteropathogens. More specifically, different genes have been recognized to be involved in mucin secretion, in regulation of the different signalling pathways resulting in anti-inflammatory effects, and in strengthening the epithelial tight junctions, which have protective effects on intestinal epithelial functionality. However, only a limited number of genes have been identified in this regard and additional studies are necessary to uncover all genes involved and to clarify the specific mechanisms at the molecular level. Several studies have detected some genes that are involved in modulating the immune response toward development of T-regulatory cells, but the exact mechanisms and genes are still missing. Altogether, genomics and molecular studies have been proved to be useful to unravel the genes and molecules involved in probiotic functionality and to recognize the regions in the genome that might be interesting for further investigation to identify the exact mechanisms involved in the beneficial effects of probiotics on human health. In this context, effective *in vitro* and *in vivo* models combined with omic approaches and assisted by mathematical models that help exploiting the complex information obtained will facilitate identification of the precise mechanisms by which probiotic microorganisms confer their health benefits. However, considering the biodiversity of these microorganisms in the gut microbiota and the fact that their mode of action is species and even strain dependent, we are still in the beginning of this field of research. Therefore, it is crucial to understand the human gut microbiome to get a comprehensive view of the genetic diversity of the gut residents and the variations in their molecular characteristics and host interactions. This will allow us to find more efficient probiotics and to develop new functional food products and therapeutic agents with improved quality.

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