Bifidobacteria on the spot:

A Genomics Approach on Population Dynamics and Interactions in the Intestinal Tract

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Thesis

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Abstract

This thesis combines comprehensive microarray-based studies contributing to a better understanding of the role of bifidobacteria in relation to the human host. It reviews recently described modes of interaction between bifidobacteria and human gastrointestinal cells and highlights the unique characteristics of the genus *Bifidobacterium* that are indicative for its role in our gut. A microarray platform has been developed that enables genomic comparison of *Bifidobacterium* species originating from our gastrointestinal tract (GIT). Based on the obtained high-resolution data, species-unique genomic sequences could be identified. A large fraction of these predicted genes encode proteins belonging to the bifidobacterial glycobiome. An unique ability of the microarray platform is to zoom in on the strain level. Direct mapping of genomic hybridization patterns was applied on different *B. breve* isolates. This revealed a relatively high genomic variation, testifying for the existence of various subspecies within the species *B. breve*. Clustering of the same hybridization patterns resulted in clear grouping of isolates originating from the same infant, indicating specific niche adaption. Additionally, DNA extracts from *Bifidobacterium* populations from different infant fecal samples were analyzed. This enabled the analysis of the bifidobacterial population dynamics in breast- and formula-fed infants. The applied microarray platform showed the potential to monitor temporal development and effects of dietary regimens. The observed differences in the composition of bifidobacterial populations could be linked to dietary effects. Additionally, mapping of hybridization patterns enabled monitoring shifts in genomic content within one bifidobacterial species in time. Sequence analysis of DNA fragments showing discriminating hybridization characteristics, resulted in the selection of genes that are either conserved or strain-specific within the species *B. breve*. Next to studying genomic variation, transcript profiling experiments in both bifidobacterial cells and human intestinal epithelial cell lines were performed. Analysis of bifidobacterial transcriptional responses provided clear proof of transcriptional activity in bifidobacterial cells isolated from infant feces. To the best of our knowledge, this is the first demonstration of *in situ* activity of bifidobacteria in the human GIT. Furthermore, our results indicate a link between transcription patterns and the infants' diet, as bifidobacteria in fecal samples from breast-fed infants showed differential transcriptional responses in comparison to those in fecal samples from formula-fed infants. Additionally, transcript sequence analysis revealed expression of genes that are homologous to genes known to be involved in folate production, testifying for the production of this important vitamin in early life. Finally, transcriptome analysis

on human intestinal epithelial cells (HIECs) showed species-specific suppression by *B. breve* M-16V of genes upregulated by TNF-α. Other *B. breve* strains showed an extreme mild or no effect on TNF-α stimulation. Although we did not observe complete suppression of the TNF effect, we could show that apoptotic and immune regulatory pathways were affected by incubation with cells of *B. breve* M-16V. In conclusion, the work presented in the thesis, which formed part of a larger IOP Genomics project, contributed to an advanced insight in the interaction between bifidobacteria and the human host. Furthermore, it resulted in the development of genome-based molecular platforms suited for analyzing genomic diversity between and within species, as well as population dynamics in complex microbial communities. We anticipate that the molecular approaches pioneered in this thesis will be instrumental in the further elucidation of the host-microbe interactions in the GIT of human an other animals.

Keywords: *Bifidobacterium*, microarray, comparative genome hybridization, *Bifidobacterium* – cell line interaction, transcriptomics

Contents





Aim and outline of the thesis

Since the day of birth, our body is subjected to a world full of microbes. Some will colonize our intestinal tract and are collectively known as microbiota. A growing body of scientific evidence supports a link between our intestinal microbiota and health status. This has caused significant interest in the host interactions of our commensal microbiota. Various studies have predicted an important role of the human commensal bacteria from the genus *Bifidobacterium* in the development of the human intestinal microbiota. Furthermore, bifidobacteria are abundant intestinal inhabitants throughout our entire healthy life. The present genomics era has led to a rapidly growing set of global and high resolution molecular tools that have been instrumental in advancing our understanding of complex biological processes. This thesis describes research that started as an IOP Genomics project on applying these genomics approaches to study the interplay between bifidobacteria and the human host. It describes microarray-based molecular techniques that revealed significant insight in the development, divergence and activity of *Bifidobacterium* populations in the intestinal tract and their interactions with human intestinal epithelial cells.

Chapter 1 provides an overview of the specific characteristics of bifidobacteria and lactobacilli, focusing on the interplay with the human host cells. Based on recent omics-based developments, it reviews the scientific findings on host–microbe interactions. Moreover, is presents a basis for defining the complete set of interacting components, collectively known as the interactome.

The construction and application of the *Bifidobacterium* mixed-species (BMS) microarray was described in **Chapter 2**. DNA fragments originating from *Bifidobacterium* species mostly found in the human intestinal tract, were arrayed on a glass slide. The goal of this work was to design a tool that enables detailed genome analysis of *Bifidobacterium* species without the need of unraveling their whole genome sequence first. The BMS-array served as a high-resolution diagnostic tool that facilitated the detection of strain- and species-specific characteristics of bifidobacteria. Moreover, its application to cognate *Bifidobacterium* spp. revealed this genus to contain species-specific diagnostic nucleotide sequences that are mainly predicted to code for its complex glycobiome next to functions involved in DNA metabolism.

A comprehensive analysis of 20 infant fecal isolates of *Bifidobacterium breve* by comparative genome hybridization using the BMS-array is presented in **Chapter 3**. The study should provide information about the applicability of the microarray platform as a tool for extensive genome comparison of isolates within one species

of bifidobacteria. A high degree of genomic variation between the isolates derived from different infants was revealed, testifying for the existence of various subspecies within the species.

A further application of the BMS-microarray platform is discussed in **Chapter 4**. The objective of the presented experiment was to test the usability of this approach for the screening of complex microbial samples. A proof-of-principle study was performed to address the composition and development of bifidobacterial populations in fecal samples, based on extracted DNA. The samples were collected periodically over a period of two years since birth and derived from infants that were breast-fed, standard formula-fed or received a prebiotic formula during their weaning period. *Bifidobacterium* population analyses were performed by direct mapping of genomic hybridization patterns. This study shows that the described platform can be used for comparative genome hybridizations and serves as a culture-independent tool to analyze the dynamics of *Bifidobacterium* populations. It was not necessary to enrich the DNA samples for bifidobacterial DNA, obviating the need for PCR amplification that may introduce errors. The obtained high-resolution data enabled us to zoom in at the species and strain level, revealing an obvious link between diet and *Bifidobacterium* populations.

Next to genomic comparisons, the BMS-microarray was also applied in determining the transcriptional responses of bifidobacteria in breast- and formula-fed human infants (**Chapter 5**). The goal of this work was to increase insight in the bifidobacterial molecular processes in relation to our diet. Total RNA was extracted from rapidly processed infant fecal samples. The obtained RNA samples were labeled and hybridized on the BMS microarray in order to analyze differences in bifidobacterial gene expression. A selection of approximately 270 clones that showed the most prominent hybridization with the extracted mRNA were further analyzed. The largest fraction (14%) was predicted to be involved in carbohydrate metabolism. This study showed a significant impact of diet on the transcriptional responses of bifidobacteria in breast- and formula-fed infants.

To accommodate gain of insight in the molecular basis for intestinal host–microbe interactions was the aim of the study described in **Chapter 6**. The genome-wide transcriptional response of human epithelial intestinal cells exposed to cells of *B. breve* was determined. To select an appropriate test system reflecting inflammatory conditions, the responsiveness to TNF-α was compared of T84, Caco-2 and HT-29 human intestinal epithelial cell lines. The highest TNF-α response was observed in HT-29 cells and this cell line was selected for exposure to the *B. breve* strains M-16V, NR246 and UCC2003. Transcriptome analysis of the exposed HT-29 cells indicated that *B. breve* strains modulate gene expression under inflammatory conditions in a

strain-specific manner.

Finally, **Chapter 7** provides a summary and general discussion of the work presented in this thesis. The correlation between bifidobacteria and human health is discussed. Furthermore, it focuses on the applicability of the BMS-microarray platform and its potential future role in research on the intriguing interaction of microbe and host.

Chapter 1

Interactomics in the Human Intestine – Lactobacilli and Bifidobacteria Make a Difference

Rolf J. Boesten and Willem M. de Vos

Scientific evidence that supports a correlation between our intestinal microbiota and health status has caused significant interest in microbe–host interaction studies. It has generated a paradigm shift from analyzing pathogens to that involving commensal and probiotic bacteria. This review summarizes the interaction mechanisms described for lactobacilli and bifidobacteria based on recent omics-based developments. This information is expected to provide new avenues for further unravelling the set of interactions that includes the interactome of microbial and host cells.

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INTRODUCTION

The microbial world within us includes an astonishing array of intestinal microbes, collectively known as microbiota, consisting of more than 1000 species and dominated by gram-positive bacteria (Eckburg et al., 2005; Rajilic-Stojanovic et al., 2007; Zoetendal et al., 2006). The dynamics of this microbial diversity in time and space has attracted considerable attention and confirmed earlier notions that the density and architecture of the microbial communities differ at the various locations in the intestinal tract. The effect of the microbiota on the intestinal tract is underlined by its important and specific metabolic, trophic, and protective functions (Guarner and Malagelada, 2003). Most notable differences in the microbiota are found when the ileum and colon are considered, where there are large differences in intestinal anatomy, nutrient processing and food transit time (see Fig.1). Recent molecular sequence-based observations indicate that the human host is colonized by a core group of microbes that show significant stability in time and are likely to have specific interactions with the human host and other microbes (Rajilic-Stojanovic et al., 2007). Some of these interactions have been studied at the molecular level in mammalian model systems, notably in germ-free mice colonized by *Bacteroides thetaiomatmicron* (Hooper et al., 2002; Sonnenburg et al., 2005; Sonnenburg et al., 2006). *Bacteroides* spp. are abundant gram-negative intestinal commensals that in some cases are also pathogens in animal species, including human. These developments extended the paradigm of pathogen-host interactions and focus the interest on commensal bacteria that have developed symbiosis in the intestinal tract (Dethlefsen et al., 2007). Specific attention has been given to *Lactobacillus* and *Bifidobacterium* spp., notably the species that are among the first colonizers of the human intestinal tract (Favier et al., 2002; Heilig et al., 2002). Their location, abundance and genomic potential of these gram-positive bacteria differs markedly, with lactobacilli predominantly present in the ileum and bifidobacteria in the colon (see Fig. 1). Hence it is of significant interest to compare and contrast the rapidly accumulating information on the molecular details of their interaction with the human host and other microbes. This is done here from an -omics perspective as it provides the basis for unravelling the complete set of host–microbe interactions that includes all interacting components, collectively known as the interactome.

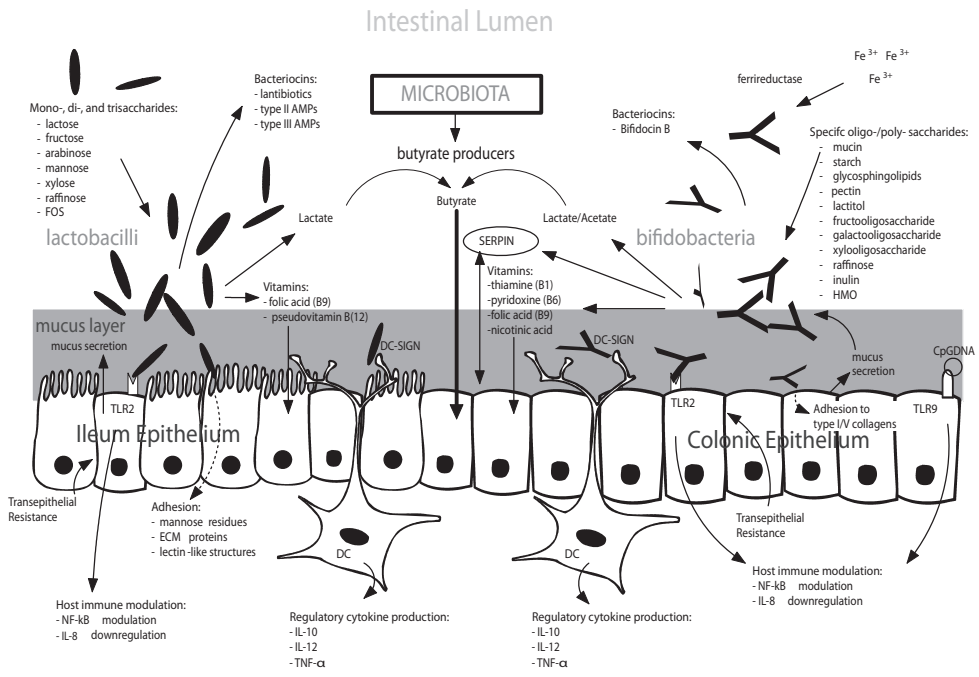

Figure 1. Schematic overview of the interactions between *Lactobacillus* spp. and the human ileum (left) and *Bifidobacterium* spp. and the human colon (right). For a further explanation see text.

THE *LACTOBACILLUS* INTERACTOME

The impact of *Lactobacillus* spp. on the human host has received considerabe attention, notably as many of their genomes have been determined and some strains are marketed as probiotics (De Vos et al., 2004; Saxelin et al., 2005). It is known that lactobacilli are notably prevalent in the human ileum but their abundance has been overestimated (Vaughan et al., 2005). This has been recently confirmed in molecular diversity studies that also showed recovery from the colonic mucosa (Heilig et al., 2002; Zoetendal et al., 2002). Hence, the present picture of the *Lactobacillus* interactome that is visualized here in the ileum, is necessarily an oversimplification, which – as all schematic representations – needs to be interpreted with care (Fig. 1).

Predictions based on the complete genome sequence of *L. plantarum*, the first *Lactobacillus* spp. analyzed at the genomic level, confirm the significant interaction with the environment as evidenced by the panoply of secreted proteins and two-component sensory transduction systems (Kleerebezem et al., 2003). Subsequent genomic analysis of other intestinal and probiotic lactobacilli confirmed this specifc

environmental interactions (Klaenhammer et al., 2005; Makarova et al., 2006; van Pijkeren et al., 2006). These and other genetic studies also reinforced the metabolic interactions that include the production of folic acid and pseudovitamin B12, a B12 derivative, that are encoded by specific gene clusters in *L. plantarum* and *L. reuteri*, respectively (Santos et al., 2008; Wegkamp et al., 2004).

The annotation of more than a dozen *Lactobacillus* genomes resulted in the identification of various sets of secreted proteins that are predicted to interact with human compounds, including extracellular matrix (ECM) proteins and mucus. The majority of these includes secreted proteins with a likely structural function that are retained at the cell envelope via covalent interactions effected by the sortase enzyme or mainly electrostatic interactions, such as the S-layer proteins (Avall-Jääskeläinen and Palva, 2005; Marraffini et al., 2006). However, none of the predicted interactions of these *Lactobacillus* proteins has been experimentally determined, apart from that of a widely distributed mucus binding proteins (Boekhorst et al., 2006). Recently, however, several sortase substrates have been found to specifically interact with the human host. A mannose-specific adhesion was identified in *L. plantarum* that binds to the mannose residues that decorate human glycoproteins (Pretzer et al., 2005). As these are also receptors for various pathogens, such as enterotoxic *Escherichia coli* (ETEC), this may form the molecular basis for competitive exclusion, one of the probiotic mechanisms. Moreover, recent evidence also indicates specific signalling of a *L. plantarum* sortase substrate and *L. acidophilus* S-layer with the DC-SIGN receptor of dendritic cells (DC) that are abundant in the ileum (Konstantinov et al., 2008; Marco et al., 2006). The observation that sortase substrates play an important role in the *Lactobacillus*-host interactome stems from observations in their expression in human and mouse models that also pointed to the involvement in the intestinal persistence of a *L. plantarum* gene for a sortase substrate (Bron et al., 2007). A last specific interaction that has been described recently, includes the signalling of specifically decorated lipotechoic acids (LTA) of *L. plantarum* to TLR2, one of the Toll-like receptors (TLRs) that enable the immunosensory cells of the host to discriminate between pathogen and commensal (O'Hara et al., 2006). It has been observed that reducing the incorporation of D-alanine in the *L. plantarum* LTA changes the response of colonized conventional mice from pro-inflammatory to anti-inflammatory (Grangette et al., 2005).

The transcriptome analysis of *Lactobacillus* spp. also allowed for further detailing the metabolic interactions with substrates and other microbes. Some lactobacilli can utilize oligosaccharides including those not metabolized by human, such as. fructo-oligosaccharides (FOS). *Lactobacillus acidophilus* was found utiliuze FOS by inducing the transcription of a specific transport and degradation system (Barrangou et al.,

2003). Similarly, *L. plantarum* showed specific gene expression but of other genes when exposed to FOS, but only degraded the short chains of FOS (Saulnier et al., 2007). These data are compatible with the possibility that at least some lactobacilli have metabolic functions in the colon where most non-digestable oligosaccharides are thought to be accumulating.

Post-genomic studies also provided insight in the quorum sensing systems that in gram-positive bacteria involve specific communication peptides. These sensing systems provide a coordinated response at high cell densities that result in specific survival strategies. Reports of multiple quorum sensing systems found in intestinal lactobacilli underline the importance of intercellular communication and its ecological impact as many are involved in the production of bacteriocins or other anti-microbial peptides (AMP) (Sturme et al., 2002). Experimental evidence for the impact of a *L. salivarius* bacteriocin on *Listeria monocytogenes* was recently established in a mouse model and if applicable to human, may constitute another probiotic mechanism (Corr et al., 2007). In *L. plantarum*, a quorum sensing system was recently discovered that is involved in the production of a novel cyclic peptide that appears to control exopolysaccharide (EPS) production and hence the cell surface properties that affected biofilm formation and adhesion to surfaces (Sturme et al., 2005).

THE *BIFIDOBACTERIUM* INTERACTOME

Members of the genus *Bifidobacterium* are numerically abundant commensals that populate the human intestinal tract since birth. Next to their ability to produce a variety of vitamins, degrade a multitude of milk, mucus and other dietary oligosaccharides, the described immune modulating and adhesion capacities testify for a specialization of bifidobacteria to a symbiotic relationship with the human host as is discussed below (Fig. 1).

With the exception of species isolated from human dental caries, sewage or insects, the majority of *Bifidobacterium* spp. are found in the mammalian intestinal tract. Bifidobacteria are known to be host-specific and studies in the human intestinal tract have shown a temporal development of the major species that include *B. adolescentis*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. longum* (Ventura et al., 2004). While dominant in early life, culture-dependent methods showed bifidobacteria to comprise up to 10% of the human adult intestinal microbiota but latest molecular estimations show lower numbers but this partly is due to the discovery of new and not-yet cultured bifidobacteria (Ben-Amor et al., 2005; Klijn et al., 2005).

A major source of energy is the fermentation of non-digestible dietary residues

and endogenous mucus by the intestinal microbiota. Moreover, some bifidobacteria are specialized in degrading human milk oligosaccharides (HMO) such as *B. longum* subsp. *infantis*, previously known as *B. infantis* (Ward et al., 2006). A specific and dedicated degradation system for human oligosaccharides was recently discovered and includes a gene cluster encoding a transporter and novel hydrolase (Kitaoka et al., 2005). Remarkably, expression analysis in baby fecal communities established this gene cluster to be specifically upregulated in the intestinal tract of young infants that are abundantly colonized by bifidobacteria (Klaassens et al., 2009). Bifidobacteria can also utilize sialic acid-containing complex carbohydrates in mucin, glycosphingolipids, and human milk (Schell et al., 2002; Ventura et al., 2004; Ventura et al., 2007). Thus, the mammalian host supplies substrates for its intestinal commensals including bifidobacteria, in a specific symbiotic relationship, that includes for instance the production of a large array of essential vitamins (see Fig.1). Although only the genome of *B. longum* NCC2705 has presently been published, its annotation predicts complete pathways for the synthesis of folic acid, thiamine and nicotinate (Schell et al., 2002). However, it is likely that the analysis of other bifidobacterial genomes will reveal the genetic information for the synthesis of other relevant vitamins (Ventura et al., 2007).

It is has been suggested that bifidobacteria owe their specific ecological success to their capacity to metabolize complex carbohydrates. Hence, gene clusters coding for complex plant-derived sugar degradation pathways are abundant in the genomes of *B. breve* and *B. longum* biotype *longum* (Ventura et al., 2007). Similarly, the first intestinal metatranscriptomics studies also showed that a large set of bifidobacterial genes that were predicted to code for sugar degradation pathways were expressed in the human infant gut (Klaassens et al., 2009).

The genome sequence of *B. longum* and that of other bifidobacteria predicts the production of 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). *Bifidobacterium adolescentis* strains adhere to immobilized type I and type V collagens, but not to other ECM proteins, and its interaction involves two cell surface proteins that bind galactose chains by their lectin-like activity (Mukai et al., 1997). It seems that multiple adherence factors from the bacterial surface, including EPS, are responsible for the adhesion of bifidobacteria to the intestinal mucosa but no molecular data have been presented (Ruas-Madiedo et al., 2006).

It has been suggested that bifidobacteria provide yet another level of symbiosis with the host by competing with pathogens but only limited information about the interacting molecules is known. Some bifidobacteria have been reported to produce

antimicrobials as a defence against pathogenic bacteria and an infant isolate was shown to produce bacteriocin-like peptides with strong inhibitory activity against *L. monocytogenes* (Moroni et al., 2006). However, molecular details are lacking and it appears that only a limited number of bacteriocins have been found to be produced by *Bifidobacterium* spp. (Ventura et al., 2007). Competitive binding has been reported between several species of *Bifidobacterium* spp. BL2928 and ETEC-expressing colonization factor antigen (CFA) II, to gangliotetraosylceramice (asialo GMT1 or GA1), a common bacterium-binding structure (Fujiwara et al., 1997). The factor produced by BL2928 bacteria in the culture supernatant fluid that inhibits the binding of *E. coli* to GA1 is a >100 kD proteinaceous substance(s) that awaits further characterization. Furthermore, bifidobacteria have developed a mechanism that enables them to efficiently harvest iron, which is an essential nutrient for many intestinal pathogens but not for lactobacilli. This is realized by a membrane-bound ferrireductase, which ensures the conversion of iron from ferric to ferrous state at the cell surface. This biologically available form of iron is subsequently transported by a divalent metal permease, which requires a functioning ATPase and a proton gradient (Kot and Bezkorovainy, 1993).

A recent genome-based finding is the discovery of the production by *B. longum* of a human-like serine proteinase inhibitor (SERPIN) (see Fig. 1). This protein was shown to efficiently inhibit eukaryotic elastase-like proteases with a stoichiometry of inhibition close to 1 (Ivanov et al., 2006). Bifidobacteria may encounter both pancreatic elastase and neutrophil elastase in their natural habitat and protection against exogenous proteolysis may play an important role in the interaction between these commensal bacteria and their host. If so it is likely that the SERPIN action also takes place in the ileum where the human serine proteases are abundant, implying that bifidobacteria also colonize the ileum.

Several bifidobacteria have been found to interact with intestinal cells and modulate the host immune system. However, the signalling pathways have not yet been discovered although it is likely that similar pattern recognition receptor (PRR) systems operate as in the interaction with lactobacilli, including DC-SIGN and TLRs (Furrie et al., 2005; Hart et al., 2004). Notable TLR candidates are TLR2 and TLR9 that react to LTA and CpG DNA, respectively. As bifidobacteria have a high G+C content – in contrast to the low G+C lactobacilli – the latter signalling via TLR9 is expected to occur. *Bifidobacterium adolescentis* and *B. longum* induced in a murine macrophage-like cell line, a more pro-inflammatory cytokine secretion, IL-12 and TNF-α, than did *B. bifidum*, *B. breve*, and *B. infantis*. In contrast, *B. adolescentis* did not stimulate the production of anti-inflammatory IL-10 as the other tested bacteria did (He et al., 2002).

HOST- MICROBE INTERACTOMICS: COMPARE AND CONTRAST

It is evident that there are several differences between the interaction components of *Lactobacillus* and *Bifidobacterium* spp. with the human host (Fig. 1). Notably, these relate to the differences between the proteins encoded by these important bacterial groups. So far no major differences are due to the colonization of different sites of the intestine, which is reassuring as the strict separation between ileum and colon is an oversimplification as explained above. There are many metabolic differences between lactobacilli and bifidobacteria and some of these are indicated (Fig. 1). However, they have in common that oligo- or polysaccharides are converted into lactic and acetic acid – these acids are subsequently impacting the microbiota and can be used, directly or indirectly by butyrate-producing bacteria.

The comparison between *Lactobacillus* and *Bifidobacterium* interaction systems is complex as only few comparative studies have been done, and none in human. Moreover, the knowledge base between the two bacterial groups is different as is illustrated by the fact that presently there are more than a dozen published *Lactobacillus* genomes but only a single *Bifidobacterium* genome. Moreover, various advanced studies have been carried out with *Lactobacillus* spp., including the identification of *L. rhamnosus* GG secreted proteins that upregulate gene expression via MAPK in intestinal cells (Tao et al., 2006), the global ileal transcriptome response to consumption of *L. rhamnosus* GG (Di Caro et al., 2005) or perfusion with *L. plantarum* (Troost et al., 2008). Similar molecular studies with bifidobacteria are lacking and only the interaction of *B. longum* and germ-free mice has been reported (Sonnenburg et al., 2006). However, the interest in bifidobacteria is expanding rapidly, notably in view of their role in early life and new genomes will appear soon, paving the way for molecular studies on the interacting components. This level of sophistication is essential for concluding whether lactobacilli and bifidobacteria really make a difference.

Chapter 2

A *Bifidobacterium* mixed-species microarray for high-resolution discrimination between intestinal bifidobacteria

Rolf J. Boesten, Frank H.J. Schuren, and Willem M. de Vos

A genomic DNA-based microarray was constructed containing over 6,000 randomly cloned genomic fragments of approximately 1-2 kb from six mammalian intestinal *Bifidobacterium* spp., including *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. catenulatum*, *B. longum* and *B. pseudolongum*. This *Bifidobacterium* Mixed-Species (BMS) microarray was used to differentiate between type-strains and isolates belonging to a set of nine *Bifidobacterium* spp. Hierarchical clustering of genomic hybridization data confirmed the grouping of the *Bifidobacterium* spp. according to the 16S rRNA-based phylogenetic clusters. In addition, these genomic hybridization experiments revealed high homology between the type-strain *B. animalis* subsp. *lactis* LMG 18314 and *B. animalis* subsp. *animalis* LMG10508 (79%) as well as between the type strains *B. longum* biotype *longum* LMG13197 and *B. longum* biotype *infantis* LMG8811 (72%). Nevertheless, discrimination between these species was possible due to the high-resolution output of the BMS-array. In addition, it was shown that the BMS-array could be used for assigning unknown *Bifidobacterium* isolates to a species group. Finally, a set of 54 diagnostic clones for *Bifidobacterium* identification was selected and sequenced to advance the understanding of the species-related differences. Remarkably, a large fraction (31%) of these was predicted to encode proteins that belong to the bifidobacterial glycobiome and another 11% had functional homology with genes involved in the protection against foreign DNA. Overall, the BMS-microarray is a high-resolution diagnostic tool that is able to facilitate the detection of strain- and species-specific characteristics of bifidobacteria.

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INTRODUCTION

Bifidobacterium species are known to be among the first and most dominant gut inhabitants in our early life (Favier et al., 2002; Xu and Gordon, 2003; Vaughan et al., 2005). Their main habitat is the intestinal tract of humans and other animals. Bifidobacteria are reported to derive their specific ecological success from their capacity to metabolize complex carbohydrates (Ventura et al., 2007b). This characteristic forms the basis for various claims on the prebiotic effect of dietary oligosaccharides that selectively stimulate growth of bifidobacteria in the human colon (for a review see Gibson *et al*., 2005) (Gibson et al., 2007). However, there is only limited information on the effect of bifidobacteria on human health (Guarner and Malagelada, 2003; Klijn et al., 2005) in spite of the fact that some *Bifidobacterium* strains are also marketed as probiotics (Juntunen et al., 2001; Parvez et al., 2006; Mohan et al., 2006).

The *Bifidobacterium* genus presently includes 34 species that are characterized by specific host-relations and can be grouped into six phylogenetic clusters (Ventura et al., 2006). Two of these contain *Bifidobacterium* spp. found in the human intestinal tract, including the *B. adolescentis* and *B. longum* clusters. Another relevant intestinal species is *B. bifidum*, which only includes human isolates but does not belong to any of the clusters but has a separate branch (Ventura et al., 2006). A fourth one, the *B. pseudolongum* cluster, is of interest in relation to the human gut because among others it contains *B. animalis* subsp. *lactis* that is isolated from fermented milk and is widely marketed as a human probiotic (Wall et al., 2007). As a consequence, this species is increasingly found in human fecal samples that are also known to contain *B. adolescentis*, *B. catenulatum*, *B. longum*, and *B. bifidum* (Matsuki et al., 1999). Although, fecal samples of healthy infants are significantly different (Matsuki et al., 1999) and apparently affected by the health status (Salminen et al., 2005; Marchesi and Shanahan, 2007), the *Bifidobacterium* spp. composition in the intestinal tract of healthy adults is stable over time (Zoetendal et al., 1998; Matsuki et al., 2004). Sequence analysis of 16S rRNA and 16S-23S spacers (Leblond-Bourget et al., 1996; Kwon et al., 2005), but also of highly conserved genes such as tuf, recA (Ventura and Zink, 2003) and groEL (HSP60 family) gene sequences (Jian et al., 2001), have been used for elucidation of the *Bifidobacterium* taxonomy. However, controversy exists with respect to the phylogenetic position of some industrial strains such as *B. animalis* that is also described as *B. lactis* (Ventura and Zink, 2002). Furthermore, the taxonomic classification of *B. longum*-related strains is still not completely resolved (Mattarelli et al., 2008).

While the number of sequenced genomes is rapidly accumulating, so far only the *B. longum* NCC2705 genome has been sequenced and annotated completely (Schell et al., 2002). Complete genomes of a variety of other *Bifidobacterium* species have reported to be completed (Liu et al., 2005; Ventura et al., 2007a), including those of *B. animalis*, *B. breve* and *B. adolescentis*, but only that of the latter one has been deposited in a public database. This may be due to their application potential, as is illustrated by the discovery of a serpin-encoding gene in the *B. longum* genome that has the capacity to inhibit eukaryotic elastase-like serine proteases and may play an important role in the interaction between these commensal bacteria and their host (Ivanov et al., 2006).

One of the outstanding characteristics of the bifidobacterial genome is the presence of numerous genes that allow acquisition, transport and metabolism of a broad range of complex dietary polysaccharides that can not be processed by the human host. This arsenal of genes encoding proteins involved in carbohydrate depolymerization, uptake and metabolism is known as glycobiome. Over 8% of the annotated genes of *B. breve* UCC2003 and *B. longum* biotype *longum* NCC2705 encode enzymes involved in the carbohydrate metabolism (Ventura et al., 2007b). Some sugar-degrading abilities are restricted to certain species or strains of a particular species (Ward et al., 2007; LoCascio et al., 2007). Comparative genome hybridization suggested that these sugar-degrading genes and those that encode restriction modification systems belong to the variable clusters within the *Bifidobacterium* genome that have been acquired via horizontal gene transfer (Schell et al., 2002; Ventura et al., 2007b). In addition, mobile elements such as prophage-like elements and plasmids, although not ubiquitous in bifidobacteria, can also cause variation between strains and species (Ventura et al., 2007a).

In addition, a *B. longum* and an unpublished *B. breve* microarray have been used as genotyping tools in comparative genome hybridizations (Parche et al., 2007; Ventura et al., 2007a). To provide an alternative in absence of published genome sequences, we developed an approach based on random clone-based microarrays (Vlaminckx et al., 2007; Leavis et al., 2007). This approach is ideally suited for determination of genomic differences of not or not yet completely sequenced genomes. A genomic DNA-based microarray was constructed, containing random-clones of six *Bifidobacterium* species relevant for the intestinal tract of humans. In this study we show the applicability of this microarray as a diagnostic tool in the analysis of diversity and function of bifidobacteria.

MATERIALS AND METHODS

Bacterial strains, culturing, DNA isolation and 16S rRNA sequencing

The origin and other information of the used bacterial strains is summarized in Table 1. All *Bifidobacterium* strains were grown anaerobically in MRS broth (Difco, Detroit, USA) supplemented with 0.05% L-cysteine hydrochloride monohydrate (Sigma-Aldrich, Steinheim, D) and incubated at 37°C. Genomic DNA (gDNA) was extracted using a protocol based on enzymatic lysis (Baess, 1974). Additionally, after precipitation the supernatant was discarded and the pellet was washed using 250 µl of 70% ethanol solution and dissolved in 100 µl 1 x TE/RNase (10 mg/ml) (Sigma-Aldrich, Steinheim, D) solution. The mixture was incubated at room temperature for 30 min and subsequently purified by ethanol precipitation and resuspended in 100 µl 1 x TE buffer. The DNA was quantified using the Agilent 2100 Bioanalyser (DNA 12000 kit, Agilent Technologies, Palo Alto, CA).

All strains were characterized by sequence analysis of their 16S rRNA genes which were obtained via PCR amplification using two DNA primers complementary to universally conserved regions of 16S rRNA. These primers were synthesized by Isogen Bioscience, Maarssen, The Netherlands - the two primers complement positions 8 to 30 (primer 8FE; 5'-CAGAGTTTGATCHTGGYTCAG), and 537-556 (primer 556R; 5'-CTTTACGCCCARTRAWTCCG) (Baker et al., 2003). Per amplification reaction 5 µl GoldStar buffer (10 x, Eurogentec, Seraing, BE), 5 µl dNTP solution (2 mM), 3 µl $MgCl_2$ sollution (25 mM), 1 µl 8FE primer (50 pmol/µl), 1 µl 556R primer (50 pmol/µl), 0.4 µl Gold Star DNA polymerase (4U/µl, Eurogentec, Seraing, BE), 2.5 µl dimethyl sulfoxide (99.9%, Sigma-Aldrich, Steinheim, D), 27 µl milliQ H_2O, and 5 µl DNA (2 ng/µl) was combined. Amplification was carried out using a DNA thermal Cycler 480 (Perkin Elmer Cetus, Wellesley, USA) according to the following program, 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 2 min ending with one cycle of 72°C for 4 min. The amplified 16S rRNA genes were loaded on a 1% agarose/1 x TAE gel for electrophoresis. 16S rRNA bands were isolated from gel and purified using a QIAquick Gel Extraction Kit according to the manufacturer's protocol (Qiagen Benelux B.V., Venlo, NL). For sequencing we used the ABI Prism® BigDye™ Terminator V3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Maarssen, NL) and the standard procedures recommended by the manufacturer. Sequencing reactions were performed using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Maarssen, NL). The 16S rRNA gene sequences were aligned and their relationships were assessed using the 3.1 version of MEGA (Molecular Evolutionary Genetics Analysis) software (www.

Table 1. Bacterial names, sources and references
–[a] not used for the construction of microarrays; * based on 16S rRNA sequence; TNO Quality of Life, Zeist[1]; Laboratory of Microbiology, Wageningen University[2]

Strain	Code	Subarray	Collection	Origin	Reference
B. adolescentis LMG 10502[T]	ADO	SA3	TNO[1]	Infant and adult feces	Reuter, 1963
B. animalis subsp. *animalis* LMG 10508[T]	ANI	SA3	TNO[1]	Rat, chicken, rabbit and calf feces	Scardovi and Crociani, 1974
B. bifidum LMG 11041[T]	BIF	SA3	TNO[1]	Infant and adult feces	Orla-Jensen, 1924
B. breve LMG 13208[T]	BRE	– [a]	TNO[1]/ WUR[2]	Infant feces	Reuter, 1963
B. breve NCIMB 8807/ UCC2003	BRE NCIMB8807	– [a]	WUR[2]	Infant feces	Reuter, 1963; Leahy *et al*., 2005
B. catenulatum LMG 11043[T]	CAT	SA3	TNO[1]	Infant and adult feces	Scardovi and Crociani, 1974
B. longum biotype *infantis* LMG 8811[T]	INF	– [a]	TNO[1]/ WUR[2]	Infant feces and vagina	Reuter, 1963; Sakata S *et al*., 2002
B. animalis subsp. *lactis* Bb-12	LAC Bb12	– [a]	WUR[2]	Fermented milk	Masco *et al*., 2004, Chr. Hansen, Hørsholm, Denmark
B. animalis subsp. *lactis* LMG 18314[T]	LAC	– [a]	WUR[2]	Fermented milk	Meile *et al*., 1997; Masco *et al*., 2004
B. longum LMG 13197[T]	LON	SA2/ SA3	TNO[1]	Infant and adult feces	Reuter, 1963
B. longum NCC 2705	LON NCC2705	– [a]	WUR[2]	Infant and adult feces	Reuter, 1963; Schell et al., 2002
B. pseudolongum subsp. *pseudolongum* LMG 11571[T]	PSE	SA1/ SA3	TNO[1]	Piglet, rat, chicken, calf feces and rumen	Yaeshima *et al*., 1992
B1 - *B. breve* *	B1	– [a]	WUR[2]	Human infant feces	This chapter
B2 – *B. adolescentis**	B2	– [a]	WUR[2]	Human infant feces	This chapter
B3 – *B. bifidum**	B3	– [a]	WUR[2]	Human infant feces	This chapter
B4 – *B. animalis* subsp. *lactis**	B4	– [a]	WUR[2]	WUR collection	This chapter
B5 – *B. animalis* subsp. *lactis**	B5	– [a]	WUR[2]	WUR collection	This chapter
B6 – B. *pseudolongum* subsp. *pseudolongum**	B6	– [a]	TNO[1]	TNO strain collection	This chapter

megasoftware.net). For alignment default settings were applied. A Neighbor-Joining bootstrap Test of Phylogeny was performed to visualize the evolutionary analysis.

Array design and construction

A genomic library was constructed from six strains representing six different *Bifidobacterium* species. These strains were obtained from the Belgian Co-ordinated Collections of Microorganisms, Ghent, Belgium, and include: *B. adolescentis* LMG 10502T, *B. animalis* subsp. *animalis* LMG 10508T, *B. bifidum* LMG 11041T, *B. catenulatum* LMG 11043T, *B. longum* biotype *longum* LMG 13197T, and *B. pseudolongum* subsp. *pseudolongum* LMG 11571T. The constructed gDNA-based microarray (Vlaminckx et al., 2007; Leavis et al., 2007) contains three random clone-based *Bifidobacterium* subarrays. The first subarray (SA1) contains a *B. pseudolongum* subsp. *pseudolongum* gDNA library, the second (SA2) a *B. longum* biotype *longum* gDNA library and the third subarray (SA3) was constructed from genomic material that originated from equivalent amounts of the six different *Bifidobacterium* species mentioned above. For the SA1 and SA2 subarrays, 10 µg of single strain gDNA was used. For subarray SA3 equimolar amounts of *B. adolescentis*, *B. animalis* subsp. *animalis*, *B. bifidum*, *B. catenulatum*, *B. longum*

biotype *longum*, and *B. pseudolongum* subsp. *pseudolongum* gDNA were mixed (10 µg in total) and used. The *B. pseudolongum* subsp. *pseudolongum* (SA1) and the *B. longum* biotype *longum* libraries (SA2) each contain 2000 clones which represent about 85% of the genomic material of each strain (calculated according to Akopyants *et al*., 2001 (Akopyants et al., 2001)). SA3 containing 2000 clones represents about 30% of the genomic material of each of the six species used for this library. DNA was precooled and sonicated (Branson 250/450 Sonifier, 6-mm microtip, Branson Ultrasonics, Danbury, USA) until an average fragment size of around 1.5 kb was obtained. Correct sizing was checked by agarose gel electrophoresis. Fragments of approximately 1500 bp were gel isolated, extracted, end-repaired and ligated into a pSMART-HCKan vector (CloneSMART Blunt Cloning Kit, Lucigen, Middleton, USA) according to the protocol of the manufacturer. Ligation products were transformed into *E. coli*, ElectroMAX™ DH10BTM Cells (Invitrogen, Breda, NL). Per library two thousand recombinant positive clones were arrayed into 96-well plates. Each well contained 100 µl TY medium plus kanamycin (30 µg/ml) (Sigma-Aldrich, Steinheim, D). After an overnight incubation at 37°C, 40 µl glycerol (40%) was added to each well and plates were stored at -80°C. Clone inserts were amplified by polymerase chain reaction using SMART-L1-NH2: 5'-CAGTCCAGTTACGCTGGAGTC and SMART-R1-NH2: 5'-CTTTCTGCTATGGAGGTCAGGTATG primers (described by the CloneSMART Blunt Cloning Kit manual, Lucigen, Middleton, USA) with 5'-C6 amino linkers (20 pmol each, Isogen, Maarssen, NL) to facilitate cross-linking to the aldehyde-coated glass slides (European Biotech Network, Dolembreux, BE). Alongside primers the PCR reactions contained 1µl bacterial culture (template DNA), 0.2 units SuperTaq polymerase with 1 x reaction buffer (HT Biotechnology, SpaeroQ, Gorinchem, NL), 0.2 mM each dNTP (Roche, Almere, NL), and 5% dimethyl sulfoxide (Sigma-Aldrich, Steinheim, D) in 50 µl reaction volume. PCR cycle conditions were 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 1 min, followed by 72°C for 10 min after cycling was completed. Insert sizes were determined by gel electrophoresis using 1% Ready-to-Run gels (Amersham, Piscataway, USA). PCR products of the correct size (1000-2000 bp) were purified by an isopropanol precipitation and the efficacy of this precipitation/purification was checked again by agarose gel electrophoresis on Ready-to-Run gels (Amersham, Piscataway, USA). Purified PCR products were arrayed into 384 wells spotting plates, concentrated, and dissolved in 3 x SSC (1 x SSC is 150mM sodium chloride plus 17 mM sodium citrate, pH 7.2, Sigma-Aldrich, Steinheim, D) solution. All PCR products were printed by using ESI three-axis DB-3 robot (versarray ChipWriter Pro, Biorad, Hercules, CA) at controlled humidity of 55% on CSS-100 silylated aldehyde glass slides (European Biotech Network, Dolembreux, BE). After drying, slides were

blocked following the manufacturer's instructions using sodium-borohydride (Sigma-Aldrich, Steinheim, D).

Fluorescent labeling and hybridization

Total gDNA samples were labeled by random primed labeling according to the protocol supplied with the BIOprime labeling kit (Invitrogen, Breda, NL) using Cy5-labeled dUTP (Amersham Biosciences, Piscataway, USA). Unincorporated dyes were removed using AutoSeq G50 columns (Amersham, Piscataway, USA) as described in the protocol of the manufacturer. DNA microarrays were prehybridized for 45 min at 42°C in prehybridization solution (1% bovine serum albumin, 5 x SSC, and 0.1% SDS). Hybridizations of labeled gDNA samples were performed overnight at 42°C in 40 µl Easyhyb buffer (Roche, Almere, NL) according to the manufacturer's protocol. Slides were washed twice in 1 x SSC, 0.2% SDS at 37°C, once in 0.5 x SSC, and twice in 0.2 x SSC at RT and dried with N_2-flow.

Microarray scan-image and quality analysis

After washing and drying, slides were scanned with a ScanArray Express 4000 scanner (Perkin-Elmer, Wellesley, USA). The obtained images were analyzed using ImaGene 5.6 software (www.biodiscovery.com). A gene identification file was constructed using CloneTracker software (www.biodiscovery.com). Spots were quantified using ImaGene 5.6 software (www.biodiscovery.com). During quantification, the ImaGene 5.6 software (www.biodiscovery.com) determined the average spot quality. Spot parameters such as shape regularity of the spots (threshold: 0.4), spot size and background signals (signal intensity >2 x background signal) were used for flagging poor and empty spots.

Clustering analysis, visualization, correlation mapping and normalization of hybridization signals

Genomic DNA samples were hybridized on a microarray. As standard normalization methods could not be applied due to the sample-related variation in hybridization patterns (Oshlack et al., 2007), we designed an alternative normalization approach. This alternative normalization was only used for those analyses which were based on absolute values. For Pearson correlation based clustering, untreated Cy5 signal intensity data were used as input, which was performed and visualized with TIGR MeV 3.1 Software (http://www.tm4.org/mev.html). For this analysis unnormalized data was used, to prevent the introduction of noise in the data set.

Correlation mapping was performed and visualized using PLS-toolbox version

4.02 (www.eigenvector.com) in MatLab version 7.3 (www.mathworks.com). A correlation value of '1' means that the hybridization patterns are similar. A correlation value of '0' means there is no similarity between hybridization patterns. Based on the gDNA fragments present on the BMS-array, the correlation values give an indication of the genomic overlap between the strains tested. The correlation values were extrapolated to a percentage of genomic overlap. A correlation range of '0 up to 1' was translated to a percentage of genomic overlap of 0% up to 100%. Due to the relatively long taxonomic distance between *B. longum* biotype *longum* and *B. pseudolongum* subsp. *pseudolongum* and their high abundance on the BMS-microarray, a negative correlation value was predicted for these species. Genomic overlap between *B. longum* biotype *longum* LMG13197 and *B. pseudolongum* subsp. *pseudolongum* LMG11571 was therefore calculated by correlation mapping of the SA1 and SA2 libraries separately (data not shown).

For the selection of diagnostic clones we normalized the obtained hybridization data using an alternative approach since this analysis was based on absolute values. Per library the median Cy5 signal intensities as generated by image analysis were sorted in ascending order. These ranked hybridization patterns were visualised in a line chart with the ranked values on the X-axis and the corresponding signal intensities on the Y-axis. The cut-off value for discriminating between non-homologous (low) and homologous (high) hybridization signals was based on the slopes of the curves. The transition point between the high and low values in the curves reflects the transition in the data set from low homology to high homology sequences. The exact position of the transition point was calculated as the first point where a significant change in the tangent line following the non-homology level occurred. To calculate this, results of negative control spots (empty spots on the array) were used for defining significant changes in signal intensity values between clones next to each other in rank. Based on two times the standard deviation of the control spot results, the transition point for each dataset was determined. All the spots with a rank higher than the transition point clone were classified as 'high homology sequence', whereas spots with signal intensities above the control spots but below the transition point were labelled as the 'low homology sequence' group. Spots that did not met these above mentioned criteria were labelled as 'non-significant' signals.

Significance Analysis for Microarrays (SAM) analysis and Analysis of Variance (ANOVA), TIGR MeV 3.1 Software (http://www.tm4.org/mev.html), was used for selection of a set of clones that are specific for one species or a group of species.

Sequencing of gDNA fragments

Plasmid isolation, purification of PCR products and high throughput sequencing was performed by GATC Biotech AG, Konstanz, Germany (www.gatc-biotech.com). Sequences were compared with public databases by using blastx and blastn. Blast analyses were performed against all sequenced genomes in the database and against the genome of *B. longum* NCC2705 and *B. adolescentis* ATCC 15703, using the NCBI website (http://www.ncbi.nlm.nih.gov/).

RESULTS AND DISCUSSION

Design, construction and application of the BMS-microarray

A *Bifidobacterium* mixed-species microarray was constructed, carrying genomic DNA (gDNA) of six *Bifidobacterium* species, derived from the adult colon, except for *B. pseudolongum* that was included to address its comparison with the intestinal *B. longum* (Table 1). This so called BMS-array was used to study genomic differences both within a single *Bifidobacterium* species and between bifidobacterial phylogenetic clusters (Fig. 1). First, gDNA of the six used *Bifidobacterium* spp. was hybridized to a microarray carrying two sub-arrays consisting of single-species libraries of *B. pseudolongum* (SA1) and *B. longum* (SA2) with a genomic coverage of 85%. The combination of these two sub-arrays allowed for distinct hierarchical clustering of the hybridization signals (Fig. 1A). *B. longum* and *B. pseudolongum* are known to cover a broad taxonomic range (Ventura et al., 2006). They show clearly distinguishable hybridization patterns on the combined SA1 and SA2 sub-arrays (Fig. 1A), confirming their relatively low genomic overlap. However, the combination of the SA1 and SA2 sub-arrays has too little discriminating power for the distinction between *B. bifidum* and species belonging to the phylogenetic *B. adolescentis* cluster, including the human commensals *B. adolescentis*, *B. animalis* and *B. catenulatum*. Therefore, we constructed a third sub-array (SA3) containing a mixed-species gDNA library, covering about 30% of the genomes of each of these four *Bifidobacterium* species, and combined it with the other sub-arrays, resulting in the BMS-microarray. Hybridization of gDNA of the six *Bifidobacterium* spp. to the BMS-microarray resulted in signals that showed a distinct hierarchical clustering and allowed separation of all human intestinal species (Fig. 1B). The clear clustering of all the different species, and the high similarity of these clusters with the defined *Bifidobacterium* clusters (Ventura et al., 2006), indicates that the arrayed DNA contains sufficient unique sequence information. Since untreated hybridization signals were used for the clustering, this

approach should be used for comparison at the array level but is not suitable for comparing signal intensities at the single spot level that requires normalization as described below.

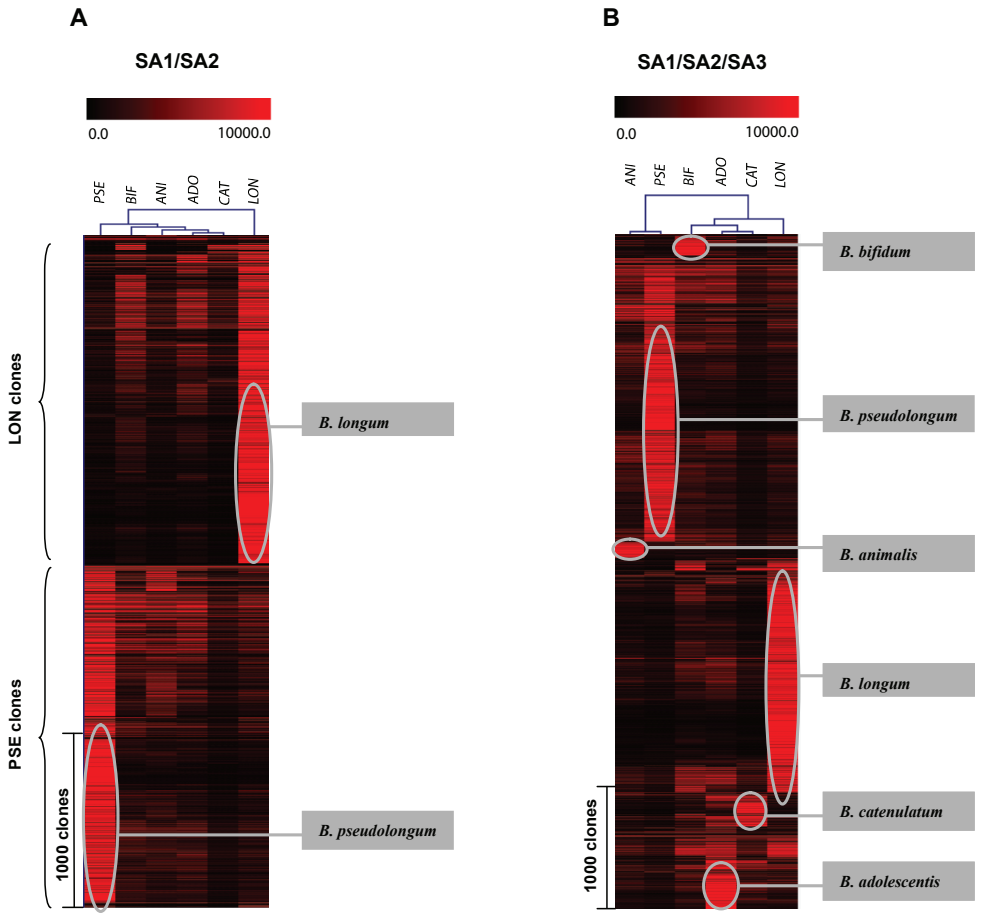


Figure 1. Hierarchical clustering (Pearson correlation, complete linkage) of Cy5 signal intensity gDNA hybridization patterns of gDNA (Y-axis) of the strains (X- axis; codes according to Table 1) used for the construction of the BMS-microarray. (A) based on the SA1 and SA2 subarrays, and (B) based on the BMS-array combining the SA1, SA2 and SA3 subarrays. Cy5 signal intensity values are indicated in red according to the color scale shown in the bar above the image. Species specific groups of clones are encircled and assigned.

Analysis of genomic diversity by hybridization pattern correlations

To test the applicability of the BMS-microarray as a taxonomic tool, we analyzed an independent set of 12 culture collection strains, three unclassified bifidobacterial

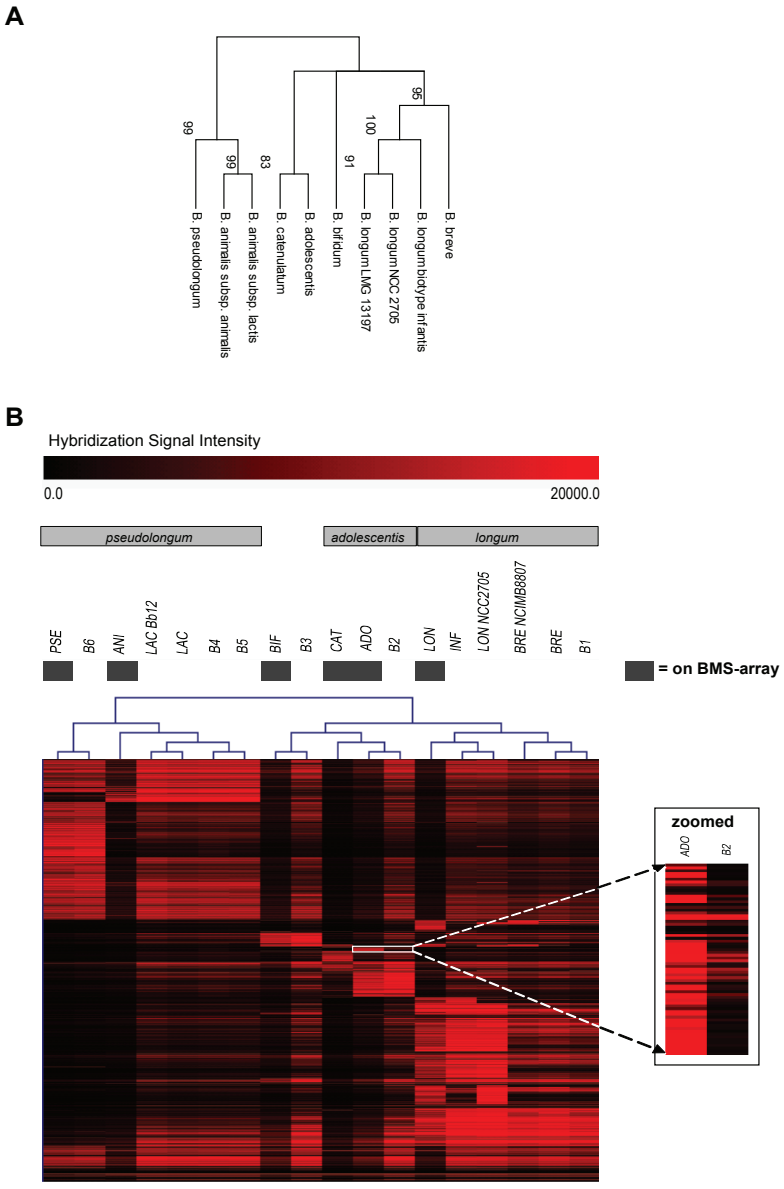


Figure 2. (A) Phylogenetic tree based on partial 16S rRNA sequences, Neighbour-Joining bootstrap Test of Phylogeny (B) Hierarchical clustering (Pearson correlation, complete linkage) of Cy5 signal intensity hybridization patterns, of *Bifidobacterium* strains (X-axis; codes according to Table 1), based on 6000 clones (Y-axis). The dark-grey bars indicate whether the sample is used for the construction of the BMS-microarray. In the light-gray bars the according phylogenetic clusters are indicated. Cy5 signal intensity values are indicated in red according to the scale shown in the bar above the image. On the right, a magnified detail of cluster that shows diversity in hybridization patterns between strains ADO and B2.

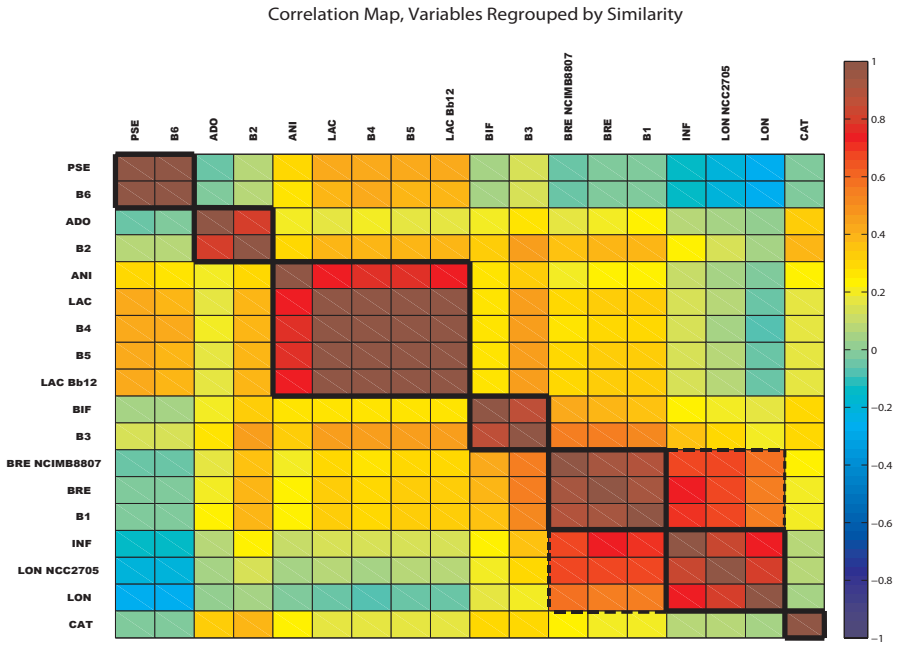


Figure 3. Correlation mapping of hybridization patterns of gDNA samples (codes according to Table 1) of tested *Bifidobacterium* strains based on the BMS-array. Colouring applied according to the scale legend on the right. Groups of strains that belong to the same species are highlighted by black lines and closely related strains by dashed lines.

strains and three fecal isolates that altogether include nine different *Bifidobacterium* species (Table 1). All strains were typed by sequence analysis of their 16S rRNA genes (Fig. 2A). Genomic DNA of each of these 18 *Bifidobacterium* strains was hybridized to the BMS-microarray (Fig. 2B). The hybridization data sets were hierarchically clustered and showed a high degree of similarity with the 16S rRNA based phylogenetic clusters described by Ventura *et al*. (2006) (Ventura et al., 2006) and the generated partial 16S rRNA sequence based phylogenetic tree (Fig. 2A). The tree obtained by hierarchical clustering of the hybridization data contains three main branches (Fig. 2). These main branches have similarity to the *Bifidobacterium* clusters defined by Ventura *et a*l. (2006) (Ventura et al., 2006). One branch is similar to the *B. pseudolongum* group, one branch is similar to the *B. longum* group, and one branch contains the *B. adolescentis* group and the separate *B. bifidum* branch (Fig. 2).

The set of *Bifidobacterium* strains tested contains fecal and other isolates with an unknown identity at the species level. Correlating the hybridization patterns of these isolates with *Bifidobacterium* type-strains enabled their rapid classification that

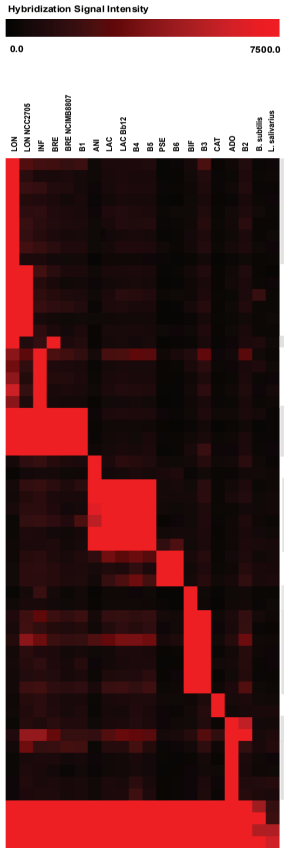
was confirmed by 16S rRNA sequencing (data not shown). However, hybridization to the BMS-array also allowed determination at the strain level as is illustrated by the fecal isolate B2 that belongs to *B. adolescentis* but detailed analysis shows its hybridization response to differ from that of the *B. adolescentis* type-strain, indicative of genomic differences (Fig. 2 close-up).

To clarify the genomic overlap between every combination of strains tested, we used a correlation mapping approach. Correlation mapping describes the similarity between the hybridization patterns (Fig. 3). This approach formed the basis of the BMS-microarray as a molecular genomic tool, which we applied to analyze the genomic diversity of a set of *Bifidobacterium* strains. Confirming the results of the hierarchical clustering, we were able to define several groups of strains with highly similar hybridization patterns (Fig. 3, highlighted squares).

Taking in account that strains with genomic homology above 70% should be interpreted as similar species (Lauer and Kandler, 1983), we identified strain B1 as a *B. breve* and strain B2 as a *B. adolescentis*, as their genomic overlaps with these species were 93 % and 81 %, respectively. The genomic overlap of the used *B. animalis* subsp. *animalis* LMG10508 and *B. animalis* subsp. *lactis* LMG18314 strains was found to be 79%, confirming that they belong to the same species. Strains B4 and B5 show all high homology to *B. animalis* subsp. *lactis* LMG18314 (98%), indicating that they are highly similar *B. animalis* subsp. *lactis* strains. Due to its high homology to *B. bifidum* LMG11041 (86%), we concluded that strain B3 belongs to *B. bifidum*. *B. longum* NCC2705 and *B. longum* biotype *infantis* LMG8811 overlapped *B. longum* LMG13197 for 80%, and 72%, respectively. This makes it debatable whether *B. longum* and *B. infantis* belong to the same species and it strengthens their grouping as different biotypes as proposed recently (Ventura et al., 2006; Mattarelli et al., 2008). Finally, in contrast with the suggestion made by their nomenclature, *B. longum* LMG13197 and *B. pseudolongum* LMG11571 showed only low genetic homology of 9% (data not shown), confirming their present taxonomic position (Ventura et al., 2006).

Characterization of species-specific sequences

The possibility was studied to use the BMS-arrays for detecting bifidobacterial sequences that are specific at the level of strain, species or phylogenetic cluster. Such sequences could be developed as molecular markers that could be useful for diagnostic discrimination and provide underlying functional information relating to their unique presence. In order to select specific clones we needed to classify spot signals as ‘absent’ (no or weak hybridization signal) or ‘present’ (strong hybridization



Specificity	BLAST
LON	gi|28377951|ref|NP_784843.1| polysaccharide biosynthesis protein [Lactobacillus plantarum WCFS1]
LON	**gi|50954295|ref|YP_061583.1| glycosyl transferase [Leifsonia xyli subsp. xylistr. CTCB07]**
LON	**gi|50954297|ref|YP_061585.1| rhamnosyltransferase [Leifsonia xyli subsp. xylistr. CTCB07]**
LON	ref|ZP_02028516.1| hypothetical protein BIFADO_00949 [Bifidobacterium adolescentis L2-32]
LON	ref|NP_695369.1| possible arabinosidase [Bifidobacterium longum NCC2705]
LON	gi|15614430|ref|NP_242733.1| hypothetical protein BH1867 [Bacillus halodurans C-125]
LON	**gi|29827685|ref|NP_822319.1| secreted protein [Streptomyces avermitilis MA-4680]**
LON	**gi|68250155|ref|YP_249267.1| putative type I restriction enzyme HindVIIPspecificity protein [Haemophilus influenzae 86-028NP]**
LON	**gi|119025199|ref|YP_909044.1| hypothetical protein BAD_0181 [Bifidobacterium adolescentis ATCC 15703]**
B. longum spp.	**gi|23464766|ref|NP_695369.1| possible arabinosidase [Bifidobacterium longum NCC2705]**
B. longum spp.	**gi|23465412|ref|NP_696015.1| hypothetical protein BL0837 [Bifidobacterium longum NCC2705]**
B. longum spp.	**gi|23466239|ref|NP_696842.1| ATP binding protein of ABC transporter forpentoses [Bifidobacterium longum NCC2705]**
B. longum spp.	**gi|23464662|ref|NP_695265.1| ATP binding protein of ABC transporter [Bifidobacterium longum NCC2705]**
B. longum spp.	**gi|23464774|ref|NP_695377.1| very narrowly conserved hypothetical protein [Bifidobacterium longum NCC2705]**
B. longum spp.	**gi|23465808|ref|NP_696411.1| hypothetical membrane protein with unknownfunction [Bifidobacterium longum NCC2705]**
LON/BRE	gi|19703892|ref|NP_603454.1| hypothetical protein FN0557 [Fusobacteriumnucleatum subsp. nucleatum ATCC 25586]
LON/INF	ref|NP_695859.1| possible cell surface protein with gram positive anchor domain [Bifidobacterium longum NCC2705]
LON/INF	gi|81428209|ref|YP_395209.1| Hypothetical prophage Isa1protein [Lactobacillussakei subsp. sakei 23K]
LON/INF	gi|38234327|ref|NP_940094.1| Putative DNA methyltransferase [Corynebacterium diphtheriae NCTC 13129]
LON/INF	gi|86741650|ref|YP_482050.1| putative transposase, IS891/IS1136/IS1341 [Frankiasp. CcI3]
LON/INF	ref|ZP_02029086.1| hypothetical protein BIFADO_01537 [Bifidobacterium adolescentis L2-32]
B. longum spp./INF/BRE	**gi|23465347|ref|NP_695950.1| hypothetical protein BL0771 [Bifidobacterium longum NCC2705]**
B. longum spp./INF/BRE	**gi|23465346|ref|NP_695949.1| hypothetical protein BL0770 [Bifidobacterium longum NCC2705]**
B. longum spp./INF/BRE	**gi|23465203|ref|NP_695806.1| hypothetical protein BL0619 [Bifidobacterium longum NCC2705]**
B. longum spp./INF/BRE	**gi|23465396|ref|NP_695999.1| possible thioredoxin-dependent thiol peroxidase [Bifidobacterium longum NCC2705]**
ANI	**gi|85375778|ref|YP_459840.1| hypothetical protein ELI_14755 [Erythrobacterlitoralis HTCC2594]**
ANI	gi|54026395|ref|YP_120637.1| hypothetical protein nfa44220 [Nocardia farcinicaI FM 10152]
ANI/B. lactis spp.	gi|23465507|ref|NP_696110.1| possible pyridoxine kinase [Bifidobacterium longum NCC2705]
ANI/B. lactis spp.	gi|119026125|ref|YP_909970.1| peptidoglycan synthetase; penicillin-bindingprotein 3 [Bifidobacterium adolescentis ATCC 15703]
ANI/B. lactis spp.	gi|119025720|ref|YP_909565.1| hypothetical protein BAD_0702 [Bifidobacterium adolescentis ATCC 15703]
ANI/B. lactis spp.	gi|23465898|ref|NP_696501.1| narrowly conserved hypothetical protein withpossible isomerase function [Bifidobacterium longum NCC2705]
ANI/B. lactis spp.	gi|23464646|ref|NP_695249.1| aspartyl-tRNA synthetase [Bifidobacterium longum NCC2705]
ANI/B. lactis spp.	gi|55822649|ref|YP_141090.1| MutT/nudix family protein [Streptococcusthermophilus CNRZ1066]
B. pseudolongum spp.	gi|23464987|ref|NP_695590.1| integral membrane protein in the upf0059 [Bifidobacterium longum NCC2705]
B. pseudolongum spp.	gi|54026396|ref|YP_120638.1| hypothetical protein nfa44230 [Nocardia farcinicaIFM 10152]
B. pseudolongum spp.	gi|19551586|ref|NP_599588.1| hypothetical protein NCgl0330 [Corynebacteriumglutamicum ATCC 13032]
BIF	gi|23464820|ref|NP_695423.1| hypothetical membrane protein with unknownfunction [Bifidobacterium longum NCC2705]
BIF	gi|42519452|ref|NP_965382.1| hypothetical protein LJ1578 [Lactobacillusjohnsonii NCC 533]
B. bifidum spp.	ref|ZP_00206596.1| hypothetical protein Blon03000683 [Bifidobacterium longum DJO10A]
B. bifidum spp.	gi|23465240|ref|NP_695843.1| dimethyladenosine transferase [BifidobacteriumlongumNCC2705]
B. bifidum spp.	**gi|119025571|ref|YP_909416.1| narrowly conserved hypothetical membrane protein [Bifidobacterium adolescentis ATCC 15703]**
B. bifidum spp.	gi|54293105|ref|YP_125520.1| hypothetical protein lpl0144 [Legionellapneumophila str. Lens]
B. bifidum spp.	gi|23466292|ref|NP_696895.1| probable long-chain-fatty-acid--CoA ligase;long-chain acyl-CoA synthetase [Bifidobacterium longum NCC2705]
B. bifidum spp.	ref|YP_909028.1| hypothetical protein BAD_0165 [Bifidobacterium adolescentis ATCC 15703]
B. bifidum spp.	gi|119026150|ref|YP_909995.1| possible lipoprotein signal peptidase [Bifidobacterium adolescentis ATCC 15703]
CAT	gi|54025888|ref|YP_120130.1| hypothetical protein nfa39180 [Nocardia farcinicaIFM 10152]
CAT	gi|23465396|ref|NP_695999.1| possible thioredoxin-dependent thiol peroxidase [Bifidobacterium longum NCC2705]
B. adolescentis spp.	**gi|119025726|ref|YP_909571.1| pullulanase [Bifidobacterium adolescentis ATCC 15703]**
B. adolescentis spp.	**gi|119026628|ref|YP_910473.1| beta-glucosidase [Bifidobacterium adolescentis ATCC 15703]**
ADO	**gi|119025072|ref|YP_908917.1| putative exodeoxyribonuclease V [Bifidobacterium adolescentis ATCC 15703]**
ADO	**gi|119026278|ref|YP_910123.1| similar to glutamine ABC transporter (ATP-bindingprotein) [Bifidobacterium adolescentis ATCC 15703]**
ADO	**gi|119026197|ref|YP_910042.1| CRISPR-associated protein Cas2 [Bifidobacterium adolescentis ATCC 15703]**
ADO	**gi|119026198|ref|YP_910043.1| CRISPR-associated DNA polymerase [Bifidobacterium adolescentis ATCC 15703]**
ADO	**gi|119026274|ref|YP_910119.1| putative ABC-type amino acid transport systemperiplasmic component [Bifidobacterium adolescentis ATCC 15703]**
conserved	**23S rDNA**
conserved	**23S rDNA**
conserved	**16S rDNA**
conserved	**16S rDNA**

Figure 4. Sequence analysis of diagnostic clones. Visualization of the Cy5 signal intensities of the 54 selected diagnostic clones (Y-axis) for all strains (X-axis; codes according to Table 1). Signal intensity values are visualized according to the colour scale on top of the figure. Per clone the specificity and gene predictions (BLAST results) are shown. Hits that are supported by BLAST analysis of both the forward and the reverse sequence fragment, or have a 100% alignment with one of these fragments, are indicated in bold.

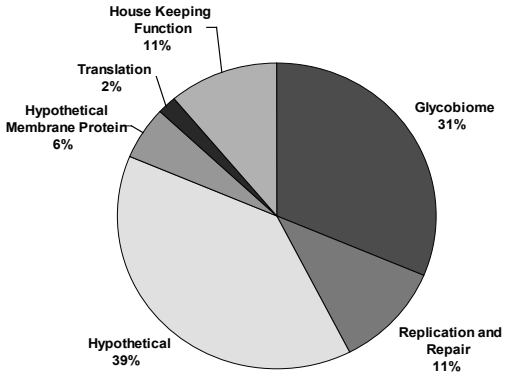


Figure 5. Distribution of KEGG ontologies of predicted proteins encoded by the 54 diagnostic sequences listed in Figure 4. The group described as 'House Keeping Function (11%)' encompasses the following ontology groups: Bacterioferrin Comigration Protein (3%), Lipid Metabolism (2%), Metabolism of Cofactors and Vitamins (2%), Signalling Molecules and Interaction (2%), and Amino Acid Metabolism (2%).

signal). Hence, we developed a signal amplitude-independent normalization method, which enabled us to discriminate between clones that contain sequences with varying degree of homology in relation to the hybridized gDNA (see Materials and Methods). This classification allowed for determining the characteristics of a single spot after hybridization with different *Bifidobacterium* gDNA samples. From a set of 800 strain- or species-specific clones that were obtained by applying the SAM tool, we selected 54 discriminating clones that showed relatively high hybridization intensities. The insert sequences of these clones were determined and compared with database entries (Fig. 4). Clones that were selected as specific for *B. longum*, *B. adolescentis* or originating from closely related strains gave BLAST-hit results with high probabilities (Fig. 4). As expected, clones specific for *Bifidobacterium* spp. with genomes that are not in the public database gave BLAST hits with lower probabilities (Fig. 4). Only two percent of the clones spotted on the BMS-array showed hybridization to other genera than *Bifidobacterium* used as control and these were found to represent 16S and 23S rRNA genes. In summary, these findings strengthen the appropriateness of our method for selection of strain- and species-specific DNA sequences.

Subsequently, we analysed the distribution of the ontology groupings of the predicted genes encoded by the 54 diagnostic clones (Fig. 5). A high percentage of predicted hypothetical proteins (39%) and predicted genes with low homology BLAST hits was found, as we used *Bifidobacterium* strains that have not or not fully been sequenced yet, while the main fraction of the diagnostic sequences was related to the bifidobacterial glycobiome (31%). As a reference, less than 9% of the

predicted proteins in *B. longum* NCC2705 represent the glycobiome consisting of an array of glycoside hydrolases (Schell et al., 2002). This indicates that bifidobacterial strains can be identified by their specific sugar-degrading abilities and extend previous observations from a study on strain-specific sugar-degrading abilities (Ryan et al., 2006). According to our findings, another variable section of bifidobacterial genomes may encode genes from the replication and repair ontology group, which provide resistance against foreign DNA (11%). These include a series of restriction-modification systems as well as CRISPR-associated functions. The latter group of genes provide immunity against foreign genetic elements via a mechanism based on RNA interference (Barrangou et al., 2007). The fact that these CRISPR-associated gene clusters have often been found on mobile genetic elements (Kunin et al., 2007) indicates that their compositions can vary between closely related species. Finally, specific housekeeping functions are recognized as species- and strain-specific (11 %) and may reflect different capacities to produce vitamins, deal with signalling, or produce specific metabolites.

In conclusion, the BMS-microarray is a high-resolution diagnostic tool that can be used to reveal strain- and species-specific characteristics of bifidobacteria. Moreover, its application to cognate *Bifidobacterium* spp. revealed this genus to contain species-specific diagnostic sequences that are mainly predicted to code for the glycobiome and functions involving DNA metabolism.

ACKNOWLEDGEMENTS

This work was supported by the Dutch Ministry of Economic Affairs through the Innovation Oriented Research Program on Genomics (IOP Genomics: IGE01016). We would like to thank Martien Caspers from TNO Quality of Life for his excellent input with respect to the data analysis and Eline Klaassens for providing *Bifidobacterium* isolates and database searches.

Chapter 3

Analysis of infant isolates of *Bifidobacterium breve* by comparative genome hybridization indicates the existence of new subspecies with marked infant-specificity

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A total of 20 *Bifidobacterium* strains were isolated from fecal samples of 4 breast and bottle-fed infants that were all characterized as *B. breve* based on 16S rRNA gene sequence and metabolic analysis. These isolates were further characterized and compared to the type strains of *B. breve* and 7 other *Bifidobacterium* spp. by comparative genome hybridization. For this purpose we constructed and used a DNA-based microarray, containing over 2,000 randomly cloned DNA fragments from the *B. breve* type strain LMG13208. This molecular analysis revealed a high degree of genomic variation between the isolated strains and allowed the vast majority to be grouped in 4 clusters. One cluster contained a single isolate that was virtually indistinguishable from the *B. breve* type strain. The 3 other clusters included 19 *B. breve* strains that differed considerably from all type strains. Remarkably, each of the 4 clusters included strains that were isolated from a single infant, indicating a niche adaptation may contribute to the variation within the *B. breve* species. Based on the genomic hybridization data, the new *B. breve* isolates were estimated to contain approximately 60-90 % of the genes of the *B. breve* type strain, testifying for the existence of various subspecies within the species *B. breve*. Further bioinformatic analysis identified several hundreds of diagnostic clones specific for the genomic clustering of the *B. breve* isolates. Molecular analysis of representatives of these, revealed the annotated genes from the conserved *B. breve* core to encode mainly house-keeping functions while the strain-specific genes were predicted to code for functions related to life style, such as carbohydrate metabolism and transport. This is compatible with a genetic adaption of the strains to their niche, a combination of infant and diet.

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INTRODUCTION

The human gut is colonized by a complex community of microorganisms, the microbiome, which are known to impact its host (Hooper and Gordon, 2001). One group of commensal bacteria, those belonging to the genus *Bifidobacterium*, is considered to play an important role in the development of our microbiome in early life, during infancy. This is mainly based on its abundance in our early life and its capability to affect our immune system (Boesten and de Vos, 2008; Favier et al., 2002; Favier et al., 2003; O'Hara et al., 2006). Representatives of the species *B. breve* are highly abundant in early life and hence received considerable attention because of its impact on human health (Haarman and Knol, 2005; MacConaill et al., 2003; Margolles et al., 2006; Menard et al., 2005). *Bifidobacterium breve* is considered to be widely distributed irrespective of host age (He et al., 2001; Matsuki et al., 1998; Turroni et al., 2009). Bifidobacteria are host specific and different species occupy different niches. For example, the most common habitat of *B. dentium* is the human mouth, whereas *B. breve* has been mainly identified from the human intestinal tract. This suggests that these species possess unique characteristics which can be applied in a specific niche. Unravelling species-specific characteristics therefore will reveal adaptation strategies for survival in specific ecological niches.

Genomic characterization of *B. breve* is essential to gain more insight in the underlying mechanisms involved in the interaction with its human host (Boesten and de Vos, 2008). Although detailed studies on the *B. breve* UCC2003 genome have been published (Ventura et al., 2007b), the genome of *B. breve* UCC2003, with a size of 2.4 Mb, is unfortunately not publically available (Leahy et al., 2005). To date several *Bifidobacterium* genomes have been sequenced. Comparing bifidobacterial genomes reveals highly interesting information on the divergence of the genes they encode (Ventura et al., 2009). This gives clues for the molecular background of their species specific characteristics and genetic adaptation to their ecological niches. For example, whole genome comparison studies showed that nearly 10% of the total bifidobacterial gene content is dedicated to sugar internalization (Ventura et al., 2007a). This is 30% higher than other intestinal bacteria such as *Escherichia coli* or *Enterococcus faecium* (Ventura et al., 2007a). Furthermore, a *B. breve* specific operon that encodes for enzymes that are involved in the breakdown of complex sugars such as starch, amylopectin and pullulan was identified (Maze et al., 2007). Next to the *B. breve* UCC2003 genome, a CRISPR-related system (CASS), which is implicated in the defence against phages and plasmids is identified in most sequenced *Bifidobacterium* genomes (Horvath et al., 2009; Ventura et al., 2009). In summary, *Bifidobacterium* species have specific characteristics which are rooted in

its genetic material, which makes genome studies fruitful.

Genome analysis clarifies intraspecies diversity and can indicate for the presence of subspecies. The proposition of reclassifying the three biotypes of *Bifidobacterium longum* as three subspecies for example, was mainly based on genomic data (Mattarelli et al., 2008). Little variation of 16S rDNA sequences and DNA-DNA hybridization similarity levels between 65 – 80% supported the unification in one species. One the other hand, genotypic data from recA, tuf, and ldh gene analysis next to RAPD-PCR, PCR-DGGE, and BOX-PCR studies provided evidence for unification of *B. longum*, *B. infantis*, and *B. suis* in three subspecies (Mattarelli et al., 2008). Besides genotypic divergence, phenotypic variation is needed for proof of the existence of subspecies. For example, the three subspecies of *B. longum* reveal considerable differences in carbohydrate metabolism (Mattarelli et al., 2008).

In this study we describe the genetic characterization of a selection of *B. breve* strains isolated from fecal samples originating from three breast- or bottle-fed infants using a DNA-based microarray specific for *B. breve*. The microarray platform serves as a tool for comparison of genome contents without the obligation to sequence complete genomes. Due to the lack of commercially available *Bifidobacterium* microarrays, we developed a clone-based *B. breve* microarray (Boesten et al., 2009). The goal of this study was to screen for possible correlations between the host origin, obtained biochemical data and DNA-DNA hybridization patterns of the fecal isolates.

MATERIALS AND METHODS

Bacterial strains, and culturing

Bifidobacterium breve LMG13208, *B. longum* biotype *longum* LMG13197, *B. longum* biotype *infantis* LMG8811, *B. bifidum* LMG11041, *B. adolescentis* LMG10502, *B. catenulatum* LMG11043, *B. animalis* subsp. *animalis* LMG10508, and *B. pseudolongum* subsp. *pseudolongum* LMG11571 strains were obtained from the Belgian Co-ordinated Collections of Microorganisms (BCCM), Ghent, Belgium. These type strains were used as reference material in this study. All other strains were isolated, characterized biochemically and identified by 16S ribosomal sequence analysis (Baseclear, Leiden, NL) at Danone Research (Wageningen, NL) (Felske et al., 1997; Haarman and Knol, 2005; Nubel et al., 1996). Isolates NR201, NR202, NR203, NR204, NR214, NR224, NR226, NR229, NR239, NR240, NR241, NR242, and NR243 were isolated from the feces of a healthy bottle-fed human infant 2.

Isolates NR231, NR238, NR207, NR212, NR225, and NR232 were isolated from two healthy breast-fed human infants, 3 and 4. Isolate NR200 is commercial strain isolated from a healthy bottle-fed infant (Danone Research, Wageningen, NL). LMG strains were cultured anaerobically in MRS broth (Difco, Detroit, USA) supplemented with 0.05% L-cysteine hydrochloride monohydrate (Sigma-Aldrich, Steinheim, D) and incubated at 37°C for 16-40 h. Culturing of the fecal isolates was performed at Danone Research (Wageningen, NL) according to conditions described by Haarman *et al*. (2005).

Measuring of bile salt hydrolysis activity, and lactic acid, ammonia, histamine, and tyramine production

Bile salt hydrolase activity was detected using a plate assay based on Christiaens *et al*. (1992), and Dashkevicz and Feighner (1989). Freshly cultured bacteria were preincubated in MRS broth with 2 mM taurodeoxycholic acid (TDCA; Sigma-Aldrich, Zwijndrecht, NL) or glycodeoxycholic acid (GDCA; Calbiochem, Merck, Darmstadt, G) and incubated for 24 h at 37°C. The bacterial cultures from the TDCA pre-culture were streaked on MRS-plates containing 0.5% TDCA and on a control MRS-plate. The bacterial cultures from the GDCA pre-culture were streaked on a MRS-plate containing 0.5% GDCA and on a control MRS-plate. Plates were incubated for 72 h at 37°C. Plates were checked for bile salt hydrolyse-active colonies with precipitates around the colonies.

L-lactate and D-lactate production was analyzed with L-Lactic acid kit (Enzyplus EZA-890, Rasio Diagnostics, www.biocontrolsys.com) and D-Lactic acid kit (Enzyplus EZA-889, Rasio Diagnostics, www.biocontrolsys.com). Bacteria were cultured in MRS-broth for 48 h at 37°C. Cultures were centrifuged (Sorvall RT-7, rotor RTH 750) for 10 min at 4000 rpm. The supernatant was diluted 50 times in demineralised water and tested for the presence of D- and L-lactate.

Ammonia production was analyzed with a spectrophotometer using previously published methods (Di Giorgio, 1974; Lin and Visek, 1991) with some modifications.

Strains were cultured anaerobic in 10 ml BHI-broth (37 g/l; Oxoid, Badhoevedorp, NL) for 48 h at 37°C. Cultures were centrifuged (Sorvall RT-7, rotor RTH 750) for 10 min at 4000 rpm. A volume of 100 µl trichloricacid solution (30%) was added to 900 µl supernatant or to 900 µl BHI-broth. From an ammonia stock solution (5 mM) a standard curve (0 – 5 mM) was prepared. 10 µl Standard, sample or blank (all in triplicate) was added to a 96 wells plate. Then 75 µl phenol reagent (50 mg/ml phenol and 0.24 mg/ml sodium nitroprusside) and 75 µl alkali-hypochlorite reagent (31.2 mg/

ml NaOH and 2.6 mg/ml sodium hypochlorite) was added. After an incubation at 37°C for 20 min, 100 µl demineralised water was added. Subsequently, the extinctions were measured at a wavelength of 620 nm using a plate reader (Spectramax 190, Molecular Devices, Sunnyvale, USA).

The production of the biogenic amines histamine and tyramine was detected via a pH increase according Le Jeune *et al*. (1995).

DNA extraction

DNA was extracted from *Bifidobacterium* LMG strains according to the following protocol earlier described (Boesten et al., 2009). The DNA was quantified using the Agilent 2100 Bioanalyser (DNA 2000 kit, Agilent Technologies, Palo Alto, CA). Genomic DNA for DNA-DNA hybridization was isolated from fecal bifidobacterial isolates according to the isolation procedure described previously (Boom et al., 1999).

Array design and construction

A genomic library was constructed from *Bifidobacterium breve* LMG13208, which was obtained from the Belgian Co-ordinated Collections of Microorganisms (Gent, B). The constructed genomic DNA-based microarray was performed as described earlier (Boesten et al., 2009). The final array contained 2000 clones which represent about 85% (calculated according to Akopyants *et al.* (2001)) of the genomic material of *B. breve* LMG13208.

Fluorescent labeling and hybridization

Total DNA samples were labeled by random prime labeling. A volume of 1 µl DNA sample was diluted in 4.25 µl H_2O and mixed with 5 µl of 2.5 x random primer (BioPrime Kit, Invitrogen, Breda, NL). The mixture was incubated at 96°C for 5 min. After cooling on ice, the samples were shortly centrifuged at maximum speed in an eppendorf centrifuge. Subsequently the following components were added while the samples were kept on ice: 0.25 µl 50 x AminoAcid-dNTP mix (AA-dNTP) (Invitrogen, Breda, NL), 1.75 µl milliQ, and 0.25 µl Klenow fragment (Invitrogen, Breda, NL). After addition the samples were incubated at 37°C for 1.5 hours for amplification. Unincorporated AA-dUTP and free amines were removed using QIAqiuck columns following the protocol described by the supplier (Qiagen Benelux, Venlo, NL). For this purpose, five times the reaction volume of PB buffer (Qiagen supplied) was added to the samples and the mixture was transferred to a QIAqiuck Spin Column that was centrifuged at 20,000 x g for 1 min. To increase the binding efficiency, the

flow through was reloaded and the column was centrifuged again at 20,000 x g for 1 min and the flow through was discarded. To elute the DNA samples from the columns they were first washed separately with 500 µl of 80% ethanol and then eluted with 30 µl of milliQ H_2O, a sept that was repeated twice as described by the manufacturer (Qiagen Benelux BS, Venlo, NL). The elutate was dried at high temperature in a speed vac for about 15-30 min. For coupling of the Cy Dye Esters to the AA-cDNA the dried cDNA samples were dissolved in 4.5 µl 0.1 M sodium carbonate buffer (Na_2CO_3), pH 9.0 during 10 min at RT while mixed a few times. Subsequently, 4.5 µl of the appropriate NHS-ester Cy Dye (Amersham, Piscataway, USA) were added. This reaction mix was incubated for 1 h at room temperature in the dark. After incubation, 10 µl of milliQ H_2O was added and the samples were centrifuged shortly. To remove the unincorporated dyes we used Autoseq G50 (Amersham, Piscataway, USA) columns according to the protocol supplied by the manufacturer.

DNA microarrays were prehybridized for 45 min at 42°C in prehybridization solution (1% bovine serum albumin, 5xSSC, and 0.1% SDS). Co-hybridizations of labeled total DNA samples were performed overnight at 42°C in 40 µl Easyhyb buffer (Roche, Almere, NL) according to the manufacturer's protocol. Slides were washed twice in 1 x SSC, 0.2% SDS at 37°C, once in 0.5 x SSC, and twice in 0.2 x SSC at RT and dried with N_2-flow.

Microarray scan-image and quality analysis

Performed as described earlier (Boesten et al., 2009). For validation of the labeling and hybridization reaction self-self hybridization reactions were performed.

Normalization, visualization, and data analysis

Genomic DNA samples were hybridized on a microarray. Significance Analysis of Microarrays (SAM), Analysis of Variance (ANOVA), and Principle Component Analysis (PCA) were performed and visualized using TIGR MeV 3.1 Software (http://www.tm4.org/mev.html). As a basis for data analysis the ^{2}log values of untreated Cy5 (sample)/Cy3 (reference LMG13208) signal intensity value ratios (R) were used. ANOVA (p-value: 0.01) was used for selecting a set of clones that were specific for one genomic cluster (http://www.tm4.org/mev.html). PCA Settings; 100% data values, components: 30, covariance, number of neighbours for KNN imputation: 10. SAM settings; Two-class unpaired, number of permutations: 100, number of K-Nearest Neighbours: 10.

Sequencing of DNA fragments

Plasmid isolation, purification of PCR products and high throughput sequencing was performed by GATC Biotech AG, Konstanz, Germany (www.gatc-biotech.com). The average clone size was 1.5 kb. All clones were sequenced in parallel using a forward (AMPL1) and a reverse (SR2) universal primer. Sequence runs resulted in stretches of on average 800 nucleotides. Sequences were compared with public databases by using blastn. BLAST analyses were performed against all currently available sequenced genomes using the NCBI website (http://www.ncbi.nlm.nih.gov/).

RESULTS AND DISCUSSION

Characterization of fecal isolates

Fecal samples were collected from four healthy human infants. Isolate NR200 is a commercial strain (Danone Research, Wageningen, NL) isolated from a healthy human infant. We want to note that these infants received different diets. Infant 2 was bottle-fed and infants 3 and 4 were breast-fed (Figure 1). Bacterial strains were isolated from these fecal samples, and a set of 19 isolates was characterized as *Bifidobacterium breve* strains, based on 16S rDNA sequencing. Biochemical analyses were performed in order to further characterize these isolates. The production of ammonia, histamine, tyramine, L-lactate, and D-lactate was measured next to bile acid deconjugation activity. These biochemical characteristics were tested because of safety criteria (Borriello et al., 2003; FAO/WHO, 2002) on the one hand, and the positive effects ascribed to bile salt deconjugation activity on the other hand. Bile salt tolerance could contribute to a higher survival in the stomach and small intestine and might be necessary for probiotic applications. Based on this biochemical analysis, all bifidobacterial isolates showed the ability to deconjugate glycodeoxycholic acid (GDCA) and taurodeoxycholic acid (TDCA). Bile salt hydrolase activity is commonly found in bifidobacteria and also in the species *B. breve*. A strong correlation has been observed between the habitat and the presence of bile salt hydrolase activity, being more often found in fecal isolates compared to other habitats (Tanaka et al., 1999). Furthermore, all isolates produced L-lactate but not ammonia, histamine, tyramine, or D-lactate. In general bifidobacteria and *B. breve* are known to produce lactic acid in the L-form, which is more easily metabolized by infants compared to the D-form. Ammonia is a potentially toxic compound which can be produced in the intestine from ureum by bacterial urease activity. Urease activity has been found in bifidobacteria but is not very common in the species *B. breve* (Crociani and Matteuzzi, 1982), which

is confirmed in this study. Histamine and tyramine are biogenic amines which can irritate the intestinal wall. They are formed from amino acids by the action of bacterial decarboxylating enzymes. The presence of this enzyme activity in bifidobacteria is not very well studied. Histamine and tyramine are not produced by the *B. breve* isolates tested in this study.

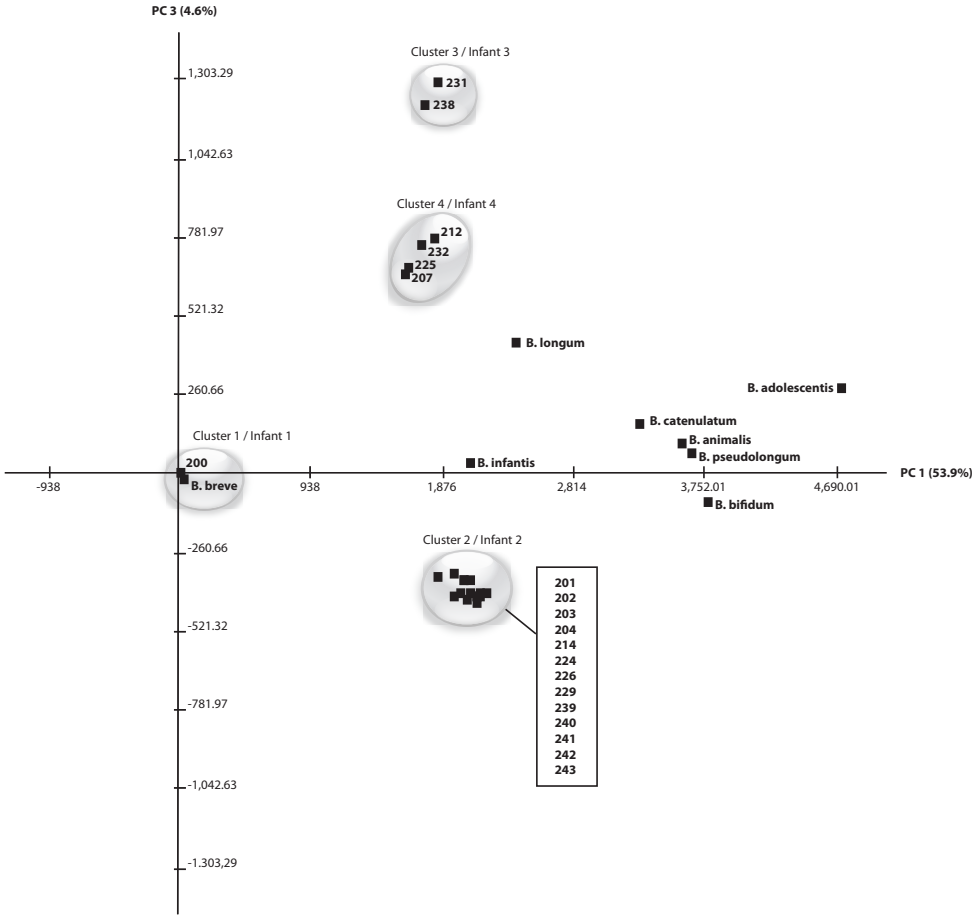

Figure 1. PCA analysis of the samples visualized in a PC1-3 plot which explains 58.5% data variation. Settings; 100% data values (1920 clones/sample), components: 30, covariance, number of neighbours for KNN imputation: 10. Clusters of *B. breve* strains and isolates are highlighted by oval shapes and coded 1, 2, 3, and 4. Data points are coded by the NR-code of the isolate or the species name, when representing a reference strain. When data points overlap, the codes are listed next to the cluster.

Genotyping of human infant fecal *B. breve* isolates

To gain more insight into the differences in genome content and functional abilities, we compared the total genomic material of the *Bifidobacterium* isolates to eight type strains of common human commensal *Bifidobacterium* species. Total DNA was isolated and hybridized on a microarray containing randomly cloned DNA fragments of *B. breve* LMG13208T. The array contained 2000 clones which together represent approximately 85% of the *B. breve* genome. Based on the draft sequence of *B. breve* DSM 20213 (GI:268158265), our array contains about 1975 genes. ^{2}Log (Cy5/Cy3) hybridization signal intensity values of the different DNA samples were analyzed by principle component analysis (PCA) (Fig. 1). Clones with 100% sample data, 1561 in total, were selected for PCA analysis in order to have reliable results. PCA plots of principle component (PC)1 and 3 (Fig. 1), visualize the variation of the data. PC1, 2, and 3, explain 53.9%, 25.9%, and 4.6% of the data variation respectively. Altogether, PC1 and PC2 predict 58.5% of the total data variation. Figure 1 shows, that NR200, which is a commercial strain, is highly similar to the *B. breve* type strain. Based on PC1 and 2, all fecal isolates cluster together relatively close to the *B. breve* type (data not shown). Interestingly, there seems to be more genetic variation within the genus *Bifidobacterium* than expected. Although all the fecal *B. breve* isolates cluster together in the PC1-2 plot, they can be split up in three groups based on the PC1-3 plot (Fig. 1). These three clusters correlated exactly with their infant origin. This means that although the feces of all studied infants contained *B. breve* species, each individual contained unique strains with significant differences in genomic composition, 30% on average (data not shown). Clearly, the genomic clusters of isolates show a large genomic diversity. This suggests the presence of possible *B. breve* subspecies. The high correlation of the hybridization patterns within one cluster suggests that the *B. breve* species is represented by one single strain in every infant. These data indicate that the population of *B. breve* within one infant does not always comprise more than one strain. Strains with the same characteristics could be competing while focussing on the same niche within the gastrointestinal tract of its host.

Our results prove that next to species assignment, the *B. breve* microarray is able to discriminate between isolates within one species. This discriminating ability results in generating sets of isolate-specific clones. Significance analysis of microarrays (SAM) was used to select 371 specific clones from the whole dataset, 23% of all clones. To study the functionalities encoded by the DNA fragments that are discriminating for the obtained clusters of *B. breve* strains we selected a subset of 43 clones for sequencing (Fig. 2). The major part of the sequenced specific clones (n=33) encoded hypothetical sequences (50%), while the rest showed homology to genes that encode for 'life style' functions (38%), such as membrane transport,

signal transduction, and the glycobiome. We have to note that an annotated *B. breve* genome is not available to date. Next to diagnostic clones we included *B. breve*-conserved clones (n=10) in our sequencing experiment. Instead of 'life style' functions, the conserved clones mainly encoded for genes involved in 'house-keeping' functions (40%), next to hypothetical proteins (40%). From these results we concluded that the genomic variation of the different groups of *B. breve* isolates is based on 'life style' characteristics. Furthermore, BLAST analysis showed that some diagnostic clones had high homology (>95%) to genes present in the genomes of other closely related *Bifidobacterium* spp. (Fig. 2). This could indicate for horizontal gene transfer (HGT) events as suggested by Delétoile *et al*. (2010).

The clones that we describe as discriminating for different strains of *B. breve* (Figure 3), and done not have high homology with other species, could be used as molecular markers. The use of molecular markers has shown to be fruitful in relation to the genetic characterization of bifidobacteria (Boesten et al., 2009).

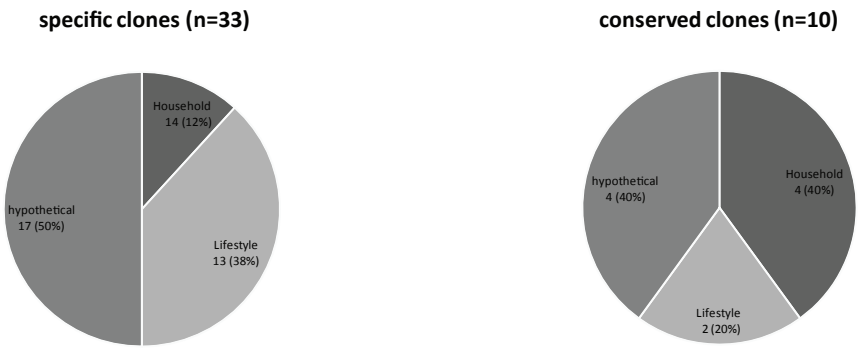

Figure 3. The composition of the KEGG orthologies (www.genome.jp/kegg/) of the sub selection of 33 specific and 10 conserved clones listed in Figure 2, according to their encoding gene homology.

ACKNOWLEDGEMENTS

We would like to thank Marleen van Nuenen, Monique Haarman, and Aldwin Vriesema for providing genomic DNA samples, and additional data, extracted from the fecal isolates. This work was supported by the Dutch Ministry of Economic Affairs through the Innovation Oriented Research Program on Genomics (IOP Genomics: IGE01016).

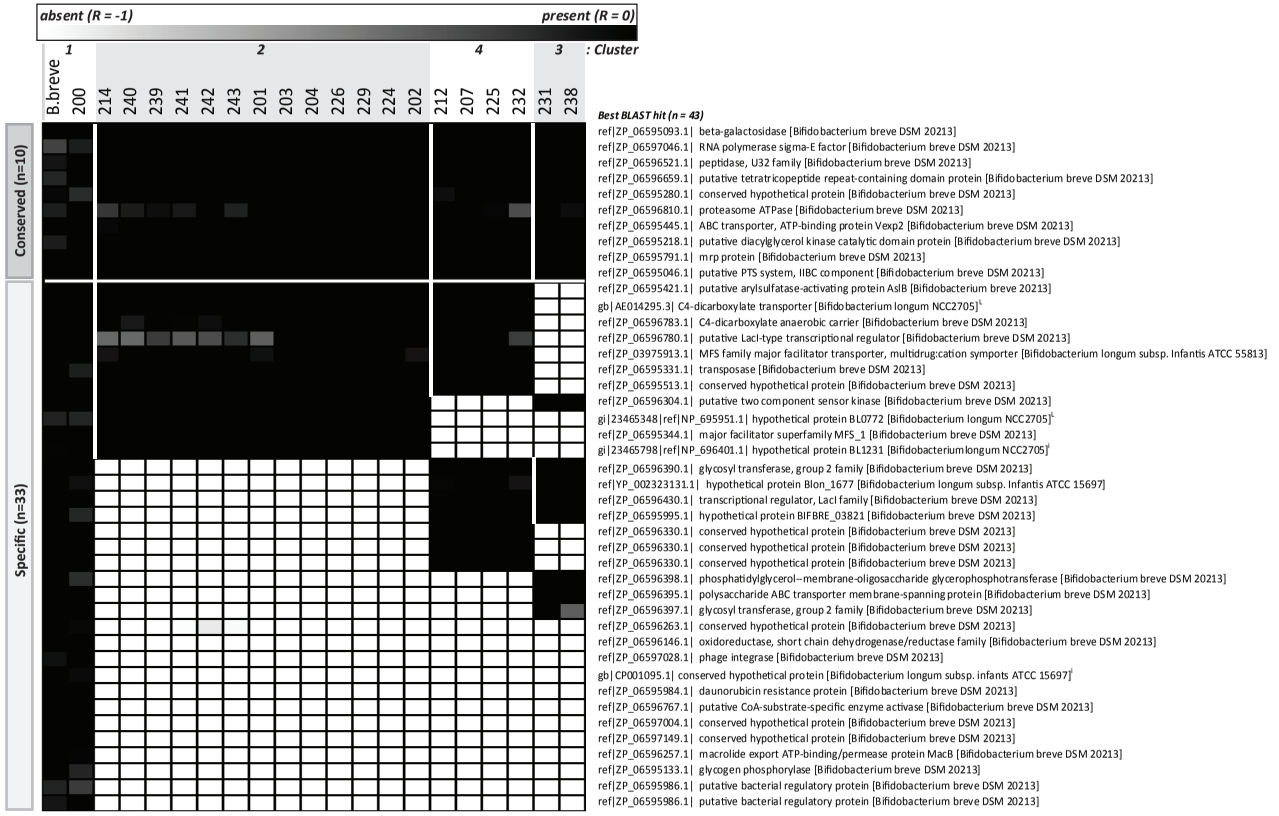

Figure 2. Heatmap, visualizing the presence or absence (according to the grey-scaled bar on top) of a sub selection of 33 specific and 10 conserved clones, from a total set of 371 specific clones (SAM analysis), with their best BLAST-hit (>95% query homology).
[L] also present in *B. longum* type strain; [I] also present in *B. longum* subsp. *infantis* type strain. The sample origins are indicated by their NR-codes on top of the matrix.





Chapter 4

Bifidobacterium population analysis in the infant gut by direct mapping of genomic hybridization patterns: potential for monitoring temporal development and effects of dietary regimens

Rolf J. Boesten, Frank H.J. Schuren, Kaouther Ben Amor, Monique Haarman, Jan Knol, and Willem M. de Vos

A bifidobacterial mixed-species microarray platform was used in a proof-of-principle study to address the composition and development of bifidobacteria in DNA extracted from fecal samples. These were collected in a time course of two years since birth and derived from human infants that were breast-fed, standard formula-fed, or received a prebiotic formula during their weaning period. A set of over 50 samples was analyzed, testifying for the throughput of the designed platform for multiple genome hybridizations. The generated data revealed that fecal samples of breast-fed infants contained a high abundance of genomic DNA homologous to *Bifidobacterium breve*. In contrast, fecal samples from standard formula-fed infants lacked detectable amounts of this *B. breve* DNA but contained genes with high similarity to *B. longum*. Remarkably, infants that received breast milk and later a prebiotic formula consisting of a standard formula milk containing a mixture of specific galacto- and fructo-oligosaccharides, continued to harbor a *B. breve*-dominant fecal population. One infant that received standard formula in combination with the additional *B. lactis* Bb12 culture, contained significant amounts of fecal DNA belonging to Bb12 but only during the period of ingestion. The microarray platform showed sufficient sensitivity to analyze the *B. breve* group at the strain level. Overall, the *B. breve* populations observed in the fecal samples of the studied infants showed a stable composition over time and were unique per infant. In conclusion, our results show the applicability of comparative genome hybridization to study bifidobacterial populations in infant fecal samples without the use of any amplification step.

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INTRODUCTION

The human gastrointestinal tract (GIT) is colonized since birth by a complex community of microorganisms that is known to impact its host (Hooper and Gordon, 2001; Vaughan et al., 2005; Zoetendal et al., 2008). The contribution of the GIT microbiota to converting dietary components into short chain fatty acids with significant caloric value, vitamins and other health-promoting compounds, has been well established. However, the interest in determining the microbial composition and its development is growing as it has been shown that the GIT microbiota has developed intimate relations with its host, communicates with its immune system, and can be affected by specific dietary components or bacteria, such as those marketed as prebiotics and probiotics (Ouwehand et al., 2005; Saxelin et al., 2005; van Baarlen et al., 2009; Zoetendal et al., 2008).

The microbial composition of the adult human gut has been subject to multiple studies and found to consist of an individual core that is relatively stable in time (Zoetendal et al., 2008). Moreover, phylogenetic analyses of adult subjects has revealed a general core of bacterial representatives shared between multiple individuals of different ages (Rajilic-Stojanovic et al., 2009; Tap et al., 2009). This was confirmed at the genomic level by recent massive parallel sequence analyses of the adult gut microbiome although at a lower level of depth (Kurokawa et al., 2007; Qin et al., 2010; Turnbaugh et al., 2009).

In contrast to the large attention for the adult microbiota, there is only limited information on the development of our intestinal microbe in early life. This is partly due to the large variability in the microbial colonization that is encountered after birth, as was observed in a longitudinal study using culture independent global analyses with a limited set of newborns in the Netherlands (Favier et al., 2002). This was confirmed in a more recent comprehensive and longitudinal studies with US babies (Dominguez-Bello et al., 2010; Palmer et al., 2007). It is likely that the first bacterial contact will be different for every individual and in most cases the intestinal inoculum for the newborn baby will derive from the mother. Deep genome sequencing studies of the mother and child intestinal microbiota appear to confirm this (Turnbaugh et al., 2009). We hypothesize that depending on the infants' environmental and host factors, certain groups of bacteria will be favored and dominate their intestinal microbiota. One group of commensal bacteria, those belonging to the genus *Bifidobacterium*, is found among the first colonizers of the baby intestine, notably when breast-fed (Benno et al., 1984; Favier et al., 2002; Harmsen et al., 2000; Penders et al., 2006). Remarkably, different results were observed in the recent studies indicating that bifidobacteria are present in only a small fraction of infants or are not numerically

dominant in babies (Hopkins et al., 2005; Palmer et al., 2007). However, this may be due to bias introduced by the methodologies of DNA extraction or PCR detection as discussed recently (Salonen et al., 2010). Alternatively, geographic origin (EU versus US), diet or both may play a role in explaining this difference that is in particular notable in view of the presumed interactions of bifidobacteria and our immune system (Boesten and de Vos, 2008; O'Hara and Shanahan, 2006). Data on the compositions of bifidobacterial populations in infants show variable results, partly reflecting the high variability between the baby microbiota. Overall, *B. longum*, *B. breve*, *B. infantis*, and *B. adolescentis* have been found in fecal samples of healthy human infants (He et al., 2001b; Matsuki et al., 1998; Turroni et al., 2009a). A broader set of species has been described for the 'adult-like' bifidobacterial microbiota including additional species of *B. catenulatum*, *B. bifidum* and *B. pseudolongum* (He et al., 2001a; Matsuki et al., 1998; Turroni et al., 2009a). Moreover, there are indications for a several new *Bifidobacterium* species in the human gut that so far have not been cultured (Ben Amor et al., 2005; Turroni et al., 2009b). The development of the infant microbial community from the time of birth to the 'adult-like' composition often follows the introduction of solid foods after weaning and its timeframe is poorly understood. Similarly, the effect of diet on the composition of the infant microbiota is also controversial. Numerous studies have found a lower abundance of bifidobacteria and a higher abundance of aerobic bacteria in the microbiota of formula-fed infants relative to breast-fed infants (Harmsen et al., 2000; Hopkins et al., 2005; Penders et al., 2006), yet other reports have found no such difference (Penders et al., 2005). An explanation could be that these studies have to cope with numerous different parameters such as differences between babies, extraction methods, sample storage, and detection platforms.

Interestingly, bifidobacteria have been detected in breast-milk samples using culture-independent molecular techniques (Collado et al., 2009; Gronlund et al., 2007; Gueimonde et al., 2007). Although it is not clear how translocation of bifidobacteria from intestine to breast-milk occurs within the mother, it has been shown that breast-milk contains similar bacteria both before and after breast-feeding, rendering the possibility of inoculation of the breast-milk by the infant less likely. Recently, it has been suggested that the colonization of breast-milk may take place by transfer of intestinal bacteria within the phagocytosing cells from the mothers gut to breast-milk (Martín et al., 2004; Perez et al., 2007). Interestingly, bifidobacterial translocation has recently been shown to occur after oral administration in a particular mouse model (Cronin et al., 2010). In addition, DNA from intestinal bifidobacteria was detected in human placenta samples. Although no bifidobacteria could be cultured, this suggests that horizontal transfer of bacterial DNA from mother to foetus may occur via the placenta (Satokari et al., 2009). These results imply that a constant

supply of bifidobacteria to the infant's intestine is thus assured during breastfeeding and will affect the development of the intestinal microbiota.

For further studying the impact of bifidobacteria or other bacterial groups, it is essential to have reliable, quantitative and identification tools with high discriminating abilities. A variety of culturing-independent microbial screening and detection techniques have been developed. Small subunit (SSU) ribosomal DNA (rDNA) sequence analysis via cloning or pyrosequencing (Andersson et al., 2008; Kwon et al., 2005; Matsuki et al., 1998), temperature/denaturing gel electrophoresis (D/TGGE) (Favier et al., 2002; Zoetendal et al., 1998) and terminal-restriction fragment length polymorphism (T-RFLP) (Zoetendal et al., 2008) are useful for identifying the dominant members of a population and for discovering new rDNA species (Nagashima et al., 2003), but inadequate for detection and quantification of rare species while they all rely on PCR amplifications. Other currently available quantitative techniques, such as fluorescence *in situ* hybridization (FISH) (Amann et al., 1990; Amann et al., 1995; Moter and Gobel, 2000), dot-blot hybridization (Hopkins et al., 2005) and real-time PCR (Haarman and Knol, 2005; Penders et al., 2005), are not easily applied to large numbers of taxonomic groups or also need an amplification reaction. Recently, 16S rRNA-based phylogenetic microarrays have been developed to study the intestinal microbial ecology of different age groups (Claesson et al., 2009; Palmer et al., 2007; Rajilic-Stojanovic et al., 2009; Zoetendal et al., 2008). Moreover, a variety of genomics approaches including comparative genome hybridization have been developed to study the functionality of bifidobacteria (Ventura et al., 2009). Previously, we described the development and application of comparative genome hybridization approach involving a Bifidobacterial Mixed-Species (BMS)-microarray platform, consisting of genomic DNA fragments that are specific for *Bifidobacterium* species and phylogenetic groups of bifidobacteria (Boesten et al., 2009). Here, we expand the use of this BMS-microarray to analyze complex *Bifidobacterium* communities in fecal samples that were obtained from six healthy human infants that were either breast-fed, standard formula-fed, or fed with probiotic of prebiotic formulas. From every infant ten fecal samples ranging from the first week to 2 years of age were analyzed for their *Bifidobacterium* DNA content and composition. Specific patterns were observed that showed the impact of the diet notably on the development of *B. breve* communities. This proof of principle study illustrates the potential of using comparative genome hybridization in fecal samples and shows population shifts at the strain level obviating the need for cultivation and amplification procedures.

RESULTS

Analysis of the bifidobacterial community based on genomic hybridization patterns

We designed a culture-independent tool for analyzing the composition of bifidobacterial communities within complex microbial samples on species and strain level by generating and mapping of genomic hybridization patterns. This tool is based on a random clone microarray containing total DNA of those bifidobacterial species that have been found in the human intestinal tract. DNA from collected fecal samples was extracted to test the applicability of the microarray platform as a molecular tool in a proof-of-principle study (Fig. 1). Fecal samples were obtained from six healthy human infants who received different diets during their weaning period. Two infants were breast-fed, two were fed with a standard formula – one of them received an additional supplement of viable *B. lactis* Bb12 cells, and two were fed with a formula containing a prebiotic mixture of galacto- and fructo-oligosaccharides (GOS and FOS, respectively). In order to study the temporal development and changes of the bifidobacterial composition, a total of 10 samples were analyzed per infant and the first sample was obtained at day 5 and the final sample at an age of 25 months (Fig. 1). The microarray results show that most fecal samples of each of the infants turned out to be *Bifidobacterium*-positive (Fig. 2). Furthermore, we generally observed a drop in hybridization signals, in general the majority (over 90 %) of the signals disappeared, from the moment the infants reached the age of 1 year. In most cases, this time-point correlates with the change of diet. Overall, during the period between 6 and 12 months of age the infants received a mixed diet. This means that next to their weaning they started to ingest solid food. This obviously affects the composition of their intestinal microbiota. To verify whether there was any effect on the total community and that of bifidobacteria in particular, we performed qPCR amplification of strategic phylogenetic groups (see below). In line with the array data the bifidobacteria qPCR values also dropped, indicating that the absolute amount of *Bifidobacterium* DNA was decreased (Fig. 2).

The presented microarray data showed that the samples from two infants who were breast-fed for at least one year, infant A and B, showed presence of DNA fragments that are highly homologous to *B. breve* DNA (Figs. 2A and 2B). The DNA samples originating from infants C and D, who were fed a standard formula, showed abundance of bifidobacterial DNA which was highly homologous to DNA of *B. longum* subsp. *longum* (Figs. 2C and 2D). Additionally, the samples isolated from the feces of infant D contained DNA that hybridized also with marker clones for the *B. animalis/B.*

lactis group. This latter observation is in concordance with the feeding trial of the viable *B. lactis* Bb12 supplement, which started from birth and ended of 12 months. The infants that received a prebiotic mixture showed a shift in the bifidobacterial composition (Figs. 2E and 2F). This shift was observed between week 8 and week 12. Interestingly, this correlates with the start of the prebiotic mixture feeding trial at week 10. From week 12, DNA homologous to *B. breve* becomes more abundant whereas DNA of other *Bifidobacterium* species disappears (Fig. 2).

Validation by qPCR analysis

To support the microarray results and to gain information on the quantitative amounts of *Bifidobacterium* species present in the fecal samples, a series of qPCR analyses were performed as summarized in heat maps (Fig. 2). Although it is a totally different approach based on specific ribosomal RNA sequences, the qPCR results were in line with the data obtained by the microarray platform. This means that when the microarray detected DNA homologous to the DNA of a specific *Bifidobacterium* species, the qPCR platform detected the presence of DNA originating from that same species. Differences between qPCR and microarray results can be explained by the fact that qPCR is a more quantitative measurement than the microarray platform. The dynamic ranges of the platforms are quite comparable and the microarray platform could detect as little as 0.1% of the DNA isolated from the fecal samples (data not shown). However, the microarray platform provided detailed information on the genetic content and shifts within one species or phylogenetic group of bifidobacteria that goes beyond the detection capacity of qPCR (see below).

Screening for genomic shifts within one *Bifidobacterium* species group in time

Due to its high resolution output, the microarray platform does not only give information on shifting of bifidobacterial species but also enables to zoom in at the strain level. Comparison of hybridization signals, results in detailed information on the shifting of bifidobacterial genomic material even within a single species, which suggests succession of strains. Using this principle, we analyzed the DNA samples and zoomed in on the composition of the *B. breve* species group of each infant (Fig. 3). We concluded that every infant contained a unique *B. breve* community, with the exception of the fecal samples of infants C and D that did not contain any *B. breve* related DNA (Fig. 3). This was observed earlier as visualized in Fig. 2. The *B. breve* populations were generally stable in time. Concentrating on the differential markers within the *B. breve* set, we detected a shift in time in the hybridization pattern of

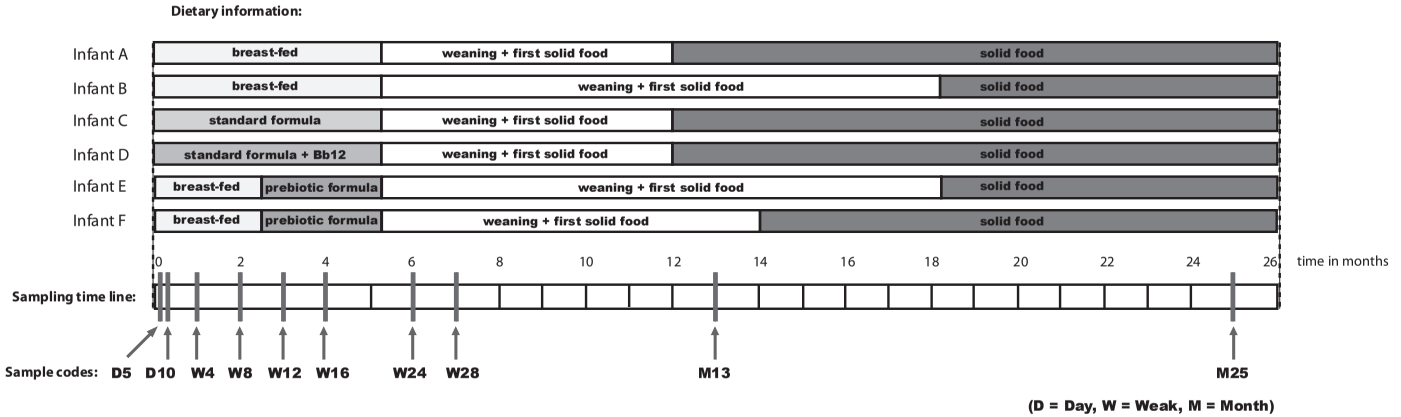

Figure 1. Schematic sample information overview. A selection of fecal samples, collected during the study described by Bakker-Zierikzee *et al.* (2005).

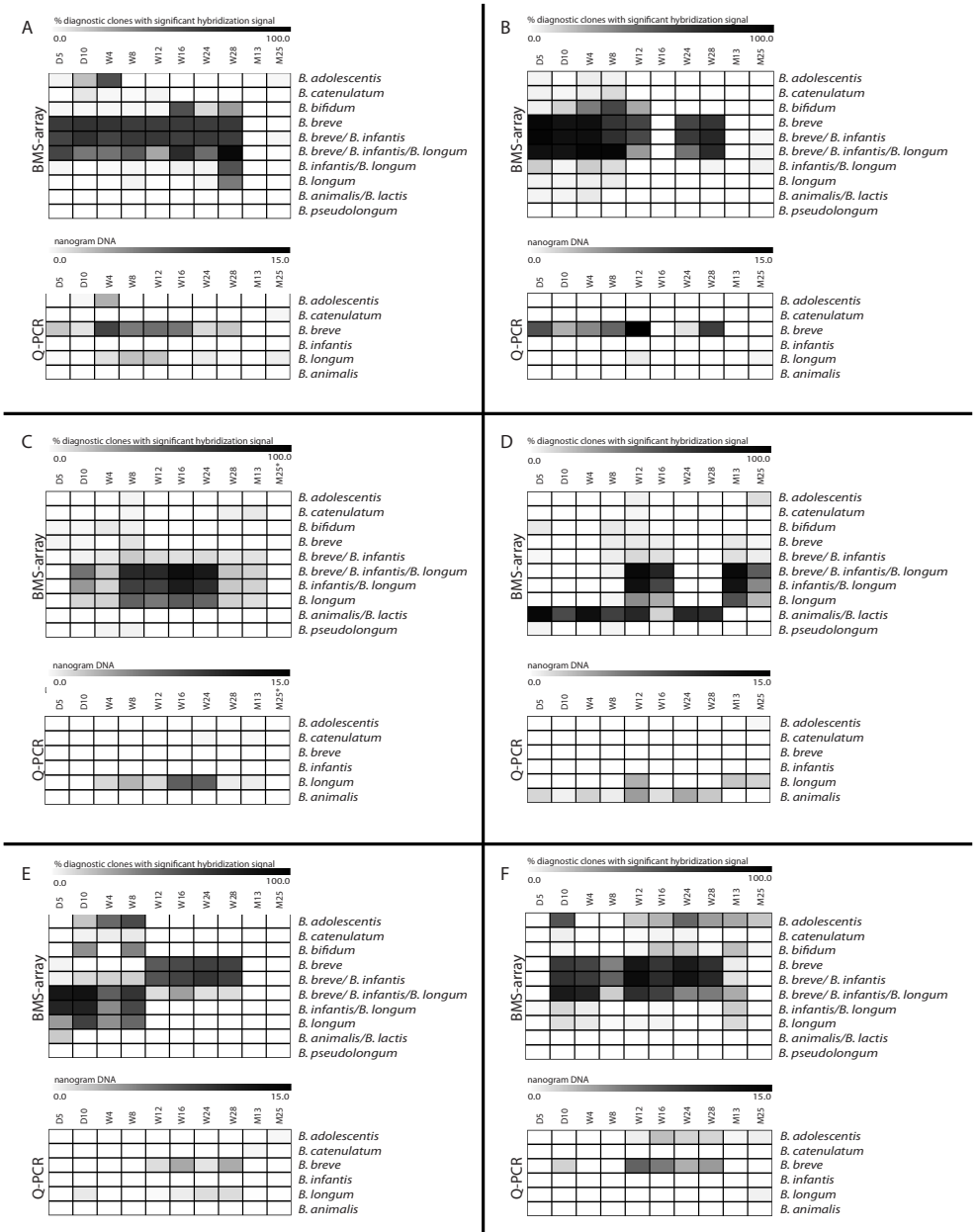

Figure 2. Heat map visualization of the microarray- and qPCR-based screening of the *Bifidobacterium* composition of fecal samples of six infants who received three different diets (Fig. 1). Time-course samples are indicated by the age of the infant (D=day, W=week, M=month). The microarray data are presented as the percentage of marker-clones, per species or species group (Table 1), that give a significant hybridization. The percentages are visualized according to the colour-bar above the heat map. The qPCR results are presented as DNA concentrations (nanograms of DNA per sample) of the different species and visualized according to the colour-bar above the heat map.The asterisk (*) indicates data not available.

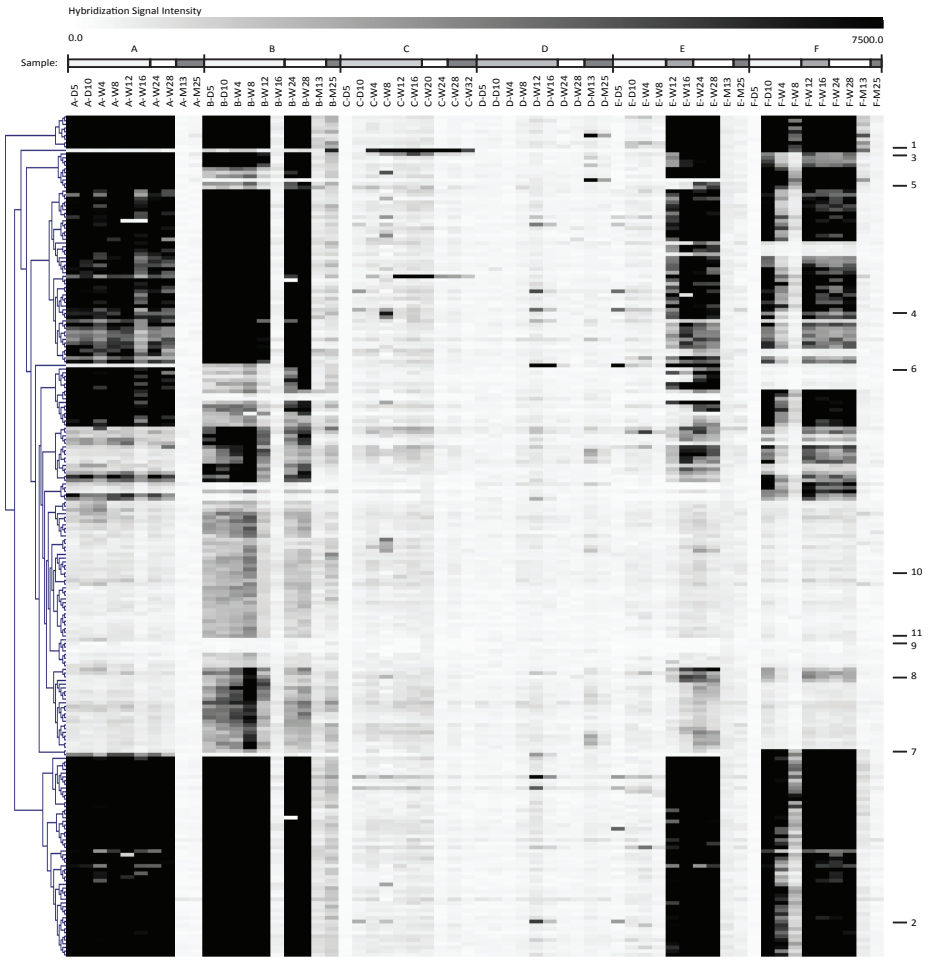

Figure 3. Hierarchical clustering (Pearson correlation) of the hybridization signals of genomic DNA fragments within the *B. breve* marker set for all fecal samples in time-course order. Signal intensities are indicated by a black-white color gradient. On the X-axis the sample codes are presented (D=day; W=week; M=month). Samples from one infant are highlighted by the bars above the codes as indicated in Figure 1.

infant B (Fig. 3). We conclude that the *B. breve* group of this infant gained and lost specific *B. breve*-specific DNA fragments at approximately 12 weeks of development. However, a core *B. breve* genome seemed to be present in both situations. We hypothesize that a *B. breve* population always contains the *B. breve* core genome plus *B. breve* strain-specific genomic fragments. To gain more insight in the genes encoded by these fragments we have sequenced a subset of 11 genomic *B. breve* markers (Fig. 4). Fragments 1 and 2 are present in all samples that are known to contain *B. breve*, based on the qPCR results. We suggest that they belong to the *B. breve* 'core-genome' (Fig. 4). Fragments 5 and 6 (Fig. 4) seem to appear in the later time-samples in infant B but were present in all time-samples of infant A. These fragments must be strain-specific. The best BLASTx hits all showed more than 98% sequence homology to genes in the published *B. breve* DSM 20213 genome (ACCG02000000) and had E-values close to zero. Fragments 9, 10, and 11 are not specific for any of the fecal isolates but only for the *B. breve* type strain that was used for defining the species-specific marker fragments (Table 1). Inspection of the other fragments showed them to code for stress related functions, proteins involved in cell envelope integrity or location hypothetical proteins. Remarkably, fragment 1 and 4 coded for a potential sugar-specific PTS transport component and a β-glycosidase that were both almost or completely absent in the babies that received standard formula. It is tempting to speculate that the used formula product lacks the sugars present in the breast milk and prebiotic FOS/GOS mixture that was used to feed the other babies.

DISCUSSION

In this study we showed that comparative genome hybridization using a bifidobacterial mixed species microarray platform can be applied as a culture-independent tool to analyse the composition and development of *Bifidobacterium* populations in fecal samples from human infants. Using this microarray platform we obtained a high resolution data output which enabled us to zoom in towards the species and strain level of the bifidobacterial population. An advantage of our approach is that we did not need to enrich our DNA samples for bifidobacterial DNA. While qPCR is more quantitative than the microarray platform, both approaches have similar sensitivity and dynamic range. Our observations showed differences in composition of fecal bifidobacterial communities in developing infants. As this study serves as a proof of principle, we want to stress that these results are based on a limited set of infants.

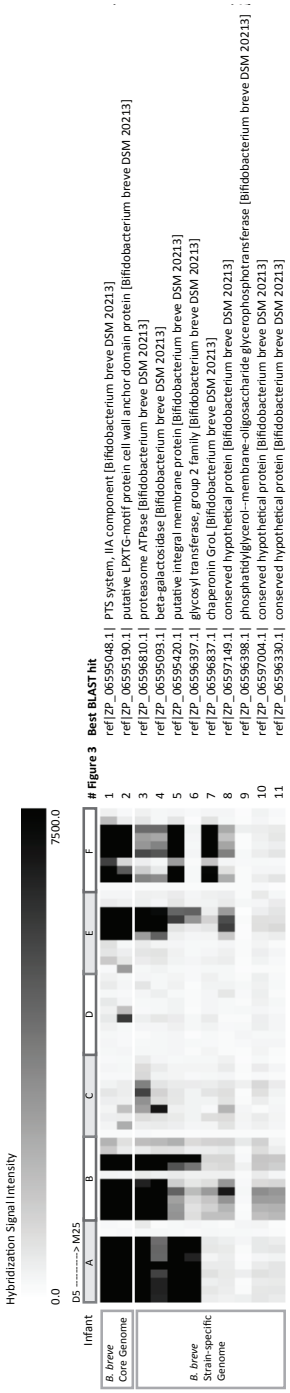

Figure 4. Hybridization signal intensities and 'best BLASTx hits' (>98% homology) of a subset of 11 *B. breve* markers that have been sequenced are visualized for each marker fragment. The sample order is similar to the order in Figure 3. Marker fragments 1, and 2 can be described as *B. breve* core genome whereas marker fragments 3 to 11 show to be strain-specific.

Nevertheless, the data suggests an obvious link between diet and *Bifidobacterium* population. Moreover, our results indicate that the prebiotic formula applied in this study affects the composition of the intestinal *Bifidobacterium* population. As indicated previously (Bakker-Zierikzee et al., 2005), the composition of the infants who received the FOS/GOS mixture seems to be shifted towards the direction of the *Bifidobacterium* community as found in infants who were breast-fed, in contrast to the standard-formula diet (Fig. 2). In line with other studies no *B. pseudolongum* homologous DNA was detected in the fecal samples tested (Benno et al., 1984; Favier et al., 2002; Favier et al., 2003; Haarman and Knol, 2005; Harmsen et al., 2000; Matsuki et al., 1999). In conclusion, we expect that in combination with a large well-defined sample set, the described comparative hybridization approach with the microarray platform will contribute to a better understanding of our intestinal microbial ecology. Furthermore, this application could be combined with next generation sequencing to generate a pan-genomic *Bifidobacterium* screening tool.

EXPERIMENTAL PROCEDURES

Sample collection, DNA isolation and qPCR analysis

A selection of fecal samples, collected during the study described by Bakker-Zierikzee et al. (2005), formed the basis

of this study (Bakker-Zierikzee et al., 2005). Detailed information on the samples is visualized in Figure 1.

For the total DNA isolation from fecal samples, a 96 wells plate (Axygen, VWR, Amsterdam, NL) was filled with 250 µl 0.1 mm zirconium beads (Brunschwig Chemie, Amsterdam, NL) washed in lysis buffer without detergents (Agowa, Berlin, D), 200 µl phenol solution (Tris pH 8.0), and 250 µl lysis buffer without detergents (Agowa GmbH, Berlin, D). Subsequently, per well a volume of a fully loaded inoculation loop, (Greiner Bio One, Alphen a/d Rijn, NL), resembling 10 µl of fecal sample was added to the mixture. After 2 min of bead beating (Mini Beadbeater, Biospec Products, Lab Services, Breda, NL) the plate was cooled on ice. Then the plate was centrifuged for 10 min at 1,700 x g and 20°C. The supernatant, about 175 µl, was transferred into a 0.5 ml 96 wells plate (Axygen, VWR, Amsterdam, NL) and 100 µl phenol solution (in Tris pH 8.0) was added. After 10 min of centrifuging at 1,700 x g and 20°C the supernatant, per well approximately 150 µl, was transferred into a 96 wells PCR plate (Bioplastics, Landgraaf, NL). The DNA was extracted by magnetic beads using the Agowa DNA extraction kit (http://www.lgc.co.uk/divisions/life__food_sciences.aspx) according to the protocol supplied by the manufacturer. The supernatant, containing the DNA, was stored at -20°C.

qPCR analysis was performed as described by Haarman and Knol (2005). Finally, the qPCR data was visualized using TIGR MeV 3.1 Software (http://www.tm4.org/mev.html).

Array design and construction

A genomic library was constructed from seven strains representing seven different *Bifidobacterium* species as described previously (Boesten et al., 2009) with the difference that the *B. pseudolongum* library has been replaced by two single strain libraries of *B. breve* LMG 13208T (BRE) and *B. catenulatum* LMG 11043T (CAT). These strains were obtained from the Belgian Co-ordinated Collections of Microorganisms (BCCM), Ghent, B. In total this microarray contains four different libraries. The libraries of BRE and CAT were constructed from genomic material and contain 2000 clones which represent about 85% (calculated according to Akopyants *et al.* (2001)) of the genomes. Except for the fragmentation of the total DNA of *B. catenulatum*, the construction of the BRE and the CAT libraries was performed as previously described in Boesten *et al*. (2009). The *B. catenulatum* DNA was fragmentized by blunt-ended partial digestion using Hae III (New England Biolabs, Westburg, Leusden, NL). 10 µl total DNA (~600 ng/ µl) was combined with 9 µl reaction buffer 2 (New England Biolabs, Westburg, Leusden, NL), 1 µl HaeIII, and 80

µl milli Q water and subsequently incubated at 37°C for 20 min. After incubation the DNA samples were loaded in 1.5% agarose/1 x TAE gel. Samples sized from 1 to 2 kb were isolated from gel during electrophoresis using Takara Recochips (TaKaRa Bio, Lonza Verviers, Verviers, BE) according to the protocol provided by the supplier. Due to the blunt-end restriction of HaeIII, end-repair as described in Boesten *et al*. (2009) was not necessary.

Fluorescent labeling and hybridization

Total DNA samples were labeled by random prime labeling. A volume of 1 µl DNA sample was diluted in 4.25 µl H_2O and mixed with 5 µl of 2.5 x random primer (BioPrime Kit, Invitrogen, Breda, NL). The mixture was incubated at 96°C for 5 min. After cooling on ice, the samples were shortly centrifuged at maximum speed in an eppendorf centrifuge. Subsequently the following components were added while the samples were kept in ice: 0.25 µl 50 x AminoAcid-dNTP mix (AA-dNTP) (Invitrogen, Breda, NL), 1.75 µl milliQ, and 0.25 µl Klenow fragment (3-9 U/µl) (Invitrogen, Breda, NL). After addition the samples were incubated at 37°C for 1.5 hours for amplification. Unincorporated AA-dUTP and free amines were removed using QIAqiuck columns following the protocol described by the supplier (Qiagen Benelux BS, Venlo, NL). For this purpose, five times the reaction volume of PB buffer (Qiagen supplied) was added to the samples and the mixture was transferred to a QIAqiuck Spin Column that was centrifuged at 20,000 x g for 1 min. To increase the binding efficiency, the flow-through was reloaded and the column was centrifuged again at 20,000 x g for 1 min and the flow through was discarded. To elute the DNA samples from the columns they were first washed separately with 500 µl of 80% ethanol and then eluted with 30 µl of milliQ H_2O, a step that was repeated twice as described by the manufacturer (Qiagen Benelux, Venlo, NL). The elutate was dried at high temperature in a speed vac for about 15-30 min. For coupling of the Cy Dye Esters to the AA-cDNA the dried cDNA samples were dissolved in 4.5 µl 0.1 M sodium carbonate buffer (Na_2CO_3), pH 9.0 during 10 min at room temperature while mixed a few times. Subsequently, 4.5 µl of the appropriate NHS-ester Cy Dye (Amersham, Piscataway, USA) were added. This reaction mix was incubated for 1 h at room temperature in the dark. After incubation, 10 µl of milliQ H_2O was added and the samples were centrifuged shortly. To remove the unincorporated dyes we used Autoseq G50 (Amersham, Piscataway, USA) columns according to the protocol supplied by the manufacturer.

DNA microarrays were prehybridized for 45 min at 42°C in prehybridization solution (1% bovine serum albumin, 5 x SSC, and 0.1% SDS). Co-hybridizations of labeled DNA samples were performed overnight at 42°C in 40 µl Easyhyb buffer

(Roche, Almere, NL) according to the manufacturer's protocol. Slides were washed twice in 1 x SSC, 0.2% SDS at 37°C, once in 0.5 x SSC, and twice in 0.2 x SSC at RT and dried with N_2-flow.

Microarray scan-image and quality analysis were performed as described previously (Boesten et al., 2009).

Diagnostic clone selection, visualization, and clustering analysis

For the selection of diagnostic clones we normalized the obtained hybridization data as described previously (Boesten et al., 2009). For each *Bifidobacterium* species we defined diagnostic clones. A diagnostic clone has a 'high homology' classification for a specific *Bifidobacterium* species and a 'low homology' classification for all other species. Due to the genetic variation between species and the different sizes of the libraries printed on the array the numbers of markers vary per species of species group. The number of diagnostic clones for every species or group of species is listed in Table 1.

For visualization of the produced hybridization values, the data was converted into percentages of marker clones that show significant hybridization signals (Boesten et al., 2009). This was performed for every marker group on the microarray (Table 1). The percentages give an indication for the rate of similarity of the genomic DNA fragments for each *Bifidobacterium* group, present in the total DNA sample isolated from the fecal sample. Finally, this data was visualized using TIGR MeV 3.1 Software (http://www.tm4.org/mev.html).

The hierarchical clustering of untreated Cy5 signal intensity data, based on a Pearson correlation, was performed and visualized with TIGR MeV 3.1 Software (http://www.tm4.org/mev.html).

Sequencing of DNA fragments

Plasmid isolation, purification of PCR products and high throughput sequencing were performed by GATC Biotech AG, Konstanz, Germany (www.gatc-biotech.com). Sequences were compared with public databases by using BLASTx and BLASTn. Blast analyses were performed against all sequenced genomes in the database using the NCBI website (http://www.ncbi.nlm.nih.gov/).

Table 1. Marker groups, the number of marker clones they include, and the strains of origin.

Marker group names	Strains of origin											Number of marker clones
	B. adolescentis LMG 10502^T (Reuter, 1963)	*B. animalis* subsp. *animalis* LMG 10508^T (Scardovi et al., 1970)	*B. animalis* subsp. *lactis* LMG 18314^T (Masco et al., 2004; Meile et al., 1997)	*B. bifidum* LMG 11041^T (Orla-Jensen, 1924)	*B. breve* LMG 13208^T (Reuter, 1963)	*B. breve* NCIMB 8807 (Leahy et al., 2005; Reuter, 1963)	*B. longum* biotype *infantis* LMG 8811^T (Reuter, 1963; Sakata S et al., 2002)	*B. longum* LMG 13197^T (Reuter, 1963)	*B. longum* NCC 2705 (Reuter, 1963; Schell et al., 2002)	*B. catenulatum* LMG 11043^T (Scardovi and Crociani, 1974)	*B. pseudolongum* subsp. *pseudolongum* LMG 11571^T (Yaeshima et al., 1992)	
B. adolescentis	*											141
B. animalis/B.lactis		*	*									68
B. bifidum				*								49
B. breve					*	*						227
B. breve/B. infantis					*	*	*					129
B. breve/B. infantis/ B.longum					*	*	*	*	*			204
B. infantis/B. longum							*	*	*			127
B. longum								*	*			167
B. catenulatum										*		1348
B. pseudolongum											*	103

* indicates which marker groups are based on which strains.

ACKNOWLEDGEMENTS

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Chapter 5

Mixed-species genomic microarray analysis of fecal samples reveals differential transcriptional response of bifidobacteria in breast- and formula-fed infants

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Although their exact function remains enigmatic, bifidobacteria are among the first colonizers of the intestinal tract of newborn infants, and further develop to abundant communities, notably in response to the diet. Therefore, the transcriptional response of bifidobacteria was studied in rapidly processed fecal samples from young infants that were fed either breast milk or a formula containing a mixture of galacto- and fructoligosaccharides were studied. The presence and diversity of the bifidobacterial fecal communities were determined using PCR-denaturing gradient gel electrophoresis and quantitative real-time PCR for specific species. Changes in total number of bifidobacteria as well as in species diversity were observed, indicating the metabolic activities of bifidobacteria in the human gut. In addition, total RNAs isolated from infant feces were labeled and hybridized to a bifidobacterium-specific microarray comprising approximately 6,000 clones of the major bifidobacterial species of the human gut. Approximately 270 clones that showed the most prominent hybridization with the samples were sequenced. Fewer than 10% of the hybridizing clones contained rRNA genes, whereas the vast majority of the inserts showed matches with protein-encoding genes predicted to originate from bifidobacteria. Although a wide range of functional groups was covered by the obtained sequences, the largest fraction (14 %) of the transcribed genes assigned to a functional category were predicted to be involved in carbohydrate metabolism, while also some were implied in exopolysaccharide production or folate production. A total of three of the above-described protein-coding genes were selected for quantitative PCR and sequence analysis, which confirmed the expression of the corresponding gene and the expected nucleotide sequences. In conclusion, the results of this study show the feasibility of obtaining insight into the transcriptional responses of intestinal bifidobacteria by analyzing fecal RNA and highlights the *in vivo* expression of bifidobacterial genes implicated in host-related functions.

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INTRODUCTION

Following birth, the virtually sterile gastro-intestinal tract of neonates become rapidly colonized by microbial communities, collectively know as microbiota, which rapidly increase in complexity (Favier et al., 2002)). The vast majority reside in the colon, where densities approach 10^{11}–10^{12} cells per gram, the highest recorded for any microbial habitat (Whitman et al., 1998). Here, hundreds of bacterial species form a bacterial community in which bifidobacterial species can constitute up to 60 % of the total population in infants (Harmsen et al., 2000). It has been shown previously that various environmental factors affect the microbiota development, including the feeding regimen of the infant (Harmsen et al., 2000). Bifidobacteria are heterofermentative, non-motile, non-spore forming rods; these gram-positive bacteria have high G+C contents in their genomic DNA and belong to the *Actinobacteria* phylum, within which they form a distinct order (Biavati and Mattarelli, 2001). At present, the genus *Bifidobacterium* includes 32 species and 9 subspecies, many of which have been isolated from fecal sources (Ventura et al., 2007a). The species mostly isolated from samples obtained from breast-fed or formula-fed infants is *Bifidobacterium breve*, followed by *B. longum* ssp. *infantis*, *B. longum* and *B. bifidum* (Marteau et al., 2001). In addition, *B. catenulatum*, *B. adolescentis*, *B. pseudolongum*, and *B. dentium* have been detected but less frequently (Haarman and Knol, 2005). It has been reported that the postnatal maturation of a balanced immune system requires a constant microbial stimulation from the developing intestinal microbiota (Cebra, 1999; Hooper, 2004). Moreover, the intestinal microbiota has been claimed to have many beneficial effects, and specifically, the bifidobacteria that have been implicated in protection against pathogens (Gibson and Wang, 1994), the normal development and maintenance of a balanced immune system (Cebra, 1999; Hooper, 2004; Schiffrin and Blum, 2002), and the exertion of positive nutritional effects for the intestinal cells and the host (O'Sullivan, 2001). In spite of the numerous studies on the diversity of bifidobacteria in the human intestine, insight in the specific activity and function of bifidobacteria in the gastrointestinal tract remains very sparse. Most studies have focused on molecular techniques targeting the 16S rRNA genes, such as PCR-denaturing gradient gel electrophoresis (PCR-DGGE) (Satokari et al., 2001), fluorescent *in situ* hybridization (FISH) (Harmsen et al., 2002), quantitative real-time PCR (qPCR) (Haarman and Knol, 2005), and more recently, DNA microarrays (Zoetendal et al, 2008), to identify and quantify the different intestinal inhabitants of the gut. However, a new era has started with the sequence characterization of bifidobacterial genomes (Ventura et al., 2009). *In silico* analysis of the total genome sequence of *B. longum* NCC2705 predicted this bacterium to be adapted to a special colonic niche (Schell

et al., 2002). Several genes are predicted to encode for transcriptional regulators, which allow quick and stringent responses to environmental changes. Moreover, some genes are predicted to code for proteins that show homology to glycoprotein-binding fimbriae, structures that may be involved in adhesion and persistence in the gastrointestinal tract (Schell et al., 2002). Unfortunately, only a few complete bifidobacterial genomes have been reported, and only the full annotation of *B. longum* NCC2705 (Schell et al., 2002), *B. longum* DJO10A (Lee et al., 2008), *B. adolescentis* ATCC15703 (Ventura et al., 2007a), *B. longum* ssp. *infantis* ATCC15697 (Sela et al., 2008), and *Bifidobacterium animalis* ssp. *lactis* (Kim et al., 2009) have been made publicly available. A significant portion of the *B. adolescentis* genome differs from the *B. longum* genome, reflecting the evolutionary difference between these species based on 16S rRNA genes. Genes coding for lacto-N-biose (LNB) phosphorylase, 1,2-α-L-fucosidase and endo-α-N-acetyl galactosamidase, which are associated with host-bacterial interaction, were absent in the *B. adolescentis* genome, suggesting an alternative strategy for this species to interact with the host (Ventura et al., 2007a).

Genomic sequences, however, only provide a static view, and insight in the expression levels of the predicted genes in the intestinal tract is not yet available. In the present study we investigated the feasibility of using rapidly processed fecal samples of infants to determine the bifidobacterial transcriptome with a DNA microarray based on 6,000 clones from a shotgun library, hereby bypassing the need for genome sequence information (Parro and Moreno-Paz, 2003). This mixed species microarray was recently developed and used to study the transcriptome of *B. longum* grown in vitro in the presence of human milk (Gonzalez et al., 2008). To improve our understanding of the interaction between bifidobacteria and the host, fecal microbiota of infants receiving solely human breast milk and those receiving an infant formula containing a mixture of galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) (Arslanoglu et al., 2007; Moro et al., 2006), were studied at the transcriptional and diversity level. The results reveal the expression of a selection of genes that may be related to the adaptive responses of these species to the physiological gastrointestinal conditions.

MATERIALS AND METHODS

Subjects

In a preliminary study, the transcriptome of fecal microbiotas from four infants ranging in age from 1 week to 10 months was evaluated to test the feasibility of the selected approach for the detecting the expression of protein-encoded genes with the clone-based microarray described previously (Boesten et al., 2009). High-quality RNA preparations obtained from infants of various ages contained only a low number of sequences complementary to rRNA (less than 10% of the total hybridizing clones; data not shown). Hence, we systematically monitored two breast-fed infants (infants 1 and 2) and three formula-fed infants (infants 3 to 5) over time in a controlled dietary intervention. During the trial, the formula-fed infants received a milk-based formula containing a mixture of GOS and long-chain FOS (lcFOS) (9:1 [wt/wt]) (Arslanoglu et al., 2007; Moro et al., 2006) that was provided by Danone Research, Centre for Specialised Nutrition, Wageningen, The Netherlands. All infants were healthy Caucasians delivered normally. Their ages at the start of the intervention were on average 8 months (breast-fed infants 1 and 2 were between 6 and 7 months old, and formula-fed infants 3, 4 and 5 were between 6 and 10 months old). They received solely breast or formula milk prior to and during the sampling, in addition to small amounts of solid food after 6 months, as is common practice in The Netherlands. The formula-fed infants received a standard formula without GOS-lcFOS prior to the trial. The infants were monitored for up to 8 weeks, and periodically, fecal samples were collected and processed as described below. Written consent was obtained from parents of each infant.

PCR-DGGE analysis and qPCR

DNA was isolated from mechanically disrupted cells (Yaeshima et al., 1992) from stool samples stored at -20°C and was purified using the QIAamp DNA stool minikit (Qiagen Sciences, Maryland, USA). PCR and DGGE analysis of the V6-to-V8 regions of the 16S rRNA genes of the total microbial community were performed as described previously using 16S rRNA gene-targeted primers 968-f/1401-r (Nubel et al., 1996). The V6-to-V8 regions of the 16S rRNA genes of the bifidobacterial population were targeted using Im26-f/Im3-r, followed by Bif164-f/Bif662-rGC (Satokari et al., 2001; Zoetendal et al., 2001).

qPCR analysis of DNA extracts was performed in triplicate as described previously (Haarman and Knol, 2005) to obtain percentages for the total bifidobacterial community as well as specifically *B. adolescentis, B. angulatum, B. animalis, B. bifidum, B. breve, B. catenulatum, B. dentium, B. infantis,* and *B. longum*.

Total RNA isolation

Fecal samples were collected at three time points and immediately mixed with RNAlater (Ambion, Austin, TX, USA) at a ratio of 1:2 [wt/vol], and the mixtures were incubated overnight at 4°C and stored at -20°C until further processing as described previously (Zoetendal et al., 2006).

Design and fabrication of a clone library-based DNA microarray

Glass spotted microarrays were constructed as described previously (Boesten et al., 2009) and contained approximately 5,000 spots of 1- to 2-kb PCR amplicons that were derived from plasmid inserts in genomic libraries of *B. longum* LMG 13197 (Reuter, 1963), *B. pseudolongum* subsp. *pseudolongum* LMG 11571 (Yaeshima et al., 1992), *B. adolescentis* LMG 10502 (Reuter, 1963), *B. animalis* subsp. *animalis* LMG 10508 (Mitsuoka, 1969), *B. bifidum* LMG 11041 (Orla-Jensen, 1924), *B. catenulatum* LMG 11043 (Scardovi and Crociani, 1974). Slides were stored in the dark under dust-free conditions until further use. Before the addition of the hybridization mix, the slides were prehybridized for 45 minutes at 42°C in a solution of 5 x SSC (1 x SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% sodium dodecyl sulfate and 10 mg/ml bovine serum albumin, washed in milliQ water and isopropanol, and then dried. Labeling, hybridization, and washing were performed as described previously (Boesten et al., 2009). In all instances, Cy3-labeled DNA sequences from the bifidobacterial strains used to create the clone library employed as a reference.

Microarray analysis

The hybridized microarrays were analyzed with a ScanArray Express 4000 scanner (Perkin-Elmer, Wellesley, USA). Fluorescent images were captured in a multi-image-tagged image file format and analyzed with ImaGene software (BioDiscovery, Marina del Rey, USA). As expected, labeled chromosomal DNA of all bifidobacterial species on the microarray showed hybridization to virtually all spots on the array. Spots that were flagged by the ImaGene software (BioDiscovery) were not included in the data analysis. In total, 5,314 spots met the set quality criteria and were included in the analysis. For each spot, the mean signal intensity was divided by the local background intensity and considered to indicate positive hybridization when it was at least 1.25 times higher than the background. The hybridization intensity values were normalized to the total intensity to enable the comparison of results from different microarrays. Spots that did not show positive hybridization to any of the cDNA samples were removed for further analysis. Approximately 500- to 800-bp regions of both the 3' and 5' ends of the inserts in selected clones were subsequently sequenced using

primers SL1 and SR2, based on pSMART (GATC Biotech, Germany) (Gonzales et al., 2008). The Smith and Waterman algorithm (Smith and Waterman, 1981) was applied to evaluate the sequences against different data sets. All sequences were analyzed against the complete protein and nucleotide sequences for all genomes with complete sequences present in the NCBI repository (as of February 2009). In addition, specific searches were performed using the complete genome sequences of *B. longum* NCC2705, *B. adolescentis* ATCC 15703, *B. longum* subsp. *infantis*, and *B. animalis* subsp. *lactis*. Following these searches, all matches with the genome sequences were analyzed for annotated features by using the protein and RNA annotation files for the genomes.

Principal Component Analysis

Principal component analysis was performed using Canoco software package 4.5 (Biometris, Wageningen, The Netherlands) to assess the impact of the diet on the hybridization of the total RNAs isolated from the fecal microbiotas. Redundancy analysis (RA) was applied in specific cases to explain the contribution of the diet (breast milk or formula) to the hybridization data (calculated as the hybridization intensity divided by the local background intensity for each clone). Community similarities were graphed by using ordination plots with scaling focused on the dietary difference. The ordination plot of species and environmental variables is characterized by biplots that approximate the weighed averages of each species with respect the environmental variable (a diet of either breast milk or formula). To test significance of the relationship of the hybridization results with the dietary group, unrestricted Monte Carlo permutation tests were performed with 499 random permutations and significance level (*P* value) of 0.05.

qPCR

Primers were designed using Primer express 1.5a (Applied Biosystems, Nieuwerkerk aan den Ijssel, The Netherlands), which also takes the presence of secondary structures, including possible primer-dimer, into account. All primers were designed to have melting temperatures of 60 to 70°C and amplicon sizes between 70 and 130 bp. The specificities of the primers to bifidobacteria were evaluated by nucleotide similarity searches with the BLAST algorithm for short, nearly exact matches at the NCBI website (http://www.ncbi.nlm.nih.gov) (McGinnis and Madden, 2004). *In silico* comparisons and PCR amplification products confirmed that primer sets were specific for both *B. longum* NCC2705 and *B. longum* LMG 13197 but did not target other organisms, including *Lactobacillus plantarum* WCFS1 1 and *Escherichia coli* (data

not shown). Target genes were LNB phosphorylase (BL1641) gene, with primerset 5'-AACCGTACAAGGACGGATTCG-3'/5'-CGGAATATCGGCGATCATGC-3'; α-L-arabinosidase (BL1665) gene, with primer set 5'-TACACGCAACGGCCAAGG/5'-CCAGCAGGACCATCTGACC-3'; and the thymidylate synthase (BL0544) gene, with primer set 5'-CACGTGCATATTTGGGATGAGTG-3'/5'-CCAGGAACGCCACTGCAC-3'. iQ Sybr green supermix (Bio-Rad) was used in all reactions. iQ5 real-time PCR detection system (Bio-Rad) was used for all qPCR analyses. Each reaction was carried out in a solution containing 5.0 µl of cDNA, 12.5 µl power Sybr green master mix (Applied Biosystems), the forward and reverse primers (2 µM each), and 6.5 µl distilled water. The PCR thermal protocol applied consisted of a 2-min 95°C denaturation step, followed by 45 repeats of a 15-sec 95°C denaturation step, a 30-sec annealing step (at a temperature defined for each primer set), and a 30-sec extension step at 72°C. A melting curve analysis was performed after final amplification period via a temperature gradient from 60 to 95°C. Standard curves for quantification were based on dilution series of DNA of *B. longum* NCC2705. PCR products were sent to GATC Biotech (Germany) for purification and sequencing to confirm specific amplification of the target gene.

RESULTS AND DISCUSSION

Temporal stability and diversity of the predominant bacterial community as determined by PCR-DGGE

The fecal microbiotas of infants is influenced by transitions between breast milk, formula and solid foods (Benno et al., 1984; Favier et al., 2002; Harmsen et al., 2000). Hence, prior to gene expression studies, we compared the diversities of the microbiotas, including bifidobacteria, in five partly age-matched infants that were breast-fed (infants 1 and 2) or subjected to a diet of formula containing prebiotic oligosaccharides (infants 3, 4 and 5). Both the expression of bifidobacterial genes (see below) and the bacterial diversity were analyzed over a period of 2 to 7 weeks, and samples were taken periodically.

The diversities of the total fecal microbiotas and the bifidobacterial population was determined by DGGE analysis of the 16S rRNA amplicons. Analysis of the diversities of the bifidobacterial communities revealed relatively simple and stable patterns both for breast- and formula-fed infants.This finding is illustrated for representative infants 2 and 5, of the same age (6 to 7 months), who received breast milk and formula, respectively, and showed the same dominant bifidobacterial species for several

weeks (Fig. 1). In contrast, the DGGE profiles representing the diversities of the total bacterial communities from the breast-fed infants were much more stable than those from the formula-fed infants. This finding is illustrated ifor the same infants whose results are presented in Fig. 2. This stability is especially notable when the sample time is considered, as the community in the formula-fed infant fluctuated considerably in a period of two weeks while that in the breast-fed infant showed virtually the same microbial composition for 6 weeks. Similar observations were made with the fecal samples from the other infants (data not shown).

As DGGE analysis of PCR amplicons is qualitative rather than quantitative (Zoetendal et al., 2008), a qPCR approach was used to determine the temporal development of the total number of bifidobacteria (Fig. 3). This analysis showed that the total bifidobacterial numbers in the breast-fed infants were higher than than those in the formula-fed infants at the start of the intervention but that the numbers in the formula-fed infants increased significantly over time, due possibly to the intake of the oligosaccharide-containing formula (Fig. 3). qPCR analyses of specific *Bifidobacterium* species indicated that there were few significant differences between breast- and formula-fed infants and that numbers of these species in the breast-fed infants were initially higher (Fig. 3). In samples from all infants, *B. animalis* and *B. dentium* were not detectable and *B. angulatum* was present in very low numbers. In addition, *B. longum* subsp. *infantis*, *B. breve*, *B. bifidum*, and *B. longum* subsp. *longum* were detected in samples from all infants, with *B. longum* subsp. *infantis* being the major species found. *Bifidobacterium adolescentis*, which is most commonly found in adults, was detected at a low proportion in samples from the formula-fed infants but not at all in those from infants receiving breast milk. In conclusion, these analyses indicated that the samples from the formula-fed infants contained fewer bifidobacteria than those from the breast-fed infants but showed higher levels of diversity among bifidobacterial species. During the period in which the infants received the oligosaccharide-containing formula, the fecal bifidobacterial diversities and quantities changed and approached those in the samples from the breast-fed infants, which is in line with previous findings (Knol et al., 2005; Moro et al., 2006).

Transcriptome of the fecal bifidobacterial population

To gain insight in the activity of the bifidobacterial population within the fecal microbiota, transcripts were profiled using a mixed-species microarray of bifidobacteria (Boesten et al., 2009). In parallel to the fecal microbial diversity analysis, samples from the same five partly age-matched infants (infants 1 to 5; see Materials and Methods) with specifed diets (breast milk or formula) were compared using the microarray at three

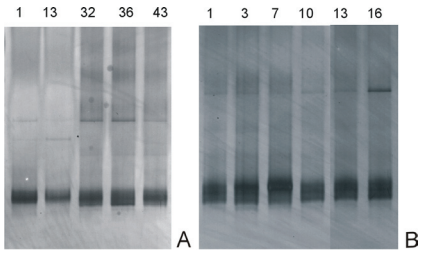

Figure 1. DGGE profiles of the fecal bifidobacteria from breast-fed infants 2 (A) and formula-fed infant 5 (B) at different time points (sampling days are indicated at the top).

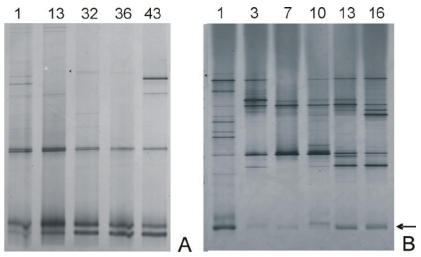

Figure 2. DGGE profiles of the fecal microbiotas of breast-fed infant 2 (A) and formula-fed infant 5 (B) at different sample points (sampling days are indicated at the top). The arrow identifies bands in the gel that correspond to the bifidobacterial populations.

time points in a 2- to 7-week peroid. A total of 4,524 clones showed positive hybridization of cDNA to at least one of the samples on the microarray, and the total sums of the fluorescent signals were comparable between arrays (data not shown). A selection of the positively hybridized clones was sequenced and showed a wide range of protein-encoding genes, indicating the metabolic activities of the fecal bifidobacterial communities and the power of this mixed-species clone library-based microarray.

It is important that the microarray consists of cloned genomic fragments derived from a mixture of six *Bifidobacterium* spp. and that the hybridization took place under stringent conditions – hence, the expression of only the cloned genes of these specific strains or genes that share significant (generally more than 80%) sequence similarity and, therefore, are predicted to have the same function as their cloned homolog could be monitored.

Statistical analysis, performed with Canoco software package, was performed to identify whether host, environment, or stochastic effects explained grouping of the studied samples. The RA showed that 44% of the difference could be explained significantly (*P*-value of 0.004) by the difference in diet, either breast milk or formula. The RA ordination plot for the hybridizations of the three samples from infants 2 to 5 visualizes the effects of the different diets (Fig. 4). The formula-fed infants differed from each other more than the breast-fed infants differed from each other, possibly because one of the formula-fed infants was 3 months older than the other infants in the group. However, all individuals within one diet group did not differ significantly from each other (*P* values above 0.05).

A set of approximately 250 significantly hybridizing clones was selected for sequencing to predict the functional identities of the genes carried on the inserts. Most of the sequences obtained (500-800 bp) did not include complete genes, but the vast majority of the inserts (90%) matched closely (E value $\leq 10^{-4}$) to already described bifidobacterial genes, and all ribosomal genes matched closely to bifidobacterial

rRNA genes. Less than 1% of the sequences were predicted to be noncoding or could not be identified (data not shown). The sequences appeared to encode a wide range of functions, such as those of components of transport systems, energy metabolism, and carbohydrate metabolism (Fig. 5). Like the taxonomic assignments, the identities of transcripts were inferred from the closest matches. These assignments are only as good as the existing database, and genes that are rare in genomes because they code for unusual or specialized traits are particularly susceptible to poor database coverage. The largest fraction of transcripts (31%) was categorized as hypothetical, and these transcripts were partly unclassified (typically having known functions but not readily placed into a role category during annotation). Among the corresponding genes may be those, encoding novel factors that allow the colonization of the human intestine by bifidobacterial species.

The inserts' sequences present in the hybridizing clones on the microarrays were functionally annotated and grouped according to the contribution of the gene expression values (signal over background) to the RA, as a measure for the impact

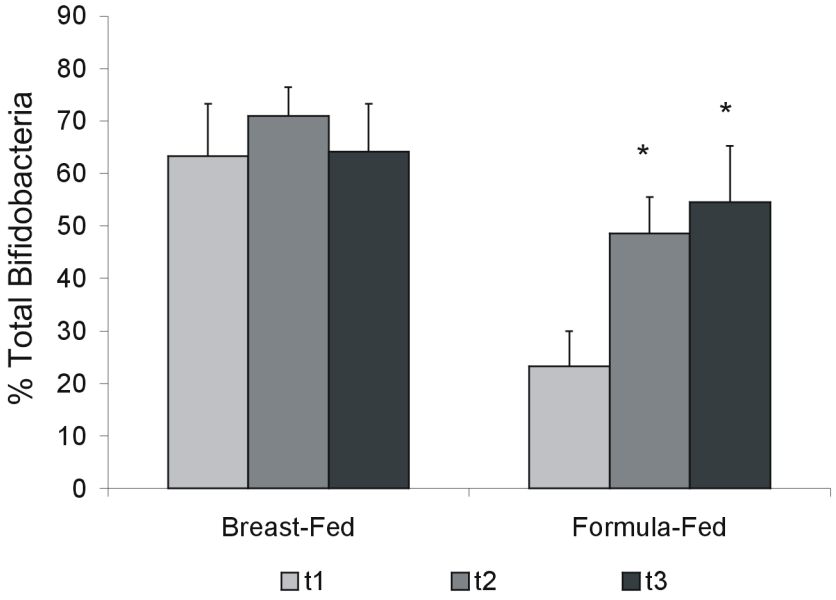

Figure 3. Total percentage of bifidobacteria in fecal samples from infants who were breast-fed (infants 1 and 2) or fed a standard formula supplemented with GOS/ lcFOS (infants 3, 4 and 5) as determined by qPCR. The bars represent the standard errors of the means. The percentage of bifidobacteria in the formula-fed but not in the breast-fed group increased significantly as compared to baseline over time (t1 start of analysis, t2 approximately 3 weeks later for breast-fed and 1 week later for formula-fed infants; and t3, an additional 1-2 weeks later) as determined by Student's *t* Test (* $p<0.05$).

of each gene on the difference between the dietary groups (see the supplementary material). The clones are listed in order of importance for the clustering of the transcriptome of the infants' microbiota according to diet (formula or breast milk) by using the hybridization signals for samples from infants 1 to 5 (Table 1). This means that the list starts with genes that have a large influence on the deviation of the infants' samples according to the dietary group. The signals for the hybridization to

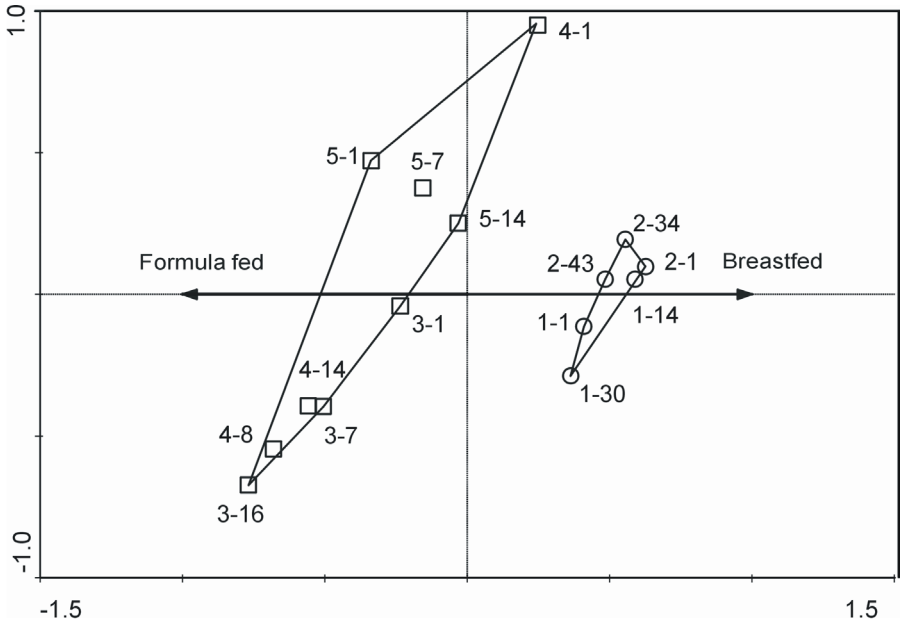

Figure 4. RA ordination plot comparing the total hybridization patterns of fecal bifidobacterial transcriptomes from breast-fed infants ((○); infant 1 and 2) and formula-fed infants ((□); infants 3, 4 and 5) at different time points. Each sample is labeled with the identification number of the infant followed by the day of sampling. The vector represents the diet variable.

the clones at the end of the list do not indicate any impact on the difference between the two dietary groups.

Active carbohydrate metabolism

The genome of *B. longum* NCC2705 includes numerous genes for carbohydrate transport and metabolism (Schell et al., 2002). These are collectively termed the glycobiome and show a preference for metabolism of di-, tri-, and oligosaccharides, pointing towards a biased utilization for complex oligosaccharides complemented

Table 1. Hybridization values for glycobiome-related genes with significant hybridization signals in samples from infants 1 to 5[a]

locustag	Function	RDA%	**1-1**	**1-14**	**1-30**	**2-1**	**2-34**	**2-43**	3-1	3-7	3-16	4-1	4-8	4-14	5-1	5-7	5-14
BAD_0708	pullulanase	12.89	39.61	41.89	33.60	67.83	92.57	64.86	12.05	2.82	nd	39.58	4.16	6.27	nd	37.04	2.28
BAD_1412	probable sugar kinase	12.25	1.89	1.89	nd	1.51	1.84	1.50	1.34	1.32	nd	2.02	1.36	1.33	nd	3.20	1.53
BAD_1527	endo-1.4-β-xylanase	9.74	nd	1.74	nd	1.31	1.58	nd	1.58	nd	nd	nd	nd	nd	nd	2.28	1.29
BAD_1605	β-galactosidase	3.01	nd	1.36	nd	nd	1.36	nd	nd	nd	1.28	nd	nd	nd	1.65	1.79	1.80
BL0146	possible arabinosidase	12.39	nd	2.43	nd	1.59	1.36	1.30	1.26	nd	nd	nd	nd	nd	2.16	nd	1.45
BL0421	narrowly conserved HP possibly involved in xylan degradation	12.81	nd	1.53	nd	nd	1.31	1.39	1.28	nd	nd	nd	1.32	nd	nd	1,33	1,45
BL0529	probable α-1,4-glucosidase; maltase-like enzyme	22.79	1.58	1.90	nd	nd	2.60	nd	2.08	nd	nd	nd	nd	nd	2.54	2.82	1.68
BL0536	sucrose phosphorylase	0.05	2.08	1.95	1.27	2.21	2.50	2.08	1.32	2.03	nd	1.36	nd	1.68	3.46	4.63	nd
BL0544	α-L-arabinosidase	24.40	nd	nd	1.37	1.30	1.46	1.10	1.38	nd	nd	1.27	nd	nd	0.99	1.59	2.40
BL0597	Glycogen phosphorylase	86.10	245.91	208.05	200.41	217.36	150.31	166.54	80.33	34.42	nd	5.77	8.25	26.81	1.94	97.23	39.30
BL0715	transaldolase	1.23	12.99	10.18	3.94	8.64	22.69	36.03	9.99	2.45	nd	24.63	1.37	3.33	46.41	63.17	25.69
BL0716	transketolase	5.75	1.33	1.27	nd	nd	1.73	nd	nd	nd	nd	1.46	nd	nd	1.29	nd	nd
BL0978	glycosyl hydrolase (*LacZ*)	90.9	30.91	46.13	43.51	41.34	59.71	51.23	10.01	1.99	nd	7.47	nd	2.95	nd	10.41	6.60
BL1104	possible glycosyltransferase	12.77	2.93	1.60	1.86	5.92	4.10	2.65	1.73	nd	nd	1.28	1.63	3.90	4.85	2.43	34.88
BL1308	lactate dehydrogenase	0.17	1.49	1.69	nd	1.26	1.52	nd	1.49	nd	nd	nd	nd	1.27	nd	4.10	1.26
BL1518	α-galactosidase	28.8	nd	1.29	nd	nd	nd	nd	1.42	nd	1.33	nd	nd	nd	1.71	1.25	nd
BL1638	solute binding protein of ABC transporter for sugars	4.28	1.33	1.44	nd	nd	1.73	nd	1.52	nd	nd	nd	nd	nd	1.52	1.82	nd
BL1639	permease of ABC transporter for sugars	14.37	nd	nd	1.37	nd	1.38	1.27	1.38	nd	nd	1.27	nd	nd	1.82	2.92	3.16
BL1641	lacto-N-biose phosphorylase	5.61	nd	nd	nd	nd	nd	nd	nd	nd	2.30	nd	nd	nd	nd	nd	nd
BL1643-44	galactose-1-phosphate uridylyltransferase; UDP-glucose 4-epimerase	0.42	nd	nd	nd	nd	1.39	nd	1.26	nd	nd	nd	nd	nd	1.31	1.36	nd
BL1674	probable glycosyltransferase	1.76	1.64	1.35	nd	1.26	2.76	1.34	2.21	1.31	nd	nd	nd	1.39	2.87	4.27	1.56

[a] Significant hybridization signals were those for which signal/background ratios were >1.25. Infants 1 and 2, for which sample designations are identified in bold, were breast fed. Sample designations indicate the infant identification number and the day of sampling. ND, not detected (no significant hybridization signal detected); HP, hypothetical protein.

with transporters for a variety of disaccharides and oligosaccharides (Parche et al., 2007). The importance of carbohydrate metabolism for the activity of bifidobacteria in the intestinal tract is also evident from this study, as carbohydrate functions are the most important category of predicted functions (Fig. 5), corresponding to 14% of the genes, a proportion greater than that determined by the sequence-based classification of carbohydrate-active enzymes (8%) (Ventura et al., 2007a). Here, the most salient features of the predicted bifidobacterial genes involved in intestinal carbohydrate metabolism (the glycobiome) are discussed briefly with specific attention for those that are highly expressed, have the greatest impact the RA analysis, or are organized into coexpressed operons (Table 1).

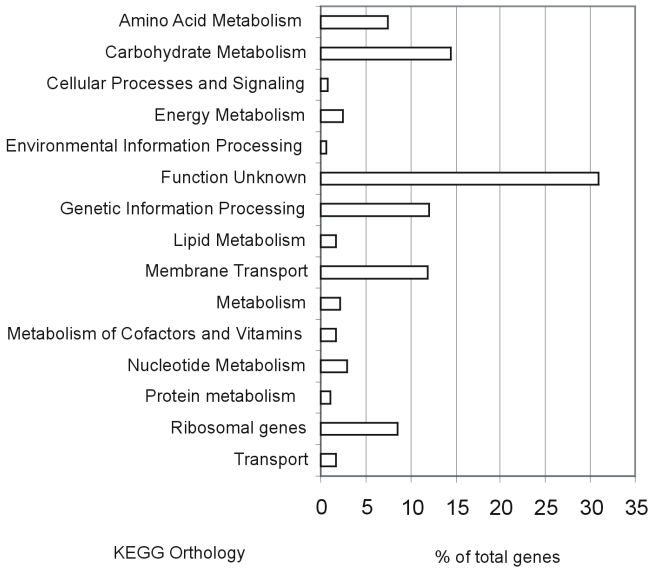

Figure 5. Classification of the predicted functions of the 250 sequenced bifidobacterial inserts present on the microarray and showing significant hybridization to the labeled total RNAs extracted from infant fecal samples. KEGG, Kyoto Encyclopedia of Genes and Genomes.

Taking the RA into account, the DNA fragment that had the greatest impact on the difference between the two diet groups included the *B. longum lacZ* gene (BL0978) that shows significant homology to the *B. longum* subsp. *infantis* HL96 gene encoding β-galactosidases I, which degrades lactose and other sugars containing a β-D-anomer-linked galactoside (Hung et al., 2001). Recent growth studies with laboratory media showed induction of the *B. longum* lacZ gene by lactose, maltose and FOS (Parche et al., 2007). In the present study, the *lacZ*-like gene showed greater expression in breast-fed infants, though a clone with a very similar gene,

BAD_1605, shows much lower hybridization signals than those for the *lacZ*-like gene, indicating the significance of the encoded β-galactosidase in intestinal sugar degradation. Similarly, the RA value for the fragment including BL0597 (coding for a glycogen phosphorylase) is high and this gene is involved in the breakdown of starch into glucose units. The α-1,6 bonds in amylopectin and pullulan can be hydrolyzed by so-called pullulanases (encoded by BAD_0708), which were previously shown to be expressed in several bifidobacterial species growing on these sugar polymers in laboratory media (O'Connell Motherway et al., 2008; Ryan et al., 2006). These latter genes show higher hybridization levels in breast-fed infants than in formula-fed infants.

A bifidobacterial α-L-arabinosidase-like gene (BL0544, or abfB) was found to be expressed in all of the infants. The *abfB* gene encodes a hemicellulose-degrading enzyme that hydrolyzes terminal α-L-arabinofuranosyl groups from arabinose-containing oligosaccharides and polysaccharides such as arabinans, arabinoxylans, and xylans, major components of plant cell walls (Saha and Bothast, 1998). It is known that α-arabinofuranosidases, together with other hydrolases with endo- and exo-activities, are required for the complete degradation of polymeric carbohydrates. These substrates are poorly digested by the host or other intestinal microbes. The sequencing of the *B. longum* genome (Schell et al., 2002) allowed the annotation of at least 14 different enzymes with a hypothetical role in the catabolism of arabinose-containing polymers, which corroborated the importance of this type of enzymes and polysaccharides in the metabolism of some bifidobacteria. The expression of the *abfB*-like gene and the influence of inducers and repressors in *B. longum* NIZO B667 have been studied, and the findings have indicated transcriptional regulation. Degenerate primers revealed widespread presence of the α-arabinofuranosidase enzym family (family 51 of glycoside hydrolases) in *B. longum, B. infantis, B. animalis* and *B. bifidum* (Gueimonde et al., 2007). A different gene, possibly coding for an arabinosidase (BL0146), was found to have hybridization levels similar to those of the *abfD*-like gene, and may also be involved in breakdown of arabinose-containing saccharides. AbfB activity indicates a selective advantage for bifidobacteria for nutritional competition and colonization of the human gastrointestinal tract as arabinose-containing polymers are abundant in the diet and known to reach the colon. The expression of arabinosidases by the bifidobacterial community could give these species an advantage to survive and colonize the human colon. As the infants that received a breast milk-based diet also showed the expression of an *abfB*-like gene, it is possible either that breast milk contains arabinose-like sugars or arabinose-decorated glycoproteins or that it contains related sugars that act as inducers of *abfB*-like expression. An alternative explanation is that milk serves a

substrate for the growth of microbes that produce arabinose-like compounds.

The novel putative operon for galactose metabolism (BL1638-BL1644) was detected in the *B. longum* clone library by hybridization of RNAs from both breast-fed and formula-fed infants. This operon is involved in the breakdown of structures present in mucin sugars (Derensy-Dron et al., 2010; Nishimoto et al., 2007; Wada et al., 2008), indicating a way for bifidobacteria to colonize the intestine. Additionally, the complex mixture of human milk oligosaccharides contains LNB structures (Kitaoka et al., 2005; Ward et al., 2008) broken down by the products of genes in this operon. BL1638 to BL1640 genes are annotated as encoding component proteins of the ABC-type sugar transporter, and BL1641 gene is annotated as encoding LNB phosphorylase. The cluster of BL1642, BL1643, and BL1644 genes, which were annotated as encoding mucin desulfatase, galactose-1-phosphate uridylyltransferase, and UDP-glucose 4-epimerase, respectively, likely codes for a metabolic pathway for mucin sugars, because galacto-N-biose is the core structure of mucin-type sugars (Derensy-Dron et al., 2010). In this pathway, galactose 1-P, formed by the phosphorolysis of LNB/ galacto-N-biose, is converted into UDP-glucose. Other genes involved in carbohydrate metabolism are predicted to encode α-1,4-glucosidase (BL0529) and α-galactosidase (BL1518), both both of which are expressed to a greater degree in formula-fed infants than in breast-fed infants. Finally, a lactate dehydrogenase (BL1308), involved in the final step in anaerobic glycolysis and formation of lactate, was found to hybridize to RNAs isolated from both in formula- and breast-fed infants, reflecting the activity of bifidobacterial sugar metabolism.

An endo-1,4-β-xylanase (BAD_1527) and a hypothetical gene involved in xylan degradation (BL0421) may be involved in breakdown of xylans by hydrolysis of 1,4-β-D-xylosidic linkages in xylans. The two genes are detected in samples from all infants and show similar hybridization signals. BAD_1412 product, a probably sugar kinase, has high levels of similarity to xylose kinases and may be involved in the breakdown of xylans as well. Sucrose phosphorylase (encoded by BL0536 gene) breaks down sucrose and is involved in energy metabolism.

Overall, the expression of the glycobiome (Table 1), especially the genes encoding pullulanes, α-1,4-glucosidase, and the glycogen phosphorylase, indicates a higher potential for carbohydrate metabolism in breast-fed infants than in formula-fed infants. This finding may very well be explained by the high diversity of complex oligosaccharides in human milk (Boehm and Stahl, 2007) that activate and/or increase the abundance of species expressing these genes.

Colonization factors

Streptococcus mutans 20381 is known to use glycosyltransferases to form polysaccharides, which are involved in the formation of biofilms and have been shown previously to be the major contributor to adherence (Wiater et al., 1999). In this study, the expression of two putative glycosyltransferases, the BL1104 and BL1674 products, was detected in most of the samples, suggesting the *in vivo* production of polysaccharides that may also play a role in biofilm formation and the colonization of the host's intestiine by bifidobacteria. A possible penicillin-binding protein (encoded by BAD_1336) is predicted to be involved in synthesis of peptidoglycan, is the major component of bacterial cell walls (Lovering et al., 2007), and may be involved in the recognition of the bacteria by the host of the bacteria. Only samples from formula-fed babies show hybridization to the clone containing BAD_1336 (see Supplementary material).

Activity of bifidobacteria within the human intestine

Several other genes and operons are worth mentioning, as these are biologically relevant and may explain functions in the intestinal bifidobacteria related to host interaction and colonization and competition within the intestinal microbiota. The gene for transaldolase (BL0715), which takes part in the non-oxidative phase of the pentose phosphate route, was expressed in all infants. Its translation product was also detected in the metaproteome of infant feces (Klaassens et al., 2007) and the proteome of *B. longum* subsp. *infantis* BI07 grown in a laboratory medium (Vitali et al., 2005). The gene for transketolase (BL0716), also involved in the pentose phosphate route characteristic of bifidobacterial metabolism, was expressed at lower levels than the gene for transaldolase.

Hybridization to the clone containing BTH_II0919, coding for a glutamine-dependent NAD^+ synthetase (EC 6.3.5.10), indicates the presence of a glutamine-rich substrate. A previous study showed increased intestinal bifidobacterial numbers upon intake of prebiotics containing glutamine-rich protein by healthy adults (Kanauchi et al., 1999). Glutamine is known to be among the nutrient requirements for the infant gut maturation as the endogenous capacity to synthesize glutamine from glutamate is not fully developed. The lower hybridization levels for samples from formula-fed infants may be due to lower glutamine levels in the formula than in breast milk (Agostoni et al., 2000).

Several genes predicted to be involved in folate biosynthesis pathways were found to be expressed, namely, those encoding dihydrofolate reductase (BL1666) and thymidylate synthase (BL1665), involved in the last step of the production of folate. Folate is involved in many metabolic pathways, such as methyl group

biogenesis and synthesis of nucleotides, vitamins, and some amino acids. It has been demonstrated that folate synthesized by bacteria in the human intestine is absorbed and used by the host (Said and Mohammed, 2006). Moreover, several bifidobacterial species have found to produce folate in laboratory media (Pompei et al., 2007). The expression of folate genes in the infants' intestinal tract indicates the *in vivo* production of this vitamin, which is beneficial to the host and can be absorbed through the large intestine (Pompei et al., 2007). Remarkably, the hybridization of folate biosynthesis-like genes was found to be slightly higher in formula-fed than breast-fed infants

The gene for a putative copper-transporting ATPase (BL0409) was expressed mostly in samples from formula-fed infants, and its translation product was also found in the proteome of *B. longum* subsp. *infantis* (Vitali et al., 2005). In a recent study, a transcriptome analysis of *L. plantarum* indicated significant expression of a gene for an orthologous putative copper-transporting protein in intestinal samples of human (De Vries, 2006) and mice (Bron et al., 2004), but the function is not confirmed.

qPCR and sequencing analysis

Using the clone insert sequences and matching sequences in the database, primers sets for qPRC were designed to target the thymidylate synthase (BL1665) gene, (BL1641), and the α-L-arabinosidase (BL0544) gene, and qPCR analysis of cDNAs was performed to confirm the specific hybridization of transcripts to the microarray. Melting curves showed specific amplification of one product for each sample primer set combination (data not shown).

The quantification of gene activity can be complicated because it is not known how many active bifidobacteria with sequences that match the amplicons spotted onto the microarray are present in the samples. The yield of RNA per sample unit was found to differ among samples and individuals because of transit time, diet, and other host-related conditions. The size of the gene fragment on the microarray can also cause variability in hybridization of samples from different origins. However, the relative copy numbers obtained with qPCR showed a similar trends similar to those demonstrated by the relative signal-to-background ratios obtained with the micro array hybridizations, confirming the presence of target mRNA (data not shown). Moreover, sequence analysis of the amplicons of qPCR confirmed amplification from the target genes BL1665, BL1641, BL0544.

Concluding remarks

This study revealed that bifidobacterial species undergo dynamic changes in infant feces on the level of persistence as well as their functional complement. To aid the

detection of the gene transcript, the number and diversity of bifidobacteria in the infants' feces was determined using molecular tools. This was complemented by transcriptome analysis using mixed-bifidobacterial species microarrays that showed a significant impact of diet on the transcriptional response of bifidobacteria in breast- and formula-fed infants.

Genome-wide transcript analyses using DNA microarrays provide opportunities for comprehensive and integrative views of bacterial activities occurring within the intestinal tract. The potential of this approach was exemplified by previous studies reporting full-genome transcriptome profiles for *Bacteroides thetaiotaomicron* and *B. longum* residing in the ceca of germ-free mice (Sonnenburg et al., 2005; Sonnenburg et al., 2006), as well as for *L. plantarum* in conventional mice (Marco et al., 2007). In the present study, the hybridization profile could be used to assess the influence of the diet on the transcriptome of fecal bifidobacterial communities from infants. Particularly, the use of a mixed-species microarray made it possible to simultaneously target several bifidobacterial species in the complex fecal microbial ecosystem. By using this array it was possible to compare fecal bifidobacterial gene expression profiles for individuals despite unique fecal microbial compositions.

Samples from breast-fed infants with stable total microbiotas and bifidobacterial populations showed variety in transcripts at different time points indicating active bifidobacterial populations. As expected, the transcriptomes of microbiotas in the formula-fed infants, with more unstable microbial diversity, also showed differences in time. The observed differences have been caused by the variation of activities of individual bifidobacterial species and/ or environmental factors such as diet. Possibly, host factors and the addition of solid food to the milk also had influence, and hence, more individuals should be compared. It is likely that specific bifidobacterial species may be a target of modulation by prebiotics, but this study did not reveal a direct link between the expressed genes and the species. The genome sequence of *B. longum* NCC2705 revealed that the chromosome includes numerous genes for carbohydrate utilization, specifically those for over 30 glycosyl hydrolases that are predicted to be involved in the degradation of higher-order oligosaccharides (Schell et al., 2002) and 19 carbohydrate transport systems were proven to be active in laboratory media (Parche et al., 2007). *Bifidobacterium adolescentis* MB 239 preferred lactose, FOS and raffinose over glucose and fructose in laboratory media, which was explained by α-galactosidase, β-galactosidase, and β-fructofuranosidase activities (Amaretti et al., 2006). The findings of this study show that at least a portion of these genes are being transcribed *in vivo* in the infant intestinal tract. This gene expression may give these bifidobacteria advantages in colonizing the infant gut and explains the growth-promoting effect of oligosaccharides which are described as prebiotics.

The sequencing of an extensive metagenomic library from the human distal gut revealed a high degree of diversity in bifidobacterial genes compared to the *B. longum* NCC2705 genome (Gill, 2006). This finding suggests the presence of multiple and related bifidobacterial strains. The present and expected sequences of several bifidobacterial genomes will open up a new era of comparative genomics and provide a basis for the setup of a molecular model that can predict the bifidobacterial-host interaction. This progress will improve the molecular studies of the functional complement with tools such as transcriptomics, described here, as well as metaproteomics (Klaassens et al., 2007) and metabolomics (Goodacre, 2007). The further development of high-throughput sequencing techniques will allow the detection and quantification of total cDNAs without the limitation of relevant probes on a microarray (Marioni et al., 2008; Wang et al., 2009).

Ultimately, this advancement will lead to a detailed understanding of the nutritional lifestyle of bifidobacteria and its impact on the host. Linking functional activities of intestinal bifidobacteria to specific groups of healthy or diseased individuals or special diets opens up leads for modulation of the intestinal bifidobacterial community, exerting health benefits.

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Supplementary Material

Appendix 1. Identified clones, listed in order of % RA. RA could explain the grouping of the samples from infants by the difference in diet, being either breast- or formula-fed.

% RDA	Library	locustag	function	KO
90,9	M	BL0978	glycosyl hydrolase (LacZ)	C
86,1	M	BL0597	glycogen phosphorylase	C, PS
81,2	P	BAD_0376	lysyl-tRNA synthetase 1	A
78,2	M	BAD_1319-20	DNA-directed RNA polymerase β chain; HP	GI, FU
76,3	P	BAD_0470	probable solute binding protein of ABC transporter system	MT
75,9	P	BAD_1130	DNA polymerase III a subunit	GI
72,9	L	BL0307-08	possible Rim-like protein involved in efficient processing of 16S rRNA; inosine-uridine preferring nucleoside hydrolase	GI, FU
69,6	L	BL0694-95	possible ABC transporter permease for cobalt; narrowly conserved protein with unknown function	T, FU
64,7	L	LSA0594	hypothetical prophage lsa1protein (*Lactobacillus sakei* ssp. *sakei*)	FU
64,3	P	BTH_II0919	glutamine-dependent NAD+ synthetase	A, CV
62,6	P	BAD_1423-24	possible secreted peptidyl-prolyl cis-trans isomerase protein; HP	FU
62,2	P	BAD_0708	pullulanase	M
52,7	L	BL1197	large HP	FU
51,9	M	BL0337	HP possibly in TetR transcriptional regulator family	FU
51,7	M	BL0440-41	glucose-6-phosphate 1-dehydrogenase; narrowly conserved HP	C, FU
50,1	P	BL1057	argininosuccinate lyase	A
49,3	L	BL0356-57	ATP synthase epsilon chain; ATP synthase subunit B	EM
49,2	M	BL0395	valyl-tRNA synthetase	GI
48,2	L	BL1275-76	possible homoserine kinase; HP with MutT domain	A, FU
48	M	BAD_0914	tyrosyl-tRNA synthetase	A, GI
46,1	P	BAD_1575-76	possible NagC/XylR-type transcriptional regulator; a-galactosidase	C, GB
44,1	L	BL1450/ BL0527	leucyl-tRNA synthetase; 4-a-glucanotransferase	A, C
43,5	M	BL1429	DNA gyrase subunit B	GI
43,5	P	BAD_1336	possible penicillin-binding protein	FU
42,5	L	BL1307	GTP-binding protein	FU
42,4	L	BL1204	DNA-directed RNA polymerase β-subunit	GI
41	P	BL1664	widely conserved protein in universal stress protein family	FU
40	L	BL0422	narrowly conserved HP	FU
39,64	L	BAD_1295	aminopeptidase N; cystathionine gamma-synthase; ATP-dependent DNA helicase RecQ	M
37,55	P	BAD_0341-42	preprotein translocase SecY subunit; adenylate kinase	N, GI

Appendix 1. Continued.

% RDA	Library	locustag	function	KO
35,85	M	BAD_0702	putative DNA methyltransferase	FU
35,58	P	BAD_1013	probably bifunctional short chain isoprenyl diphosphate synthase	LM
35,28	M	BL0409	copper-transporting ATPase	EM
35,28	L	BL0353-54	HP; possible secreted peptidyl-prolyl cis-trans isomerase protein	FU, GI
34,87	L	BL0197-98	possible ATP binding protein of ABC transporter; hypothetical membrane protein with unknown function	MT
33,84	L	BL0722	narrowly conserved HP	FU
32,3	L	BL1251	probable glutamyl-tRNA synthetase	A
31,6	P	BAD_0061	probable ferredoxin/ferredoxin-NADP reductase	M
31,59	M	BL1435	narrowly conserved HP	FU
31,55	M	LJ1578	HP (*Lactobacillus johnsonii* NCC 533)	FU
31,1	L	BL1251	probable glutamyl-tRNA synthetase	A
30,84	L	LVIS_2267	ATP-dependent exoDNAse (exonuclease V) β subunit	FU
30,63	L	BL0726	widely conserved hypothetical transport protein	FU
30,54	L	BL0919-20	possible efflux transporter protein	MT
30,31	L	BL0976	galactoside symporter (lacS)	T
29,99	L	BL1117	narrowly conserved HP	FU
29,72	M	BAD_0833-37	HP	FU
28,8	L	BL1518	a-galactosidase	C
28,13	P	SCO7398	membrane transport protein	MT
27,31	L	BL1169-70	probable permease of ABC transporter system for sugars	MT
27,08	L	BL0674	possible cell surface protein with gram positive anchor domain; probable permease of ABC transporter system for sugars	FU
26,29	L	BL1164	probable solute binding protein of ABC transporter system for sugars	MT
25,79	M	BAD_1191-92	ABC transporter; ATP-binding protein of ABC transporter	MT
25,72	L	BL1673	possible lactaldehyde reductase	C
24,44	M	BAD_1132	possible lipoprotein signal peptidase	GI
24,4	L	BL0544	a-L-arabinosidase	C
24,4	L	BL0917-18	HP with possible helix turn helix motif; HP with probable serine/threonine-protein kinase domain	FU
23,42	L	BL0962	hypothetical membrane protein with possible acetylase function	C
23,37	M	BAD_1257-60	HP; similar to glutamine ABC transporter (ATP-binding protein)	FU, MT
22,86	M	BL1170	probable permease of ABC transporter system for sugars	MT
22,79	L	BL0529	probable a-1,4-glucosidase; maltase-like enzyme	C
22,25	L	BL0871-72	possible ABC transporter component	MT

Appendix 1. Continued.

% RDA	Library	locustag	function	KO
22,06	L	BL1757-60	BglX; HP; HP with possible nucleotidyltransferase domain; HP possibly involved in exopolysaccharide production	C, FU
21,71	M	BAD_1445	HP	FU
21,05	P	BL0662	HP	FU
19,79	P	BL0009	HP	FU
19,66	L	EF2229	HP	FU
19,31	M	BL1498	HP	FU
19,05	L	BL0268	ATP binding protein of ABC transporter similar to Vex1 (VexP1) of S. pneumoniae	MT
18,44	L	BL1280-81	succinyl-diaminopimelate desuccinylase; ribonuclease G	A, GI
18,16	L	BL1291	50S ribosomal protein L1	GI
17,86	M	BL1148-50	deoxyguanosinetriphosphate triphosphohydrolase-like protein; alanine racemase; probable amino acid transporter	N, A, MT
17,59	L	BL0837-38	HP; LacI-type transcriptional regulator	FU, C
17,41	P	BP1364	putative amino-acid ABC transporter, periplasmic amino acid-binding protein	MT
16,96	M	BL0196-98	HP; possible ATP binding protein of ABC transporter	FU, MT
16,58	M	BAD_0490-92	cystathionine β-synthase	EM, A, GI
16,35	L	BL0951-53	formate acetyltransferase; HP; glutamine-dependent NAD(+) synthetase	C, FU,A
16,22	L	BL1571-73	50S ribosomal protein L13; 30S ribosomal protein S9; probable glycogen operon protein GlgX	GI, C
16,17	M	BAD_0525-26	probable phosphoribosylglycinamide formyltransferase 2; possible glycosyltransferase	C, N
16,11	L	BL1069-70	possible permease protein of ABC transporter for cobalt; ATP binding protein of ABC transporter	MT
15,84	L	BL0850-51	conserved hypothetical transmembrane protein with unknown function; conserved HP similar to MazG	MT, FU
15,59	L	DIP1756	Putative DNA methyltransferase (*Corynebacterium diphtheriae* NCTC 13129)	FU
15,24	P	BAD_0262	HP	FU
14,37	L	BL1639	permease of ABC transporter for sugars	MT
14,25	L	BL1534	hypothetical integral membrane with weak similarity to proteins in BioY family	FU
14,02	M	BL0485	DNA topoisomerase I	GI
13,46	L	BL0435-36	possible ammonium ion transporter; FtsY signal recognition particle	MT, GI
13,43	L	BL1287-90	preprotein translocase SecE subunit; probable transcription antitermination protein; HP; 50S ribosomal protein L11	GI, FU
13,17	L	BL1114-15	DNA polymerase V; S-adenosyl-L-homocysteine hydrolase	GI, A
12,89	M	BL1292	morphine 6-dehydrogenase	M
12,81	L	BL0421	narrowly conserved HP possibly involved in xylan degradation	C

Appendix 1. Continued.

% RDA	Library	locustag	function	KO
12,77	L	BL1104	possible glycosyltransferase	C
12,74	M	BL1251-52	probable glutamyl-tRNA synthetase; possible phosphodi-esterase	A
12,66	L	BL1665-66	thymidylate synthase; dihydrofolate reductase	CV
12,39	L	BL0146	possible arabinosidase	C
12,38	M	BAD_1556-58	LacI-type transcriptional regulator; putative ABC transport system membrane protein; haloacid dehalogenase-like hydrolase	C
12,35	L	BL1026	peptidyl-tRNA hydrolase	MT
12,25	P	BAD_1412	probable sugar kinase	C
12,16	M	BAD_1188	galactoside symporter	T
12,07	M	RHA1_ro03515	possible transcriptional regulator (*Rhodococcus* spp. RHA1)	FU
12	M	nfa39180	HP (Nocardia farcinica IFM 10152)	FU
10,93	L	BL1127-29	HP; probable metal uptake regulator similar to ferric uptake regulator protein; phosphoribosylaminoimidazole carboxylase ATPase subunit	FU, GI, N
10,77	M	BAD_0956-57	probable aminotransferase; similar to Mycobacterium tuber-culosis polyphosphate glucokinase	A, C
10,71	P	BL1695-96	ATP binding protein of ABC transporter for pentoses; prob-able ABC transport system permease protein for sugars	FU
10,71	L	BL0158	very narrowly conserved HP	MT
10,65	P	BL0067-68	carbamoyl-phosphate synthase small subunit; CarB	N, A
10,33	L	BL1068-69	possible rRNA methylase; possible permease protein of ABC transporter for cobalt	GI, MT
10,25	L	BL1549-50	50S ribosomal protein L10; 50S ribosomal protein L7/L12	GI, PM
10,24	L	BL1616	translation initiation factor IF-2	GI
10,23	L	BL0012-13	HP weakly similar to putative transcriptional regulator from Streptomyces; proline/betaine transporter	FU, T
10,23	L	BL0033-34	ATP binding protein of ABC transporter; probable solute binding protein of ABC transporter system possibly for sugars	MT
10,18	L	BL0820-21	HP; possible thioredoxin-dependent thiol peroxidase	FU, GI
10,04	L	Francci3_4210	Integrase	GI
9,89	M	BL0417	HP	FU
9,84	M	BL0630	glutamate dehydrogenase	A, EM
9,74	L	BAD_1527	endo-1,4-beta-xylanase	C
9,27	L	BL0342-43	possible permease of ABC transporter; possible permease protein of ABC transporter system	MT
9,11	L	BL0592-93	narrowly conserved HP	FU
8,83	M	BAD_1124	1-(5-phosphoribosyl)-5- [(5-phosphoribosylamino) methylide-neamino] imidazole-4-carboxamide isomerase	A
8,58	L	BAD_0608/ BL0338	pyrroline-5-carboxylate reductase; aspartate ammonia-lyase	A

Appendix 1. Continued.

% RDA	Library	locustag	function	KO
8,51	L	BL1772	sugar kinase in PfkB family	C
8,5	M	BH1867	HP (Bacillus haloduransC-125)	FU
8,4	L	BL1673-74	possible lactaldehyde reductase; probable glycosyltransferase	C
8,26	M	BAD_1179	CRISPR-associated protein Cas2	FU
8,22	M	BAD_0210	HP	FU
8,18	L	BL1276	HP with MutT domain	FU
8,05	L	BL0450-51	ATP binding protein of ABC transporter; possible permease protein of ABC transporter system	MT
7,91	M	BAD_0708	pullulanase	M
7,85	M	DVU2019	HP	FU
7,78	L	Lxx04970	rhamnosyltransferase (*Leifsonia xyli* subsp. *xyli* CTCB07)	C
7,75	P	APECO1_536	putative tail component of prophage	FU
7,47	L	BL1732	methionine aminopeptidase	GI
7,41	L	BL1164-65	probable solute binding protein of ABC transporter system for sugars;	MT
6,86	P	BAD_0254-55	JadJ; propionyl-CoA carboxylase beta chain	A, C
6,58	L	BL0771-72	HP	FU
6,48	P	BAD_0746-47	GTP-binding protein lepA; probable oxygen-independent coproporphyrinogen III oxidase	C
5,86	M	SAV7543	helicase	FU
5,77	L	BL1396-97	probable cation-transporting ATPase; aconitate hydratase	FU, C, EM
5,76	L	BL1169-70	probable permease of ABC transporter system for sugars; probable permease of ABC transporter system for sugars	MT
5,75	L	BL0716	transketolase	C
5,71	L	BL0552	probable ferredoxin/ferredoxin-NADP reductase	M
5,66	L	BL0163-64	HP related to thiamine biosynthesis lipoprotein ApbE; probable permease protein of ABC transporter system	CV, MT
5,63	M	BAD_1120	ATP-dependent helicase	GI
5,61	L	BL1641	Lacto-N-biose phosphorylase	C
5,32	M	BL1230	HP	FU
5,1	L	BL1126-27	probable solute binding protein of ABC transporter system; HP	MT, FU
4,97	M	BAD_0267	possible protease	EM
4,93	P	BAD_0470-71	probable solute binding protein of ABC transporter system; probable permease protein of ABC transporter system	MT
4,76	L	BL0466-67	HP	FU
4,76	L	Reut_B4371	AMP-dependent synthetase and ligase	C, EM
4,76	M	BL0206	hypothetical membrane protein with unknown function	FU
4,76	M	AAur_3512	bacterioferritin comigratory protein (thioredoxin reductase)	GI

Appendix 1. Continued.

% RDA	Library	locustag	function	KO
4,76	L	BL0099-100	HP with possible acylase domain; DNA repair protein RecO	FU, GI
4,76	L	BL0992	30S ribosomal protein S1	GI
4,76	M	BL1804	HP	FU
4,76	L	BL0604	phosphoenolpyruvate carboxylase	FU
4,7	L	BL0675	possible cell surface protein similar toFimA fimbrial subunit	FU
4,62	L	BL0157-58	narrowly conserved HP	FU
4,36	L	BL0795-96	glutamate-ammonia-ligase adenylyltransferase; choloylglycine hydrolase	A, LM
4,34	L	Lxx04950	glycosyl transferase (*Leifsonia xyli* ssp. *xyli* CTCB07)	C
4,28	L	BL1638	solute binding protein of ABC transporter for sugars	MT
4,23	L	BL0092	probable DNA helicase II	C
4,04	M	BAD_1512	putative cell wall-anchored protein	FU
3,75	L	BL0387	HP	FU
3,74	L	FN0557	HP	FU
3,37	L	BL1537	Fas	LM
3,22	P	BAD_1612	widely conserved protein with eukaryotic protein kinase domain	FU
3,18	P	BL0670-71	ribonucleotide-diphosphate reductase alpha subunit; ribonucleotide-diphosphate reductase beta subunit	N
3,11	M	BCE_1227	S-layer protein, putative (*Bacillus cereus* ATCC10987)	FU
3,09	L	BL1148	deoxyguanosinetriphosphate triphosphohydrolase-like protein	N
3,07	L	BL0655-56	HP; 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase; Cmk; 4-(cytidine-5′-diphospho)-2-C-methyl-D-erythritol kinase	FU, LM
3,05	L	BL1134-35	HP; ATP binding protein of ABC transporter	FU, MT
3,01	L	BAD_1605	β-galactosidase	C
2,81	L	CE1244	putative transposase	FU
2,71	M	BAD_1200	HP	FU
2,71	L	BL0116	glycyl-tRNA synthetase	C
2,59	L	BAF40155	IS3 family transposase	FU
2,54	L	BL1692-93	ATP binding protein of ABC transporter for pentoses; probable repressor protein in (NagC/XylR) family	MT
2,37	L	BL1276-77	HP with MutT domain; ATP-binding protein of ABC transporter system	FU
2,25	L	BL0485	DNA topoisomerase I	GI
2,14	P	BAD_1412	probable sugar kinase	FU
2,13	L	BAD_0708	pullulanase	M
2,05	L	BL1732	methionine aminopeptidase	GI
2,01	M	BAD_1230-32	helicase; HP; probable type II restriction enzyme similar to Sau3AI	FU

Appendix 1. Continued.

% RDA	Library	locustag	function	KO
1,99	L	BL1319-20	UDP-N-acetylmuramoylalanyl-D-glutamyl-2, 6-diaminopimelate--D-alanyl-D-alanyl ligase; phospho-N-acetylmuramoyl-pentapeptide-transferase	A
1,97	P	AAur_1329	putative dolichyl-phosphate-mannose-proteinmannosyltransferase family protein	FU
1,95	M	BL0387	HP	FU
1,92	L	BL1110-11	HP; possible Na(+)/H(+) antiporter	FU
1,79	L	BL1220-21	narrowly conserved HP; glycine cleavage system H protein	FU
1,76	L	BL1674	probable glycosyltransferase	FU
1,73	L	BL1723-24	oligoribonuclease; possible helicase	FU
1,7	P	BL0141	possible solute binding protein of ABC transporter	FU
1,56	L	BL0529	probable a-1,4-glucosidase; maltase-like enzyme	C
1,55	L	BL1647-48	HP	FU
1,52	M	BAD_0972	possible solute binding protein of ABC transporter	FU
1,48	L	BL0284	FemAB-like protein possibly involved in interpeptide bridge formation in peptidoglycan	GB
1,4	M	BL1422	anthranilate phosphoribosyltransferase 1	A
1,37	M	BAD_0054-56	putative exodeoxyribonuclease V; response regulator of two-component system; queuine tRNA-ribosyltransferase	EP
1,34	M	nfa44220	HP (Nocardia farcinica IFM 10152)	FU
1,28	P	BAD_0858-59	probable ribosomal-protein-alanine N-acetyltransferase; HP	GI
1,23	L	BL0715	Transaldolase	C
1,23	L	BL1535-36	JadJ; propionyl-CoA carboxylase beta chain	LM
1,19	L	NTHI1839	putative type I restriction enzyme HindVIIP specificity protein (Haemophilus influenzae 86-028NP)	M
1,18	P	BAD_1120	ATP-dependent helicase	GI
1,11	P	BAD_1016	HP	FU
1,1	M	BL0027-28	narrowly conserved HP; probable DEAD box-like helicase	FU
1,07	M	BAD_1614-15	dimethyladenosine transferase; HP	GI
1,06	L	BL1752	anaerobic ribonucleoside triphosphate reductase	N
1	L	BL0111	hypothetical metabolite transport protein possibly for a sugar	FU
0,96	P	BL0143-44	permease of ABC transporter possibly for oligosaccharides	MT
0,91	L	BL1152-53	S-ribosylhomocysteinase; ATP-dependent DNA helicase RecQ	A
0,89	M	BL0934	possible pyridoxine kinase	CV
0,87	P	BL1455	HP	FU
0,85	L	BL1098	elongation factor G	GI
0,84	L	BL0949-50	narrowly conserved HP; pyruvate formate-lyase 1 activating enzyme	FU
0,8	L	BL0613	probable integral membrane transporter	FU
0,74	L	BAD_1065	putative ABC transport system integral membrane protein	FU

Appendix 1. Continued.

% RDA	Library	locustag	function	KO
0,74	L	BAD_1177	putative transposase	FU
0,73	M	BAD_0243	glutamate 5-kinase	A
0,68	L	BL0768-70	HP	FU
0,61	L	BL1532-33	HP	FU
0,58	L	BL1002-04	HP with similarity to ImpA of *Salmonella* involved in UV protection and mutation; HP with phosphoribosylpyrophosphate transferase domain and similarity to OrfS annotated as putative ComF protein; HP with similarity to eukaryotic phosphomannomutase	FU
0,57	M	BAD_0315	probable repressor in the Rok (NagC/XylR) family	FU
0,57	L	BL1443	HP	FU
0,53	L	BL1523	sugar permease of ABC transporter system	MT
0,48	M	BL0006-07	cold shock protein; histidine kinase sensor of two-component system	GI, EP
0,44	L	BL0157	narrowly conserved HP	FU
0,42	L	BL1643-44	galactose-1-phosphate uridylyltransferase; UDP-glucose 4-epimerase	C
0,41	L	BAD_1188	galactoside symporter	T
0,39	L	BL0092-93	probable DNA helicase II; narrowly conserved HP	C
0,37	L	BL0901-02	probable ATP binding protein of ABC transporter; histidine kinase sensor of two-component system	MT
0,33	L	BAD_0181	HP	FU
0,32	L	BL1377-78	possible pyridoxal-phosphate-dependent aminotransferase; probable sugar transporter	CV, T
0,31	M	BAD_1028	DP-diacylglycerol--glycerol-3-phosphate 3- phosphatidyltransferase	LM
0,21	L	BL0675	possible cell surface protein similar to FimA (fimbrial subunit of *Actinomyces naeslundii*)	FU
0,2	L	BL1518	a-galactosidase	C
0,19	L	BL0418-20	ribose-phosphate pyrophosphokinase; HP in upf0001; probable extracellular protein possibly involved in xylan or arabinan degradation	N
0,17	L	LSL_1522	HP	FU
0,17	P	BL1308	lactate dehydrogenase	C
0,16	L	BL0619	HP	FU
0,16	L	BL1181-82	HP with weak C-terminal similarity to TraG; FtsX-like protein involved in cell division	FU
0,16	M	BL1803-04	HP	FU
0,14	P	BL0995	ATP binding protein of ABC transporter	MT
0,13	L	BL1246	hypothetical membrane protein with unknown function	FU
0,12	L	BL0949-50	narrowly conserved HP; pyruvate formate-lyase 1 activating enzyme	FU
0,1	P	BL0536	sucrose phosphorylase	C

Appendix 1. Continued.

% RDA	Library	locustag	function	KO
0,1	L	BL1795	hypothetical proteasome-associated protein;	GI
0,1	L	BL0617-18	widely conserved HP with duf21 and CBS domains; HP in DPS family	FU
0,07	L	BL1679-80	hypothetical secreted protein with D-Ala-D-Ala carboxypeptidase 3 (S13) domain; narrowly conserved HP similar to MesJ	FU
0,05	P	BL0536	sucrose phosphorylase	C
0,05	L	BL0950-51	pyruvate formate-lyase 1 activating enzyme; formate acetyltransferase	GI
0,05	L	BL1795-96	hypothetical proteasome-associated protein; possible inositol monophosphatase	FU
0,03	L	BL0059-60	narrowly conserved HP; HP with limited similarity to C-terminal part of trans-aconitate methyltransferase	FU
0,01	L	BL0072	HP	FU
0	L	BL0457-58	narrowly conserved HP; narrowly conserved HP with duf24	FU
0	L	BL0146	possible arabinosidase	C

L: *B. longum* library, P: *B. pseudolongum* library, M: mixed bifidobacterial species library. KO: KEGG Orthology, A: Amino Acid Metabolism, C: Carbohydrate Metabolism, EM: Energy Metabolism, EP: Environmental Information Processing, FU: Function Unknown, GI: Genetic Information Processing, GB: Glycan Biosynthesis and Metabolism, LM: Lipid Metabolism, MT: Membrane Transport (Environmental Information Processing), M: Metabolism, CV: Metabolism of Cofactors and Vitamins, N: Nucleotide Metabolism, R: ribosomal, T: transport, PS: Cellular Processes and Signaling, PM: protein metabolism.

Chapter 6

Bifidobacterium breve — HT-29 cell line interaction: modulation of TNF-α induced gene expression

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To provide insight in the molecular basis for intestinal host-microbe interactions, we determined the genome-wide transcriptional response of human intestinal epithelial cells (HIECs) following exposure to cells of *Bifidobacterium breve*. To select an appropriate test system reflecting inflammatory conditions, the responsiveness to TNF-α was compared of T84, Caco-2 and HT-29 cells. The highest TNF-α response was observed in HT-29 cells and this cell line was selected for exposure to the *B. breve* strains M-16V, NR 246 and UCC2003. After one hour of bacterial pre-incubation followed by two hours of additional TNF-α stimulation, *B. breve* M-16V (86%), but to a much lesser extent strains NR246 (50%) or UCC2003 (32%), showed a strain-specific reduction of the HT-29 transcriptional response to the inflammatory treatment. The most important functional groups of genes that were transcriptionally suppressed by the presence of *B. breve* M-16V, were found to be involved in immune regulation and apoptotic processes. About 54% of the TNF-α induced genes were solely suppressed by the presence of *B. breve* M-16V. These included apoptosis-related cysteine protease caspase 7 (CASP7), interferon regulatory factor 3 (IRF3), amyloid beta (A4) precursor protein-binding family A member 1 (APBA1), NADPH oxidase (NOX5), and leukemia inhibitory factor receptor (LIFR). The extracellular IL-8 concentration was determined by an immunological assay but did not change significantly, indicating that *B. breve* M-16V only partially modulates the TNF-α pathway. In conclusion, this study shows that *B. breve* strains modulate gene expression in HT-29 cells under inflammatory conditions in a strain-specific way.

INTRODUCTION

It has been well established that our body is a natural habitat for numerous microbes. Its barrier with the surrounding environment, the surface ectoderm, is a common site for colonization. The abundant presence of microbes results in a molecular interplay with the epithelial cells. A major site of microbial interaction is our intestinal tract. Besides its tremendous surface this is due to the unique environmental conditions within. Notably bacteria but also other commensal microbes are able to colonize this unique location and together form our intestinal microbiota (Eckburg et al., 2005; Rajilic-Stojanovic et al., 2009; Zoetendal et al., 2006). There is considerable interest in the understanding the mechanisms involved in beneficial host-microbe interactions and an increasing number of studies are underpinning the effects of the commensal microbes (Boesten and de Vos, 2008; Guarner and Malagelada, 2003; Ouwehand et al., 2002).

To sustain homeostasis in the gut, discrimination between pathogenic and commensal microbes by our immune system is of vital importance. To facilitate this, the human host contains two types of defense, the innate and adaptive immune systems (for a recent review see Medzhitov, 2007). In short, the innate immune recognition is germ-line encoded and mediated by pattern recognition receptor (PRR) systems (Janeway, 1989). The adaptive system enables immunological memory and is mediated by antigen receptors (Schatz et al., 1992). The development of highly specific antibodies by the assembling of genes encoding antigen receptors is stimulated by the activated innate system (Schatz et al., 1992). Basically, the initial recognition of pathogens is performed by the innate immune system. Via the PRRs, it screens for foreign material by searching for microorganism-associated molecular patterns (MAMPs). Examples of bacterial MAMPs are specific cell-envelope components, such as lipopolysaccharides (LPS), peptidoglycan and lipotechoic acids, as well as cell-envelope proteins. Pathogenic bacteria are known to induce inflammatory reactions in human host cells, in contrast to commensals that are recognized as self. As both pathogenic and commensal bacterial cells contain MAMPs, the final outcome of a host-response relies on multiple distinct interactions between PRRs and MAMPs, in combination with associated co-receptors.

The detailed analysis of MAMP-PRR interactions contributed to the present insight in the underlying molecular mechanism of this system. The best characterized class of PRRs are TLRs and their interactions with bacterial surface molecules have been well-documented and include LPS signaling via TLR4 (Akira et al., 2006; Takeda et al., 2003). Under homeostatic conditions, the expression and signaling of an epithelial PRR system may be suppressed. However during inflammation or infection, apical

TLR expression will be induced, enabling bacterial signaling (Abreu et al., 2002). Many of the host inflammatory responses are controlled by the transcription factor nuclear factor (NF-)κB (O'Hara and Shanahan, 2006). Upon activation, through a wide range of stimuli such as TNF-α, IL-1β, or *Escherichia coli* LPS that signals via TLR-4, NF-κB translocates to the nucleus (Hayden and Ghosh, 2004; Li and Verma, 2002). At that place, it controls the transcription of a series of genes involved in acute responses to tissue damage and in chronic intestinal inflammation (Riedel et al., 2006). TNF-α is an important player in the innate and adaptive immune defense. It controls cellular functions at multiple levels. TNF-α signaling leads to diverse downstream events, such as the regulation of NF-κB, MAPK, Jun N-terminal kinase (JNK) and caspases, which in turn influence cell-death and cell-survival decisions (as reviewed by Mathew, 2009). TNF-α mediated functions that have been best characterized are related to apoptosis and inflammation (Aggarwal, 2003).

The vast majority of intestinal commensals are Gram-positive bacteria that do not contain LPS but may affect epithelial activation and TLR signaling via other PPR systems than TLR4. This includes TLR2 that recognizes lipoproteins and lipoteichoic acids, which are integrated in a peptidoglycan layer of Gram-positive bacteria (Schwandner et al., 1999; Vizoso Pinto et al., 2009; Yoshimura et al., 1999). In contrary to the LPS receptor TLR4, TLR2 induces both pro-inflammatory and anti-inflammatory cytokine synthesis (Netea et al., 2004). Interestingly, many PRR ligands are expressed by commensal bacteria, yet the healthy gut does not evoke inflammatory responses to these gut inhabitants. This can be explained by the ability of these bacteria to inhibit translocation of NF-κB to the nucleus and thereby prevent the induction of inflammatory responses. Several different mechanisms to prevent this translocation have been elucidated (O'Hara and Shanahan, 2006). These include inhibition of epithelial proteasome function, stabilization of NF-κB counter-regulatory factor IκBα or prevention of nuclear export of the NF-κB subunit, p65, through a peroxisome proliferator-activated receptor (PPAR)γ-dependent pathway (Kelly et al., 2004; Neish et al., 2000; Petrof et al., 2004).

A number of studies have described the ability of commensal bacteria to modulate the inflammatory response of its host. Common model systems for interaction studies are human intestinal epithelial cell (HIEC) lines. HIECs are considered to form an integral part of the innate immune system, constituting important targets for bacteria and cytokines. Using such a cell line model, it was demonstrated that *Lactobacillus rhamnosus* GG and *L. reuteri* are able to modulate the TNF-α induced release of IL-8 in Caco-2 and in T84 and HT-29 intestinal epithelial cells, respectively (Ma et al., 2004; Zhang et al., 2005). Similarly, *Bifidobacterium bifidum* and *B. longum* showed the capacity to inhibit the LPS-induced NF-κB activation in HIECs (Riedel et al.,

2006). Several comprehensive overviews of the studies on the induction of immune modulation molecules and effector substances by lactic acid bacteria have recently been published (Winkler et al., 2007). The effects on immune modulation molecules have been described as strain- and species-specific and are executed via several different mechanisms (Candela et al., 2008; Sonnenburg et al., 2006).

In summary, many studies indicate that commensal bacteria are capable of modulating the host response under basal or inflammatory conditions. In this study we exploited a global transcriptomics approach in HIECs to provide a molecular basis for the interaction of human epithelial cells with *B. breve* strains that included the model strain UCC2003 (Ventura et al., 2009) and two industrially-relevant strains, M16-V and NR264. TNF-α stimulation was used to generate an NF-kB dependent inflammatory response in the HIEC lines. The results indicate significant but strain-dependent modulation of gene expression in HT-29 cells under inflammatory conditions.

MATERIALS AND METHODS

Bacterial strains, culturing, and co-incubation

All *Bifidobacterium* strains, *B. breve* UCC2003 (Cork University, IR), *B. breve* M-16V (Morinaga Milk Industry Co., Ltd., Zama, JP), and *B. breve* NR264 (Numico Research Culture Collection) were grown anaerobically in MRS broth (Difco, Detroit, USA) supplemented with 0.05% L-cysteine hydrochloride monohydrate (Sigma-Aldrich, Steinheim, G) and incubated at 37°C for 16 – 40 h. Human intestinal epithelial cell lines (HIECs), Caco-2 cells (ATCC destination HTB 37, Manassas, USA), T84 (ATCC, Manassas, USA; passages 57-64), and HT-29 (HTB-38; ATCC, Manassas, USA; passages 136-150) were grown in T75 Falcon flasks (Becton Dickinson, Franklin Lakes, USA) respectively on MEM/NEAA, McCoy's 5a Medium or DMEMF12 glutamax (Invitrogen, Breda, NL), supplemented with 10% Fetal Bovine Serum (Greiner Bio-One, Longwood, USA) and penicillin (100 IU/ml) and streptomoycin (100 µg/ml) (Invitrogen, Breda, NL) (pen/strep) in a humidified incubator at 37°C under 5% CO_2 and 95% air. Cells were grown to confluency. Co-incubations of HIECs and bifidobacteria were performed with supplemented medium without pen/strep. Bacterial cultures were washed in cell line culture medium prior to the co-incubation with the HIECs. A volume of 10 ml bacterial culture (5 x 10^7/ml) was added to the T75 flasks that contained a monolayer of HIECs and 10 ml of supplemented medium without pen/strep. Negative controls, without bacteria, were included in this study.

After one hour of pre-incubation, inflammatory conditions were induced by addition of human recombinant TNF-α (10 ng/ml) (Endogen, Perbio Science, Ettenleur, NL) to the co-culture of HIECs and bacteria and subsequent incubation for 2, 4 and 6 hours. Negative controls, without TNF-α, were included in this study. For ELISA analysis, 24-wells plates were used instead of T75 Falcon flasks (0.5 ml bacterial suspension per well). Prior to and after co-incubation bacterial culture samples were inoculated on MRS (0.05% L-cysteine hydrochloride monohydrate) agar plates for determination of bacterial numbers. After 40 h of anaerobic incubation at 37°C, CFUs were counted.

Immunological assay of IL-8

IL-8 was quantified in supernatants with the Human IL-8 CytoSets™ (Biosource, Invitrogen, Breda, NL), performed as per the vendor's instructions (Biosource) with the following adaptations: a complete TMB solution (Pierce, Thermo Fisher Scientific, Etten-Leur, NL) was used instead of the one described in the protocol.

RNA isolation from HIECs

Cells were harvested by scraping after adding 2 ml of Trizol (Invitrogen, Breda, NL) to the cell monolayer. After scraping the cells were collected in a 2 ml Eppendorf tube, snap-frozen in liquid N_2 and stored at -80°C. Before RNA isolation the cell mixture was defrosted by incubation at room temperature (RT). The thawed cell suspension was homogenized by pipetting and vortexing and incubated at RT for 5 min. Subsequently, 1:5 volumes of chloroform were added at RT and the mixture was shaken vigorously for 15 seconds. Then the samples were cooled on ice for 5 min and centrifuged at 18,000 x g and 4°C for 15 min. After centrifuging the water phase was transferred into a new 1.5 ml Eppendorf tube and placed on ice. The samples were purified and DNase treated using an RNeasy kit (Qiagen, Venlo, NL) according to the protocol of the supplier. Subsequently, the RNA samples were concentrated and purified using Vivaspin500 concentrator tubes (VivaScience, Sigma-Aldrich, Steinheim, G) according to the protocol of the manufacturer.

Labeling

An amount of 25 µg of total RNA was pipetted in an Eppendorf tube and dried down in a Speed Vac at low temperature. Subsequently, 3 µl of oligo (dT) 15primer (50 µM) was added. A volume of 15.5 µl 10U RNasin containing milliQ H_2O was added and the samples were put on ice for 10 min while mixed by vortexing a few times. Subsequently, the samples were incubated at 70°C for 10 min. After cooling at room

temperature for 10 min the samples were centrifuged. Subsequently, per sample the following reagents were added on ice: 6.0 µl 5 x First Strand buffer, 3.0 µl 0.1 M DTT, 0.6 µl 50 x AA-dNTP mix (25 mM dATP, dCTP, dGTP, 15 mM dTTP, 10 mM AA-dUTP), 2.0 µl Superscript II RT enzyme (200 U/µl). The reaction mix was mixed well and incubated for 3 h at 42°C. The RNA template material was hydrolyzed by addition of 3 µl of 2.5 M NaOH. The mixture was mixed well and incubated at 37°C for 30 min. After the incubation 3 µl of 2.5 M HAc was added to neutralize the pH. Unincorporated AA-dUTP and free amines were removed using QIAquick columns (QIAgen Benelux BS, Venlo, NL). Five times the reaction volume of PB buffer (QIAgen supplied) was added to the samples and the mixture was transferred to a QIAqiuck Spin Column. Then the column was centrifuged at 18,000 x g for 1 min. The flow through was reloaded and the column was centrifuged again at 18,000 x g for 1 min. After centrifuging, the flow through was discarded. To wash the samples 500 µl of 80% ethanol was added to the column and they were centrifuged at 18.000 x g for 1 min. After centrifuging, the flow through was discarded and the washing with 80% ethanol was repeated for two more times. Subsequently, the empty collection tubes were dried by centrifuging an additional 1 min at 18,000 x g. For elution of the sample material, 30 µl of milliQ H_2O was carefully added to the centre of the columns after they were transferred to a new 1.5 ml Eppendorf tube. These were incubated for 1 min and centrifuged at 18,000 x g for 1 min. The elution step was repeated and the total volume of 60 µl of purified sample was dried at high temperature in a Speed Vac for about 15-30 min. For coupling of the Cy Dye Esters to the AA-cDNA the dried cDNA samples were dissolved in 4.5 µl 0.1 M sodium carbonate buffer (Na_2CO_3), pH 9.0 during 10 min at room temperature while mixed a few times. Subsequently, 4.5 µl of the appropriate NHS-ester Cy Dye in dimethylsulfoxide (Amersham, Piscataway, USA) were added. This reaction mix was incubated for 1 h at RT in the dark. After incubation 10 µl of milliQ H_2O was added and the samples were centrifuged shortly. To remove the unincorporated dyes we used Autoseq G50 columns (Amersham, Piscataway, USA) according to the protocol supplied by the manufacturer.

Microarray design and construction

The human microarray slides used contain 19,200 spots (16,659 70-mers, Operon human version1 (Operon Biotechnologies, G), and 2541 control spots). The microarrays were printed (Genomics Laboratory, UMC Utrecht, Department for Physiological Chemistry, Stratenum, Utrecht, NL) onto Corning UltraGAPS slides (Corning Incorporated, USA). Scanning of the slides was performed at 10 micron resolution. Array layout: 48 subgrids of 20 x 20 spots.

Prehybridization

The slides were washed at room temperature in a washing buffer containing 2*SSC and 0.05% SDS by shaking vigorously for about 1 min. After washing, the slides were blocked in at 42°C preheated borohydride buffer containing 2 x SSC, 0.05% SDS and 0.25 sodium borohydride (Sigma-Aldrich, Steinheim, G) for 30 min. Subsequently, the slides were washed by dipping 5 times in room temperature milliQ H_2O. After blocking the slides were prehybridized for 45 min at 42°C in prehybridization buffer containing 5 x SSC, 25% formamide (Merck, Darmstadt, G), 0.1% SDS and 1% bovine serum albumin (Sigma-Aldrich, Steinheim, G). After prehybridization the slides were washed by dipping five times in room temperature milliQ H_2O. Subsequently, the slides were blow dried using compressed nitrogen.

Hybridization

Per six slides 250 µl of 0.22 filter sterile 2 x hybridization buffer containing 50% formamide, 10 x SSC and 0.2% SDS, was mixed with 10 µl Salmon sperm DNA (5 mg/ml) and 4 µl Yeast tRNA (25 µg/ul; Invitrogen, Breda, NL) and preheated. A volume of 55 µl of preheated 2 x hybridization buffer was combined with 23 µl Cy3 labeled cDNA, 23 µl Cy5 labeled cDNA and 9 µl milliQ H_2O. This probe mixture was incubated at 95°C for 5 min, centrifuged and placed at 42°C. A volume of 110 µl of the probe was transferred to the slide by pipetting and covered with a LifterSlip (VWR, Soulbury, UK). Finally, 20 µl of milliQ H_2O was added to each well in the hybridization chamber. The chamber was closed and placed in a water bath at 42°C for 16-20 h.

Washing of the slides

Before washing the LifterSlip was removed from the slide in low-stringency washing solution containing 1 x SSC and 0.2% SDS. Subsequently, the slides were incubated for 4 min in this low-stringency washing buffer. For the following high-stringency wash, the slides were transferred to a high-stringency washing buffer containing 0.1 x SSC and 0.2% SDS and incubated for 4 min. The slides were finally washed in 0.1 x SSC for 4 min to remove remnants of SDS. This step was repeated once, but the second time they were shaken vigorously for 1 min. The slides were blow dried using compressed nitrogen.

Microarray scan-image and data pre-processing

After washing and drying, slides were scanned with a ScanArray Express 4000 scanner (Perkin-Elmer Nederland, Groningen, NL). The obtained images were

analyzed using Imagene 5.6 software (BioDiscovery, Marina del Rey, CA). A gene identification file was constructed using CloneTracker software (www.biodiscovery.com). Spots were quantified using ImaGene 5.6 software (www.biodiscovery.com).

During quantification, the ImaGene 5.6 software determined the average spot quality. Spot parameters such as shape regularity of the spots (threshold 0.4), spot size and background signals (signal intensity >2 x background signal) were used for flagging poor and empty spots. For validation of the labeling and hybridization reaction self-self hybridization reactions were performed

Data analysis

Microarray data (Cy5/Cy3 ratios) were LOWESS normalized using ArrayNorm 1.7.2 Software (http://genome.tugraz.at) (default settings). A 80% data filter was used as a cut-off criterion for further analysis. Principal Component Analysis (PCA) (default settings), Hierarchical Clustering Analysis (HCA) by determining the Euclidean Distances in the complete linkage clustering, Significance Analysis for Microarrays (SAM) (Two-class unpaired) and Analysis of Variance (ANOVA) (critical *P* value of 0.01) of the normalized data was performed using TIGR MeV 3.1 Software (www.tm4.org/mev.html).

RESULTS

Comparison of TNF-α induced global transcriptional responses in intestinal cell lines

In a pilot experiment to select the most responsive model system, different confluent monolayers of T84, Caco-2 and HT-29 cells were stimulated with TNF-α. These three HIECs were selected as they represent the most commonly used intestinal cell lines with different degree of differentiation. Subsequently, after two and six hours of incubation, these HIECs were harvested for RNA extraction and the sample transcripts were determined by hybridization to genome-wide microarrays. Hierarchical clustering analysis (HCA) and principle component analysis (PCA) of the generated data, indicated that the parameter ‘cell type’ predicted most of the gene expression values subsequently followed by the ‘time of harvest’ and finally ‘TNF-α stimulation’ (data not shown). Although the overall gene expression showed extensive variation between the cell types, these results confirm the difference in sensitivity to TNF-α. The HCA enabled us to identify sub-clusters of genes that were up-regulated by TNF-α stimulation. Based on significance analysis of microarray data

(SAM) analysis, 25 genes showed differential expression after stimulation with the cytokine. PCA analysis clearly visualized these differences with the responsiveness of HT-29 being the highest, followed by that of Caco-2 cells, while T84 cells were the least responsive and only showed increased gene expression after 6 hours of incubation (Figure 1).

To confirm functional activation of the nuclear factor kappa-B (NF-κB) signalling pathway, IL-8 mRNA and protein concentrations in the supernatant were measured. After two and six hours respectively 16.0 and 1.96 fold up-regulation of IL-8 mRNA expression was observed in HT-29 cells compared to non in T84 and Caco-2 cells.

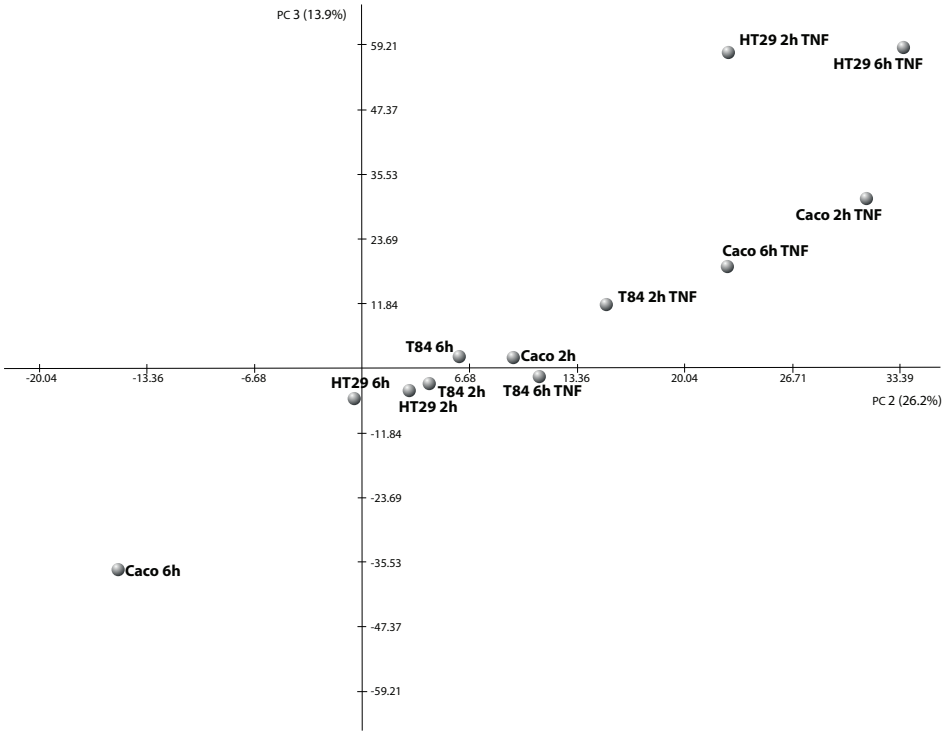

Figure 1. PCA-plot (PC2-3) based on expression data of inflammatory genes (n=25) after in vitro stimulation of Caco-2 (Caco), HT-29 (HT-29), and T84 (T84) cell line monolayers with TNF-α (TNF-) for two (2h) or six (6h) hours. Genes were selected by SAM analysis (TNF-stimulated versus non-TNF-stimulated cells) based on a total of 4710 genes (80% data filter). PC1, PC2, and PC3 explain 28.9%, 26.2%, and 13.9% of the data variation respectively. Settings; components: 25, covariance, number of neighbours for KNN imputation: 10

This was in line with the concentrations of extracellular IL-8 protein detected in their supernatant. After two, six, and 24 hours respectively 500, 2000, and 15000 pg/ml IL-8 protein was measured in HT-29 cells. The extracellular IL-8 level of T84 reaches

its maximum of 120 pg/ml after 24 hours. No extracellular IL-8 was measured in the supernatant of the Caco-2 cells.

Caco-2 cells were responsive to TNF-α but only a few inflammatory genes were differently expressed in comparison to the control situation. Mainly, NF-κB was up-regulated after two hours of stimulation with TNF-α. On the other hand, the HT-29 and T84 cells showed much more interesting gene expression patterns. T84 cells showed up-regulated expression of NF-κB genes and interferon-gamma (IFN-γ) receptor. The expression levels were changed after two hours and more increased after six hours of TNF-α stimulation. MAPK genes were up-regulated at 6 hours of stimulation. However, IL-8 expression was not induced. Obtained ELISA data determined a slide increase of extracellular IL-8, but only 24h after the stimulation. This could be an cumulative effect of either active secretion or passive leakage of IL-8 proteins from the T84 cells.

HT-29 cells showed a strong induction of NF-κB, IL-8, and IFN-γ genes after two hours of stimulation. At six hours of TNF- α stimulation this up-regulation of NF-κB and IL-8 gene expression was decreased compared to the situation after two hours of stimulation, but MAPK signal transduction routes were activated. At two hours of stimulation with TNF-α there were more genes differently expressed than after six hours. In conclusion, HT-29 are more early responsive than the other two cell lines and show an pro-inflammatory gene expression pattern after stimulation with TNF-α. Therefore, the HT-29 cells were used for the inflammatory host-microbe interaction model.

Transcriptome analysis of modulating effect of *B. breve* M-16V

Using TNF-α stimulated HT-29 cells as a test system, we studied whether *B. breve* strains were capable of immune-modulating HIECs. For this purpose, a confluent monolayer of HT-29 cells was pre-incubated for one hour with either *B. breve* UCC2003, *B. breve* M-16V, or *B. breve* strain NR246 followed by an additional TNF-α stimulation of two hours. Subsequently, the global transcriptional response was determined by hybridizing isolated RNA to genome-wide microarrays. Using an 80% data cut-off filter, 5461 genes were selected for further analysis. SAM analysis was applied in order to select genes that were affected by TNF-α stimulation of the HT-29 cells. The data showed that the transcription of 107 genes was differently regulated in the tested conditions and with the applied selection criteria (Table 1). HCA enabled clustering of these genes in three main groups. One group contained 15 genes that were upregulated in all conditions, either bacterial presence, TNF-α stimulation, or both. A second group contained 58 genes that were characterized

by an upregulation in all TNF-α stimulated conditions except for the coincubation with bacterial M-16V cells. Furthermore, a third group included 34 genes that showed solely upregulation when stimulated with TNF-α and could be suppressed by all three *B. breve* strains. This microarray dataset confirmed the pilot study by showing that TNF-α stimulation resulted in the upregulation of a pro-inflammatory gene repertoire in the HT-29 cells (Table 1). Evidence of activation of chemokine signaling, apoptotic, and MAPK pathways was found. IL-8 gene expression was up-regulated by TNF-α stimulation, but the presence of bifidobacteria up to two hours of coincubation did not reduce the IL-8 transcription. Hence, we determined the level of extracellular IL-8 by an immunological assay and observed that the TNF-α induced secretion of IL-8 was also unaltered in the two hour presence of bacteria. TNF-α stimulation resulted in 7000 (SD=63), 12000 (SD=736), and 19000 (SD=233) pg/ml extracellular IL-8 protein after respectively two, four, and six hours of incubation. In comparison, TNF-α stimulation in presence of *B. breve* M-16V showed extracellular concentrations of 8000 (SD=135), 13000 (SD=1307), and 22000 (SD=50) pg/ml, after the same time of incubation, indicating no significant difference. Also the two other strains did not alter the TNF-α induced IL-8 secretion by HT-29 cells while the bifidobacterial cells alone, in absence of TNF-α stimulation, did not induce IL-8 secretion by epithelial cells (results not shown). The colony forming units (CFU) of the bifidobacterial strains during the incubation with the HT-29 cells were determined as an indication for the bacterial viability. At time-point zero and after three hours of incubation, the average number of CFU/ml was stable (approximately 5.0×10^7), indicating that the treatments did not affect viability in this time frame.

Despite the lack of effect of the *Bifidobacterium* strains on the amount of extracellular IL-8 protein, HCA in combination with SAM of the obtained gene expression data showed divergent modulating effects of the *B. breve* strains on gene expression of HT-29 cells upon the TNF-α stimulation. Based on the earlier described TNF-α affected genes (Table 1), a PCA plot was constructed to visualize the effects of the different *B. breve* strains on the expression of these genes (Figure 2). The PCA plot clearly showed that the gene expression patterns of the HT-29 cells incubated with *B. breve* M-16V, NR246, UCC2003 were relatively close to that of the negative control. Furthermore, the TNF-α stimulated HIECs displayed a totally different pattern characteristic of an inflammatory reaction. The gene expression of the TNF-α stimulated HT-29 cells co-incubated with UCC2003 cells and those solely stimulated with TNF-α were highly similar, indicating that there was little effect of the UCC2003 cells. The HT-29 cells that were co-incubated with NR246 and stimulated with TNF-α were found on a significant distance from the controls in the PCA plot. This suggests a mild suppression of the gene expression induced by TNF-α. Remarkably,

Table 1. From the total set of human 5461 genes (80% data filter), 107 genes were selected by SAM analysis based on control (Control) versus TNF-stimulation (TNF-). Hierarchical clustering analysis resulted in three main clusters - '1', contains genes that are upregulated in all TNF-stimulated conditions (n=15); '2', contains genes that are upregulated by TNF-stimulation, but suppressed by the presence of M-16V (n=58); '3', contains genes that are upregulated by TNF-stimulation, but are suppressed by the presence of all three *B. breve* strains (n=34). The table shows the difference in expression with respect to the expression in the TNF-stimulated HT-29 cells (Δ TNF). The different conditions are listed in the header - TNF-α stimulation (TNF-), *B. breve* M-16V co-incubation (M-16V), *B. breve* NR246 co-incubation (NR246), *B. breve* UCC2003 co-incubation (UCC2003), and HT-29 without TNF-α stimulation or bacterial co-incubation (control). KEGG orthologies according to the following codes - A: apoptisis, C: cellular processes, CCRI: cytokine- cytokine receptor interaction, CGD: cell growth and death, CM: carbohydrate metabolism, CP: cytoskeleton proteins, EIP: environmental information processing, GBM: glycan biiosynthesis and metabolism, GIP: genetic information processing, IS: immune system, LM: lipid metabolism, M: metabolism, MS: MAPK signaling, n/a: not available, OS: organismal systems, PC: pathways in cancer, SMI: signal molecules and interaction, ST: signal transduction, T: transcription factor

	Co-incubation with B. breve cells					*TNF stimulation and co-incubation with B. breve cells*				
Clusters (HCA-euclidean dist)	**UCC2003**	**M-16V**	**NR246**	**control**	**TNF**	**TNF/UCC2003**	**TNF/NR246**	**TNF/M-16V**	**Gene description**	**KEGG Orthology**
1	⇩0.21	⇧1.65	⇧3.88	⇩-0.77	⇨1.00	⇩0.47	⇨0.72	⇨1.03	jun B proto-oncogene (JUNB), mRNA	T
	⇨1.38	⇨1.36	⇧1.76	⇩-0.05	⇨1.00	⇩0.36	⇧2.05	⇨0.59	mRNA; cDNA DKFZp564C2478 (from clone DKFZp564C2478); complete cds	n/a
	⇩0.38	⇩-0.09	⇩0.46	⇩-0.20	⇨1.00	⇨1.11	⇩0.07	⇩-0.28	clone 23860 mRNA sequence	n/a
	⇩0.17	⇨0.91	⇩0.09	⇩0.14	⇨1.00	⇨1.12	⇩0.25	⇩0.17	actin, alpha 2, smooth muscle, aorta (ACTA2), mRNA	CP
	⇨0.68	⇨1.31	⇨1.01	⇩-0.15	⇨1.00	⇧1.54	⇨1.48	⇧1.70	interferon gamma receptor 1 (IFNGR1), mRNA	**ST, CR, EIP**
	⇨0.69	⇨1.28	⇨0.91	⇩-0.01	⇨1.00	⇨1.27	⇨1.06	⇨1.40	tumor necrosis factor, alpha-induced protein 2 (TNFAIP2), mRNA	**IS, GIP**
	⇨0.55	⇨1.01	⇨0.50	⇩0.05	⇨1.00	⇨1.32	⇨1.05	⇨0.98	HCG I mRNA	n/a
	⇩0.36	⇨0.81	⇨0.55	⇩-0.12	⇨1.00	⇨1.02	⇨1.10	⇨1.35	syndecan 4 (amphiglycan, ryudocan) (SDC4), mRNA	EIP, SMI
	⇩0.20	⇨0.88	⇨0.73	⇩-0.34	⇨1.00	⇨1.46	⇨1.04	⇨1.15	GRO1 oncogene (melanoma growth stimulating activity, alpha) (GRO1), mRNA	n/a
	⇩0.39	⇨0.71	⇩0.28	⇩0.13	⇨1.00	⇨1.23	⇨1.16	⇨0.99	MAD, mothers against decapentaplegic homolog 3 (Drosophila) (MADH3), mRNA	EIP
	⇨0.62	⇨0.74	⇨0.55	⇩0.14	⇨1.00	⇨1.42	⇧1.69	⇨1.32	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105) (NFKB1), mRNA	**EIP, IS, CGD, A**
	⇨0.96	⇨0.59	⇩0.28	⇩-0.46	⇨1.00	⇧1.83	⇨1.40	⇨1.29	v-rel reticuloendotheliosis viral oncogene homolog B (RELB), mRNA	GIP, ST, MS
	⇩0.30	⇩0.82	⇩0.07	⇩-0.52	⇨1.00	⇧1.55	⇨1.20	⇨1.46	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (NFKBIA), mRNA	**GIP, T, IS**
	⇩0.36	⇨0.61	⇩-0.07	⇩-0.04	⇨1.00	⇨1.10	⇨0.88	⇨0.64	B-cell CLL/lymphoma 3 (BCL3), mRNA	GIP, T
	⇩0.29	⇨0.58	⇩0.19	⇩-0.28	⇨1.00	⇨1.12	⇨0.99	⇨1.13	interleukin 8 (IL8), mRNA	**GIP, IS, EIP, SMI**
2	⇩0.24	⇩0.16	⇩0.13	⇩-0.45	⇨1.00	⇩0.18	⇨1.47	⇩-0.43	anti-Mullerian hormone receptor, type II (AMHR2), mRNA	EIP, ST, SMI
	⇩0.15	⇩0.21	⇩0.40	⇩0.33	⇨1.00	⇨1.02	⇩0.44	⇩0.46	U5 snRNP-specific 40 kDa protein (hPrp8-binding) (HPRP8BP), mRNA	n/a
	⇩0.46	⇨0.82	⇨0.63	⇩-0.01	⇨1.00	⇧1.57	⇧1.50	⇧1.79	sequestosome 1 (SQSTM1), mRNA	n/a
	⇩0.49	⇩0.48	⇩0.29	⇩-0.04	⇨1.00	⇨0.88	⇨0.72	⇨0.82	inositol polyphosphate-1-phosphatase (INPP1), mRNA	EIP, CM, ST
	⇩0.49	⇨0.63	⇩0.27	⇩0.24	⇨1.00	⇨0.60	⇨0.76	⇩-0.12	phosphatidylinositol glycan, class Q (PIGQ) , mRNA	M, GBM
	⇨0.69	⇩0.30	⇩0.07	⇩0.16	⇨1.00	⇨0.55	⇨0.61	⇩-0.17	KIAA0996 protein	n/a
	⇩0.33	⇩0.26	⇩0.29	⇩0.39	⇨1.00	⇩0.33	⇩0.44	⇩0.39	mRNA for KIAA1261 protein, partial cds	n/a
	⇩0.22	⇩0.35	⇩0.47	⇩0.04	⇨1.00	⇨0.51	⇨0.56	⇩0.15	map kinase phosphatase-like protein MK-STYX (LOC51657), mRNA	n/a
	⇨0.51	⇩0.48	⇨0.52	⇩0.05	⇨1.00	⇨0.82	⇨0.76	⇩0.20	senescence-associated epithelial membrane protein (SEMP1) mRNA, complete cds	n/a
	⇩0.15	⇩0.30	⇩0.39	⇩-0.08	⇨1.00	⇩0.48	⇨0.72	⇩0.19	KIAA0969 protein	n/a
	⇨0.51	⇩0.50	⇨0.66	⇩0.08	⇨1.00	⇩0.25	⇩0.17	⇩-0.27	sulfotransferase, estrogen-preferring (STE), mRNA	M
	⇩0.17	⇩0.01	⇩0.02	⇩-0.19	⇨1.00	⇨0.82	⇩0.30	⇩-0.12	Fc fragment of IgG, low affinity IIIa, receptor for (CD16) (FCGR3A), mRNA	n/a
	⇩0.13	⇩0.23	⇩0.21	⇩-0.36	⇨1.00	⇨0.69	⇩0.42	⇩-0.23	mRNA for KIAA1331 protein, partial cds	n/a
	⇩0.00	⇩-0.40	⇩-0.18	⇩-0.18	⇨1.00	⇨0.79	⇨0.64	⇩-0.04	interferon regulatory factor 3 (IRF3), mRNA	**GIP, IS, T**
	⇩0.07	⇩-0.10	⇩0.08	⇩-0.15	⇨1.00	⇨0.53	⇨0.80	⇩-0.07	caspase 7, apoptosis-related cysteine protease (CASP7), mRNA	**M, C, CGD, A**
	⇩0.16	⇩0.02	⇩0.12	⇩0.05	⇨1.00	⇨1.08	⇨0.53	⇩-0.08	sodium channel, voltage-gated, type II, alpha 2 polypeptide (SCN2A2), mRNA	n/a
	⇩0.31	⇩-0.03	⇩-0.03	⇩-0.22	⇨1.00	⇨0.70	⇩0.32	⇩0.27	mRNA for myosin-I beta	C, CP
	⇩-0.01	⇩0.11	⇩-0.12	⇩-0.41	⇨1.00	⇨0.60	⇩0.12	⇨0.54	TRAIL receptor 2 mRNA, complete cds	**EIP, SMI, CCRI, CC**
	⇩0.19	⇩0.08	⇩-0.32	⇩-0.16	⇨1.00	⇨1.13	⇨0.96	⇩0.20	1-acylglycerol-3-phosphate O-acyltransferase 1 (AGPAT1), mRNA	M, LM
	⇩-0.02	⇩-0.20	⇩-0.32	⇩-0.16	⇨1.00	⇨0.86	⇨0.98	⇩0.23	cDNA FLJ13753 fis, clone PLACE3000353	n/a
	⇩0.05	⇩-0.16	⇩-0.19	⇩0.03	⇨1.00	⇨0.79	⇨0.85	⇩0.09	genomic DNA, integration site for Epstein-Barr virus	n/a
	⇩-0.02	⇩-0.77	⇩-0.45	⇩-0.21	⇨1.00	⇧1.58	⇨0.56	⇩0.11	(MAR11) MUC5AC mRNA for mucin (partial)	n/a
	⇩0.14	⇩-0.48	⇩-0.11	⇩-0.09	⇨1.00	⇨0.97	⇨0.98	⇩0.16	transcription factor (ICBP90), mRNA	T
	⇩-0.13	⇩-0.49	⇩-0.28	⇩-0.17	⇨1.00	⇨1.06	⇨0.85	⇩0.16	cleavage and polyadenylation specific factor 1, 160kD subunit (CPSF1), mRNA	GIP
	⇩-0.03	⇩-0.47	⇩-0.23	⇩-0.02	⇨1.00	⇨1.14	⇨1.08	⇩0.23	hexosaminidase B (beta polypeptide) (HEXB), mRNA	M, CM, GBM, C, G
	⇩0.06	⇩-0.44	⇩-0.01	⇩-0.02	⇨1.00	⇨0.95	⇨0.93	⇩0.01	mRNA; cDNA DKFZp566P2324 (from clone DKFZp566P2324)	n/a
	⇩-0.12	⇩-0.65	⇩-0.33	⇩-0.07	⇨1.00	⇨1.01	⇨0.71	⇩0.05	amyloid beta (A4) precursor protein-binding, family A, member 1 (X11) (APBA1), mRNA	**C, ST**
	⇩-0.03	⇩-0.74	⇩-0.37	⇩-0.20	⇨1.00	⇨0.98	⇨0.78	⇩-0.04	clone 24462 mRNA sequence	n/a
	⇩-0.09	⇩-0.42	⇩-0.56	⇩-0.14	⇨1.00	⇨0.97	⇨0.93	⇩-0.02	leukemia inhibitory factor receptor (LIFR), mRNA	**EIP, ST, CCRI, SMI**

	-0.01	-0.47	-0.39	-0.11	1.00	0.81	0.78	-0.14	clone RP1-100N22	n/a
	-0.08	-0.52	-0.33	-0.10	1.00	1.04	0.94	-0.12	PR domain containing 4 (PRDM4), mRNA	OS
	-0.04	-0.48	-0.32	-0.10	1.00	1.02	0.96	-0.12	G protein-coupled receptor 92 (GPR92), mRNA	GIP, SMI
	-0.07	-0.72	-0.48	-0.21	1.00	0.97	1.02	-0.08	NG3 protein (NG3), mRNA	EIP, SMI
	-0.05	-0.89	-0.12	-0.32	1.00	0.73	0.67	-0.01	NADPH oxidase, EF hand calcium-binding domain 5 (NOX5), mRNA	**C, IS**
	-0.22	-0.96	-0.15	-0.60	1.00	0.88	0.70	-0.15	mRNA for KIAA1453 protein, partial cds	n/a
	-0.01	-0.75	-0.45	-0.57	1.00	0.51	0.83	-0.07	hypothetical protein FLJ21156 (FLJ21156), mRNA	n/a
	-0.10	-0.81	-0.19	-0.31	1.00	0.38	0.62	-0.08	neuropeptide Y receptor Y2 (NPY2R), mRNA	EIP, SMI
	0.09	-0.80	-0.16	-0.38	1.00	0.30	0.36	-0.04	hypothetical protein DJ167A191 (DJ167A191), mRNA	n/a
	0.34	-1.20	-0.05	-0.68	1.00	0.71	-0.09	-0.10	butyrate-induced transcript 1 (HSPC121), mRNA	n/a
	0.16	-1.43	-0.21	-0.93	1.00	0.95	-0.61	-0.27	Kruppel-like factor 15 (KLF15), mRNA	GIP, T
	0.43	-1.23	-0.06	-0.43	1.00	0.46	0.17	-0.72	H2B histone family, member A, clone MGC:2561 IMAGE:2989839, mRNA, complete cds	GIP
	0.42	-1.31	-0.41	-1.16	1.00	0.43	0.08	-1.06	mRNA for KIAA1037 protein, partial cds	n/a
	0.63	-0.92	-0.09	-0.68	1.00	0.96	-0.17	-0.51	PAC 86C11 on chromosome 6p2131-221	n/a
2	0.66	-0.95	-0.05	-0.52	1.00	0.99	0.39	-0.33	LIM domain only 6 (LMO6), mRNA	n/a
	0.31	-1.35	-0.01	-0.89	1.00	1.40	0.62	-0.12	hypothetical protein PP5395 (PP5395), mRNA	n/a
	-0.23	-0.75	-0.06	-0.37	1.00	0.89	0.07	0.55	clone bsmneg3-t7 immunoglobulin lambda light chain VJ region, (IGL) mRNA, partial cds	EIP, SMI
	0.04	-0.29	-0.50	-0.53	1.00	0.37	0.49	-0.02	FYVE and coiled-coil domain containing 1 (FYCO1), mRNA	n/a
	0.25	-0.38	-0.26	-0.35	1.00	0.67	0.38	-0.04	collagen, type XV, alpha 1 (COL15A1), mRNA	GBM
	0.15	-0.45	-0.49	-0.07	1.00	0.46	0.31	-0.12	leucine-rich neuronal protein (LRN), mRNA	n/a
	0.00	-0.60	-0.30	-0.31	1.00	0.64	0.28	-0.23	chromosome 16 open reading frame 44 (C16orf44), mRNA	n/a
	-0.33	-5.63	-1.28	-2.46	1.00	3.27	0.97	-0.01	clone RP11-150A6 on chromosome 6	n/a
	-0.22	-1.51	-0.79	-1.39	1.00	0.23	-0.04	-0.70	cDNA FLJ20213 fis, clone COLF1971, highly similar to AB007921 mRNA for KIAA0452 protein	n/a
	-0.39	-1.62	-0.43	-0.64	1.00	0.64	0.21	-0.53	chromosome 21 open reading frame 62 (C21orf62), mRNA	n/a
	-0.41	-1.49	-0.81	-0.94	1.00	0.64	0.05	-0.96	melanopsin (MOP) gene, complete cds	n/a
	-0.91	-3.78	-0.53	-2.98	1.00	0.00	-0.22	-0.06	interleukin 6 signal transducer (gp130, oncostatin M receptor) (IL6ST), mRNA	**EIP, ST, SMI, CCRI**
	-1.68	-2.25	-0.36	-4.96	1.00	1.54	0.95	-0.79	multiple PDZ domain protein (MPDZ), mRNA	n/a
	0.02	0.06	-0.04	0.00	1.00	0.59	0.39	-0.32	hypothetical protein FLJ11618 (FLJ11618), mRNA	n/a
	0.02	0.02	0.00	-0.11	1.00	0.01	0.05	-0.03	mRNA for pak5 protein	n/a
	-0.10	-0.30	-0.23	-0.93	1.00	0.52	0.37	-0.79	mRNA; cDNA DKFZp434J1521 (from clone DKFZp434J1521); complete cds	n/a
	-0.07	-0.29	-0.20	-0.97	1.00	-0.74	0.18	-0.24	hypothetical protein FLJ11336 (FLJ11336), mRNA	n/a
	0.43	-0.22	-0.18	-0.66	1.00	0.05	-0.27	-0.06	heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1 (HS3ST3B1), mRNA	M, GBM
	0.34	0.33	-0.18	-0.65	1.00	-0.20	-0.49	-0.63	heat shock 27kDa associated protein (FLJ22623), mRNA	n/a
	-0.25	0.44	-0.34	-0.52	1.00	0.05	0.28	-0.03	wingless-type MMTV integration site family, member 10A (WNT10A), mRNA	EIP, ST, PC
	1.12	-0.56	-0.01	-0.40	1.00	0.30	0.72	-0.17	chromosome 16 open reading frame 7 (C16orf7), mRNA	n/a
	0.50	-0.49	-0.31	-0.30	1.00	0.12	0.78	-0.14	platelet-activating factor acetylhydrolase, isoform Ib, beta subunit (30kD) (PAFAH1B2), mRNA	n/a
	0.37	-0.07	0.38	0.04	1.00	0.02	0.43	-0.12	ABO blood group (ABO), mRNA	M, GBM
	0.72	0.01	0.37	-0.62	1.00	0.58	0.57	-0.04	solute carrier family 5 (sodium/glucose cotransporter), member 2 (SLC5A2), mRNA	C
	-0.41	1.41	-1.96	-3.13	1.00	-0.78	-0.41	-1.86	brain tumor associated protein NAG14 (NAG14) mRNA, complete cds	PC
	0.54	0.66	-0.09	-0.41	1.00	-1.19	0.76	-0.99	GKAP/SAPAP interacting protein (SHANK) mRNA, partial cds	n/a
	-0.03	0.80	-0.32	-0.68	1.00	-1.37	-0.22	-0.45	KIAA0644 gene product (KIAA0644), mRNA	n/a
	0.20	-0.25	-0.10	0.11	1.00	-0.19	-0.30	-0.31	platelet-activating factor acetylhydrolase 2 (40kD) (PAFAH2), mRNA	n/a
	-0.07	-0.10	0.04	0.12	1.00	-0.76	-0.06	0.19	cDNA FLJ10071 fis, clone HEMBA1001702	n/a
	0.08	0.13	0.03	0.02	1.00	-0.03	0.01	0.15	phosphomannomutase 1 (PMM1), mRNA	n/a
	0.14	1.02	0.29	0.11	1.00	-0.32	0.22	-0.25	nucleotide binding protein 1 (MinD homolog, E coli) (NUBP1), mRNA	GIP
	-0.70	-3.28	-0.70	-1.69	1.00	-0.19	0.55	0.34	mRNA full length insert cDNA clone EUROIMAGE 1968422	n/a
3	3.03	9.30	5.06	8.19	1.00	5.35	4.35	0.65	lodestar protein mRNA, complete cds	n/a
	1.04	-0.48	0.04	-4.70	1.00	3.03	-0.73	-1.23	small inducible cytokine subfamily A (Cys-Cys), member 21 (SCYA21), mRNA	n/a
	0.24	0.18	0.08	-0.37	1.00	1.55	0.04	-0.66	2,4-dienoyl CoA reductase 2, peroxisomal (DECR2), mRNA	C
	-0.38	-1.51	-0.23	-1.62	1.00	-2.29	-1.07	0.39	mRNA for KIAA1205 protein, partial cds	n/a
	0.32	-1.91	-0.48	-3.05	1.00	-2.03	-0.61	-0.26	DNA for immunoglobulin alpha heavy chain from a case of alpha heavy chain disease	n/a
	0.72	-2.41	-1.41	-2.90	1.00	-2.48	-0.59	-1.43	mRNA; cDNA DKFZp586L012 (from clone DKFZp586L012)	n/a
	-0.07	-0.79	-0.44	-0.33	1.00	-0.14	0.17	-0.13	clone RP3-402N21	n/a
	0.12	-1.75	-0.82	-1.09	1.00	0.02	0.09	0.06	clone HQ0663 PRO0663 mRNA, partial cds	n/a
	0.00	-0.90	-0.78	-0.78	1.00	-0.65	-0.50	-0.43	NDRF gene for neuroD-related factor, complete cds	n/a
	-0.19	-0.65	-0.17	-0.47	1.00	-0.49	-0.08	-0.35	cDNA: FLJ23027 fis, clone LNG01826	n/a
	-0.11	-2.82	-1.25	-2.18	1.00	0.70	-2.46	-1.66	clone FLB7723 PRO2055 mRNA, complete cds	n/a
	0.09	-2.29	-0.52	-1.24	1.00	0.03	-2.00	-0.92	hypothetical protein LOC51260 (LOC51260), mRNA	n/a
	-3.08	-6.81	-2.18	-5.48	1.00	-0.19	-5.89	-0.62	putative opioid receptor, neuromedin K (neurokinin B) receptor-like (TAC3RL), mRNA	n/a
	-1.48	-1.75	-1.02	-1.59	1.00	0.02	-2.50	0.34	small inducible cytokine B subfamily (Cys-X-Cys motif) (SCYB13), mRNA	n/a
	-0.13	-0.14	-2.23	-1.67	1.00	-0.81	-1.58	0.84	chorionic somatomammotropin hormone-like 1 (CSHL1) transcript variant 3, mRNA	n/a
	-2.03	-1.38	-1.31	-2.84	1.00	1.00	-1.84	1.35	tumor endothelial marker 1 precursor (TEM1), mRNA	C, CGD
	-0.31	-0.48	-0.55	-0.84	1.00	0.19	-0.54	-0.01	hypothetical protein FLJ13955 (FLJ13955), mRNA	n/a

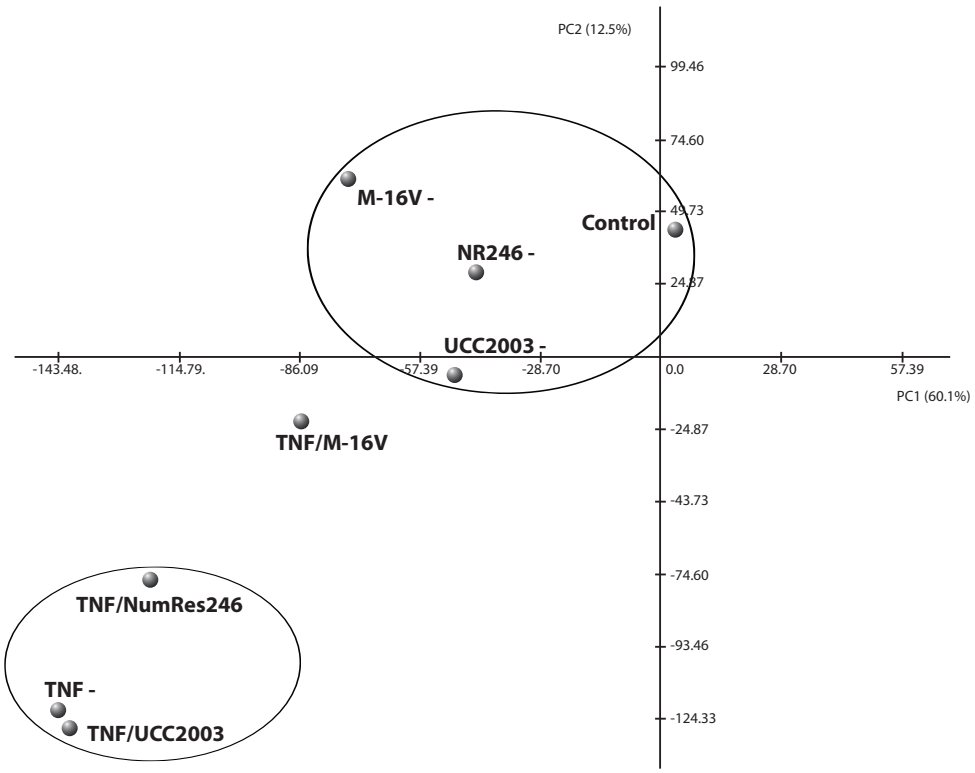

Figure 2. PCA-plot (PC1, 2) based on average expression data (n=2) of genes selected as described in the legend of Figure 2. PC1, PC2, and PC3 explain 60.1%, 12.5%, and 10.5% of the data variation respectively. Settings; components: 107, covariance, number of neighbours for KNN imputation: 10

the TNF-α stimulated HIECs co-incubated with M-16V were not located close to the other TNF-α stimulated samples in the PCA but revealed an expression pattern overlapping with that of the control incubation without TNF-α. This is indicative of the suppression of the effect induced by TNF-α-by these cells. These results indicate that the expression of a cluster of genes that is affected by TNF-α stimulation, is modulated by the presence of *B. breve* M-16V, but not by *B. breve* NR246 or *B. breve* UCC2003. This provides support for the existence of strain-specific effects of bifidobacteria on HT-29 cells.

Analysis of functional groups that are modulated

All genes that are found to be affected by TNF-α including their KEGG orthology grouping, are listed in Table 1. Remarkably, the transcriptional regulation of a subset of 58 genes could be influenced by the presence of M-16V. Some genes that were

clearly influenced can be linked to inflammation and apoptosis. *B. breve* M-16V, and not the other two strains, prevented TNF-α induced up regulation of apoptosis-related cysteine protease caspase 7 (CASP7), interferon regulatory factor 3 (IRF3), NADPH oxidase (NOX5), and amyloid beta (A4) precursor protein-binding family A member 1 (APBA1), genes which are involved in activation of apoptotic and immune regulatory pathways (Chenevier-Gobeaux et al., 2006; Korfali et al., 2004; Palmerini et al., 2001; Park et al., 2006; Reimer et al., 2008). Furthermore *B. breve* M-16V suppressed the induction of leukemia inhibitory factor receptor (LIFR), involved in cell cycle arrest (Tomida, 2000) and IL-6ST, which was also suppressed by NR246 and UCC2003, involved in pro-inflammatory cytokine signaling (Radtke et al., 2010). Notably, The TNF-α induced gene, TRAIL receptor 2, was one of the few genes to be suppressed by NR246. According to its KEGG orthology this gene is involved in TNF-related apoptosis.

DISCUSSION

In this study we determined the genome-wide transcriptional response of human epithelial intestinal cells (HIECs) following exposure to cells of *Bifidobacterium breve*. TNF-α stimulated HT-29 cells were selected for exposure to the *B. breve* strains M-16V, NR246 and UCC2003. After one hour of bacterial pre-incubation followed by two hours of additional TNF-α stimulation, *B. breve* M-16V, to a much lesser extent strain NR246, but not UCC2003, showed a strain-specific reduction of the HT-29 transcriptional response to the inflammatory treatment. The results underline the complexity of the cell-cell signalling interaction network between intestinal epithelial cells and bifidobacteria. *Bifidobacterium breve* M-16V selectively prevented TNF-α induced gene expression of HT-29 human intestinal epithelial cells. *Bifidobacterium breve* NR246 and *B. breve* UCC2003 were less effective and showed little or no specific modulation. Although 86% of the TNF-α induced genes were suppressed by *B. breve* M-16V, transcription of general NF-κB signalling molecules and IL-8 was not affected. *Bifidobacterium breve* M-16V however interfered with specific TNF-α induced genes involved in immune regulation, and apoptosis. *Bifidobacterium breve* M-16V has been described as a potential anti-allergic strain with respect to ovalbumin-allergic asthma and food allergy in mice (Hougee et al., 2010; Schouten et al., 2009). In similar experiments, no anti-allergic effect of *B. breve* NR246 could be observed (L.E.M. Willemsen, personal communication), supporting our present findings that this strain showed a mild anti-inflammatory effect. We could not

detect a clear effect of the presence of bifidobacteria on HIEC gene expression under non-inflammatory conditions. Next to HT-29 cells, incubation of Caco-2 cells with bifidobacteria did not result in a significant change of gene expression in the HIECs data (not shown). Even not a light inflammatory response was measured as described previously for a set of *Lactobacillus* strains (Vizoso Pinto et al., 2009). We suggest that under stress-free conditions the bifidobacteria did not modulate the HIECs as there was no need change their local environment. As inflammatory conditions might provoke them to perform immune-modulating activities, TNF-α induced immune stress was used to study the effect of three different bifidobacterial strains on HIEC in an inflammatory host-microbe interaction model. TNF-α induces an inflammation reaction in the HIECs *in vitro* and also is known to affect epithelial signalling and integrity in patients affected with inflammatory bowel disease (Riedel et al., 2006; Weber et al., 2010). To mimic the *in vivo* situation of a colonized intestinal tract, the HIECs were pre-incubated with bacteria before stimulation with TNF-α. A pre-incubation period of one hour is long enough to enable the HIECs to adapt to the presence of the bacteria and sufficiently short to prevent acidification of the culture medium. While gene-expression is regulated within seconds, the subsequent two hour additional stimulation with TNF should result in a measurable effect on transcriptome level. It is described that the sensitivity of HIECs to cytokines differs per cell type (Schuerer-Maly et al., 1994). Therefore, we screened the three most commonly used HIECs ─ Caco-2, HT-29 and T84 - for their sensitivity for TNF-α. Our results indicated a clear response of HT-29 cells to extracellular TNF-α stimulation of the IL-8 secretory pathway in correspondence with increased IL-8 transcription strongly present after 2 hr of incubation. This was less prominent for Caco-2 and T84 cells and IL-8 secretion was very low or not observed. In the HT-29 most genes were differentially expressed after two hours of stimulation. HT-29 showed a strong induction of NF-κB genes which was tightly regulated since it was decreased after six hours of stimulation while MAPK signal transduction routes were activated at this time point. This correlates with the described response of HT-29 to TNF-α, which triggers inflammatory pathways (Gross et al., 1995). From our results, we concluded that TNF-α stimulated HT-29 cells gave a clear response on transcription, which is measurable by microarray analysis and correlated with IL-8 protein secretion. Based on this characteristic of a rapid response, the HT-29 cell line was chosen as model cell line in co-incubation experiments with the set of bifidobacteria.

It has been described that inflammation or infection can induce apical TLR expression in HIECs (Muzio et al., 2000). Bifidobacteria may affect NF-κB signalling amongst others by binding to these TLR receptors. Stimulation of HT-29 cells with TNF-α followed by co-incubation with *B. longum* Bar33 whole cells was found to

decrease of IL-8 production, although this response was dose-independent (Candela et al., 2008). In contrast *L. rhamnosus* GG and not *B. breve* M-16V was found to enhance the pro-inflammatory immune response in a trans-well coculture model of HT-29 and activated PBMC (Hoffen et al., 2010). However, *B. breve* M-16V and not *B. infantis* NR251, *B. animalis* NR252, *B. animalis* NR253, *L. plantarum* NR8, or *L. rhamnosus* NR6 effectively decreased ovalbumin-sensitization in mice (Hougee et al., 2010). Overall, these experiments suggested that commensal microbes are strain-specific in their ability to regulate the delicate balance between necessary and excessive immune responses (Winkler et al., 2007). This is supported by our observations in which *B. breve* M-16V, NR246, and UCC2003 were able to subsequently suppress 86%, 50%, and 30% of the TNF-induced set of genes (Table 1). No clear pathways could be identified from the set of genes suppressed by UCC2003 and NR246. M-16V on the other hand showed suppression of encoded proteins involve apoptotic and immune activation pathways. Dampening of these pathways by *B. breve* M-16V may help to regulate the inflammation. Interestingly, the TNF-α signaling was only partially influenced since transcription of NFκB and MAPK signaling proteins and IL-8 transcription and translation remained unaffected.

In conclusion, *B. breve* M-16V was found to partially modulate the TNF-α induced inflammation of HIEC. These modulating abilities were strain-specific since two other *B. breve* strains were ineffective. Overall, these findings will contribute to a better understanding of the underlying mechanisms of interaction between bifidobacteria and HIECs. Moreover, genomic comparison of the *B. breve* genomes with that of the model strain UCC2003 will advance further insight in the molecular determinants involved in this strain-specific signalling.

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Chapter 7

General discussion & concluding remarks

This thesis combines comprehensive microarray-based studies contributing to a better understanding of the role of bifidobacteria in relation to the human host. An general overview on the described modes of interaction between bifidobacteria and human gastrointestinal cells is presented in **Chapter 1**. It focuses on the unique characteristics of the genus *Bifidobacterium* in comparison with that of the genus *Lactobacillus* that are indicative for the role of their members in our intestines. **Chapter 2** describes the development of a microarray platform that enables genomic comparison of *Bifidobacterium* species originating from our gastrointestinal tract (GIT). Based on the obtained high-resolution data, species-unique genomic sequences could be identified. The ability to zoom in on strain level, using this platform, was tested in **Chapter 3**. This work showed a relatively high genomic variation between the different *B. breve* isolates, using direct mapping of genomic hybridization patterns. Clustering of hybridization patterns resulted in clear grouping of isolates originating from the same infant. The predicted difference in gene content between the new isolates was approximately 60-90%, testifying for the existence of various subspecies within the species *B. breve*, or even for new *Bifidobacterium* species. Analysis of the bifidobacterial genomic variation was studied in a broader sense in **Chapter 4**. This Chapter describes the analysis of genomic variations between total *Bifidobacterium* populations from different infant fecal samples. The applied microarray platform showed the potential to monitor temporal development and effects of dietary regiments. The observation of different compositions of bifidobacterial populations could be linked to dietary effects. Mapping of the hybridization patterns enabled monitoring shifts in genomic content within one bifidobacterial species in time. Sequence analysis of DNA fragments showing discriminating hybridization characteristics resulted in a selection of genes that are conserved or strain-specific within the species *B. breve*. Next to studying genomic variation, transcript profiling experiments in both bifidobacterial cells and human intestinal epithelial cell lines were performed (**Chapters 5 and 6**). Clear proof of transcriptional activity in bifidobacterial cells isolated from infant feces was presented in **Chapter 5**. To the best of our knowledge, this is the first demonstration of *in situ* activity of bifidobacteria in the human GIT. Furthermore, our results indicate a link between transcription patterns and the infants diet. Bifidobacteria in breast-fed infants showed differential transcriptional responses in comparison those in formula-fed infants. Additionally, transcript sequence analysis revealed the expression of genes that are homologous to genes that are known to be involved in folate production, testifying for the production of this important vitamin in early life. A view from the other side was presented in **Chapter 6**. This transcriptomics study focused on the effect of different *B. breve* strains on HT-29 human intestinal epithelial cells (HIECs).

An inflammatory environment was mimicked by stimulating the HT-29 cells with the pro-inflammatory cytokine TNF-α. Co-incubation of HIECs with bifidobacterial cells during TNF-stimulation resulted in the observation of an species-specific suppression of genes upregulated by TNF-α. Although we did not observe complete suppression of the TNF effect, we could show that apoptotic and immune regulatory pathways were affected by incubation with cells of *B. breve* M-16V.

In the following sections the relations between the work presented in this thesis and other relevant studies and recent publications will be discussed in more detail. The bifidobacterial taxonomy will be summarized and used as reference for the interpretation of the observed genomic variation between new *B. breve* isolates and the results of the genomic comparisons. It will be discussed how the species-specific DNA fragments may contribute to an improved understanding of species-specific characteristics and effects. Moreover, an overview of various observations on bifidobacterial populations in both infants and adults will presented and linked to our own data. Furthermore, the new insights in the bifidobacterial effects discovered in the present work will be compared to recent findings in other interaction studies (such as reviewed in **Chapter 1**). Finally, an overview of the recently completed and currently ongoing genome projects, together with a general comparison of next-generation sequencing platforms summarizes the current state of the art and provides new avenues for studying the functionality of bifidobacteria and other microbes in the human GIT.

THE GENUS *BIFIDOBACTERIUM*

An exhaustive overview of the history and phenotypic characteristics of the genus *Bifidobacterium* has been recently published (Lee and O'Sullivan, 2010). Currently, around 31 species have been assigned to this genus including recent additions such as *B. denticolens* and *B. inopinatum* isolated from human dental caries and *B. psychraerophilum*, originating from pig intestines. Different phylogenetic analyses using different methods and models, affirm that the genus *Bifidobacterium* contains different groups of species (Favier et al., 2002; Ventura et al., 2006). These have been named based on the first described species in the group and include the following groups: the *B. adolescentis* group, the *B. pullorum* group, the *B. asteroids* group, the *B. boum* group, the *B. pseudolongum* group (for a detailed overview see Fig. 1). The species *B. breve* and *B. longum* form a couple, as well as *B. minimum* and *B. psychroaerophilum*, although the latter grouping is less well supported.

Bifidobacterium bifidum, B. magnum, B. scardovii and *B. subtile* form distinct branches (Fig.1). Recently, a phylogenetic re-analysis was reported by using a multilocus sequencing approach (Ventura et al., 2006). This confirmed the phylogenetic structure of the genus at almost all levels and improved the discrimination of related species. Other molecular methods addressed taxonomic issues in the genus *Bifidobacterium* concerning the species *B. longum* and *B. infantis*. These are now considered to belong to one of the three biotypes of *B. longum* that include the *infantis* type, the *longum* type, and the *suis* type (Sakata et al., 2002). Another debated issue is the relationship between *B. animalis* and *B. lactis*, and the latter was recently reclassified as *B. animalis* subsp. *lactis*. This had an impact on the taxonomic position of the frequently used probiotic *Bifidobacterium* strain Bb12 that systematically correct should be termed *B. animalis* subsp. *lactis* Bb12. Finally, a further differentiation was made between *B. indicum, B. coryneforme* and *B. asteroids*. While *B. indicum* and *B. asteroids* could be easily distinguished, *B. indicum* and *B. coryneforme* could not (Felis and Dellaglio, 2007). Detailed DGGE analysis of 16S rRNA amplicons confirmed the high relatedness of these species (Temmerman et al., 2002). However, multilocus sequence analysis has shown to successfully differentiate *B. indicum* and *B. coryneforme* (Ventura et al., 2006).

With the exception of species isolated from human dental caries, sewage or insects, the majority if bifidobacteria species are found in the GIT of mammals. Bifidobacteria are known to be host-specific. Species found in the human GIT are highlighted in Figure 1 (Leahy et al., 2005). Other *Bifidobacterium* species have been isolated from pig, honeybees, chicken, lamb, calf, rabbit and rat intestinal tracts (Leahy et al., 2005).

Bifidobacteria are likely to owe their specific ecological success to their capacity to metabolize complex carbohydrates. It therefore comes as no surprise that genes for complex sugar metabolism are abundantly present in the genomes of *B. breve* and *B. longum* biotype *longum* (Ventura et al., 2007). According to the sequence-based classification of carbohydrate-active enzymes (CaZy), over 8% of the annotated genes of these bifidobacterial genomes are predicted to code for enzymes involved in the metabolism of carbohydrates. These include a variety of glycosyl hydrolases for utilization of diverse, and in most cases not identified, plant-derived dietary fibers or complex carbohydrate structures, such as human milk oligosaccharides (HMOs). Relatively few of these glycosyl hydrolases are predicted to be secreted, including those that are thought to hydrolyze arabinogalactans and arabinoxylans (Schell et al., 2002). Instead, most of the bifidobacterial glycosyl hydrolases are predicted to be intracellular, and the genes that encode them are almost without exception associated with genes predicted to encode systems for the uptake of structurally

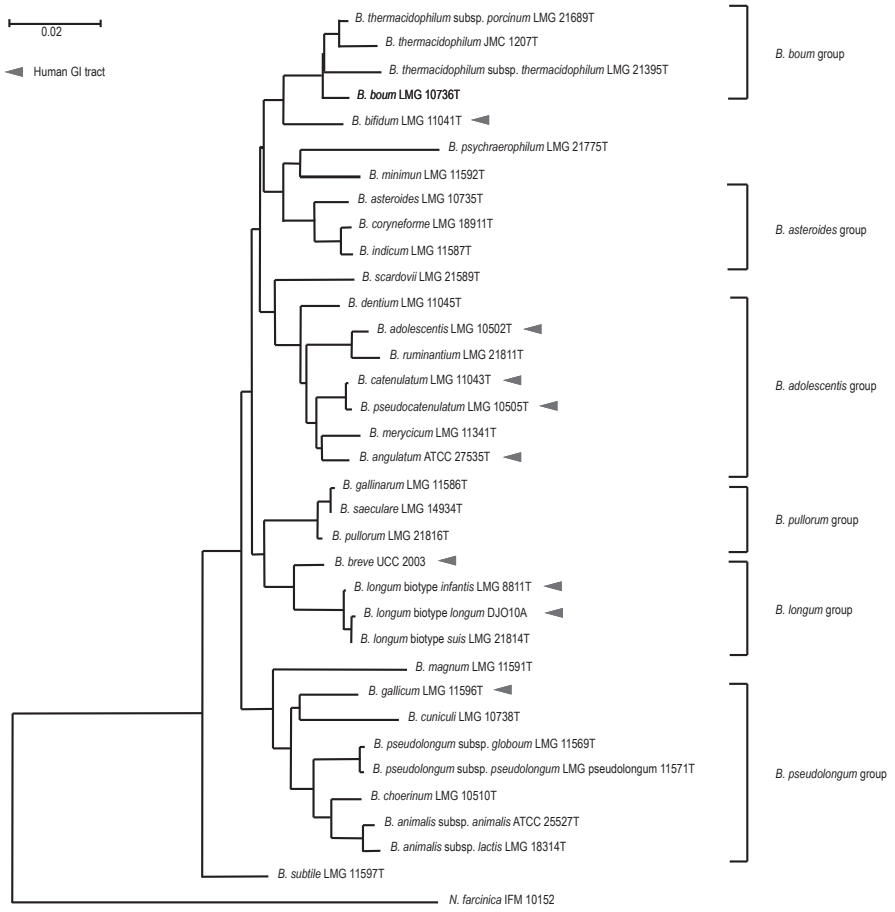

Figure 1. Phylogenetic tree of the genus *Bifidobacterium,* based on 16S rDNA sequence analysis (adapted from Ventura et al., 2006). Species that have been isolated from the human GIT are marked by arrows

diverse carbohydrate substrates. Moreover, carbohydrate-modifying enzymes may also shape the overall metabolic state of the colon to sustain a microbiota that indirectly provides the host with about 10% to 15% of its calories from the degradation of complex carbohydrates through short-chain fatty acids (Vaughan et al., 2005). Bifidobacteria can also utilize sialic acid-containing complex carbohydrates in mucin, glycosphingolipids, and human milk. Some of the details of this important degradation pathway have recently been elucidated (Turroni et al., 2010). Thus, the mammalian host supplies substrates for intestinal commensals including bifidobacteria, in a remarkable symbiotic (or altruistic) relationship (Corfield, 1992; Nakano et al., 2001). Starch and amylopectin are other examples of polysaccharides which may escape

digestion in the upper human GIT and which are plant-derived high-molecular-weight carbohydrates.

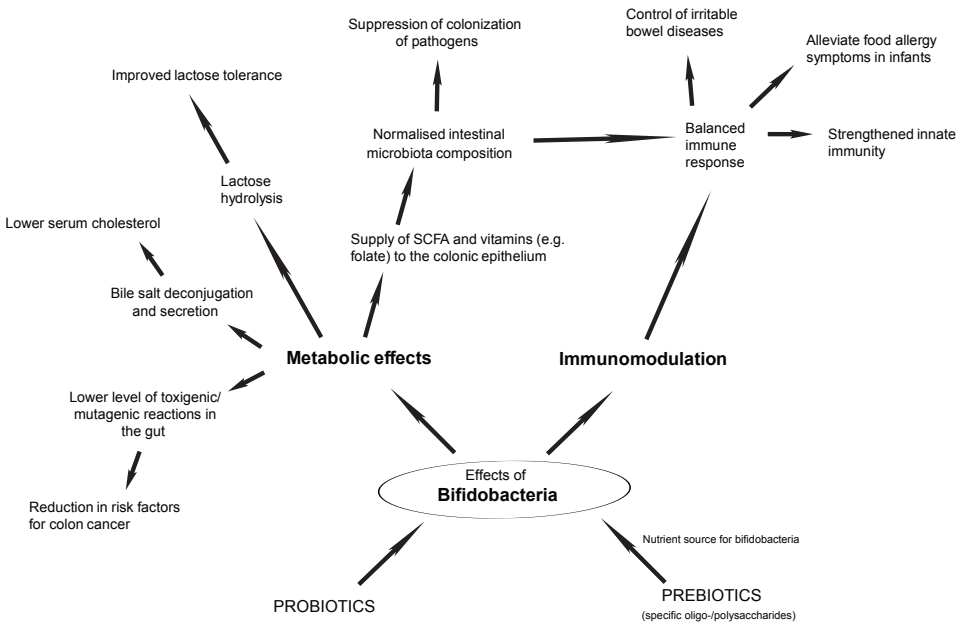

Figure 2. Overview of the effects of bifidobacteria (after Parvez et al., 2006)

The ability to degrade these sugars appears to be restricted to certain species or to certain strains of a particular species, including *B. breve* and *B. adolescentis* (Ryan et al., 2006). This is a clear example of adaptation to a specific ecological niche, namely that of the human GIT. The potential impact that bifidobacteria may have on human health resulted in the recommendation of bifidobacteria as dietary supplements already over hundred years ago (Mayer, 1948; Parvez et al., 2006; Saavedra and Tschernia, 2002; Tissier, 1906). Freeze-dried bifidobacterial preparations, sometimes with in combination with specific lactibacilli such as *Lactobacillus acidophilus*, have been used for the treatment of GIT disorders (Prevot, 1971; Reuter, 1969). Currently, bifidobacteria are added to numerous foods and numerous probiotic activities have been claimed (Figure 2). In order to confirm these claims and to provide more insight in the interaction of bifidobacteria and their human host numerous studies have been performed. **Chapter 1** summarizes the interaction mechanisms described from bifidobacteria. For example, colonization of gnotobiotic mice with bifidobacteria and *Bacteroides* revealed that the presence of bifidobacteria expanded the diversity of polysaccharides degraded by *Bacteroides*, suggesting a synergistic effect of

bifidobacteria with other intestinal microbiota on digestion of polysaccharides which are indigestible by the host (Sonnenburg et al., 2006). In addition, recently performed RNA-based stable isotope probing experiments showed that *B. adolescentis* is actively involved in inulin and lactose metabolism in a human intestinal model system (Kovatcheva-Datchary, 2010). Another possible role of bifidobacteria in the large intestine is the production of water-soluble vitamins, such as many of the B group of vitamins, as shown previously (Deguchi et al., 1985) and also substantiated by genome analysis. Additionally, the first study to provide proof of transcriptional activity in fecal bifidobacteria (**Chapter 5**), showed presence of mRNA sequences implicated to be involved in folate production. An important role of bifidobacteria in the large intestine is believed to be modulation of certain bacterial groups that may be detrimental to the host. Numerous studies support their competitive abilities against other intestinal microbes (Asahara et al., 2004; Hopkins and Macfarlane, 2003). In addition, *in vivo* studies have shown that bifidobacteria can produce antimicrobial compounds such as organic acids (Scardovi et al., 1970), iron-scavenging compounds (O'Sullivan, 2008), and bacteriocins (Cheikhyoussef et al., 2008; Lee et al., 2008; Yildirim et al., 1999). An intriguing function of bifidobacteria in the intestine may be in protection against some immune-based disorders, as numerous studies have shown them to stimulate a host innate immune response (O'Hara et al., 2006; Sonnenburg et al., 2006). These inflammatory and immune defense responses are proposed to be triggered by recognition of commensal bifidobacteria or its components via Toll-like receptors (TLRs) of the host innate immune system (Rakoff-Nahoum et al., 2004). In summary, the pivotal role of bifidobacteria in the development of the intestinal microbial community in our early life, our immune system, and its involvement in maintaining microbial stability within our intestinal tract, has greatly increased the interest in microbe–host interaction studies.

DYNAMICS OF THE INTESTINAL BIFIDOBACTERIAL POPULATION

The dynamics of the bifidobacterial population during human life has received great interest (for a summary see Figure 3). Various studies have shown that, on average, the *Bifidobacterium* population of a young infant contributes 40-60% to the total fecal microbiota. However, high variations were observed while some formula-fed infants contained no detectable fecal bifidobacteria compared to exclusively breast-fed infants that showed faecal bifidobacterial populations that constituted 90% of the total bacterial load (Harmsen et al., 2000). The most abundant species to be found in the

infant intestinal tract are *B. breve, B. longum* subsp. *longum*, and *B. longum* subsp. *infantis*. Next to these three, *B. catenulatum, B. adolescentis*, and *B. bifidum* are also often detected. Many *Bifidobacterium* population studies have been performed with respect to human fecal samples. The overall bifidobacterial compositions, as published by various research groups through different geographical regions, are presented in Table 1. Although there is some variation, the three most abundant species are consistent throughout the different studies and in line with the work presented in **Chapter 4**. An interesting observation is the difference in breast-fed and formula-fed infants. It was recently reported that the average bifidobacterial diversity was greater in breast-fed infants in comparison to infants that received a formula without additional prebiotic oligosaccharides (Roger et al., 2010). This outcome was different from the findings described in **Chapter 3** that indicate the opposite. This difference could be explained by the nature of the different formulas as the formula milk that increased the bifidobacterial diversity (as reported in **Chapter 3**) was supplemented with a mixture of galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS). This is supported by the data presented in **Chapter 4** that also showed infants that received formula milk supplemented with a FOS/GOS mixture to contain a more diverse bifidobacterial populations than those receiving a standard formula without these oligosaccharides.

The overview of recent studies on the bifidobacterial development in early life revealed the existence of geographical factors (Table 1). Characteristic species in adult populations have found to include *B. longum* subsp. *longum, B. catenulatum*, and *B. adolescentis*. By comparing the various studies, it appeared that *B. adolescentis* was mainly detected in Japanese subjects. This could be an example of a geographic effect based on local diet habits and/or genetic differences.

Individual variation in gut microbiota diversity and functional genome content is greater between infants than adults (Kurokawa et al., 2007). These variations may be due to random colonization events, differences in immune responses to the colonizing microbes, changes in host behavior, or other aspects of host life style (Dethlefsen et al., 2006; Palmer et al., 2007). As infant microbiomes are less diverse, we hypothesize that differences in composition have a significant impact on the total microbial intestinal community. However, detailed information on the development of the infant microbiome and the role of the shaping factors mentioned above is lacking. Recently, a 2.5 year case study was reported that related life events to microbiome composition and functions (Koenig et al., 2010). A gradual over time increase of the phylogenetic diversity was observed. Furthermore, major taxonomic groups showed abrupt shifts in abundance corresponding to changes in diet or health, such as fever and antibiotic treatment. This is not new and has been well documented for several

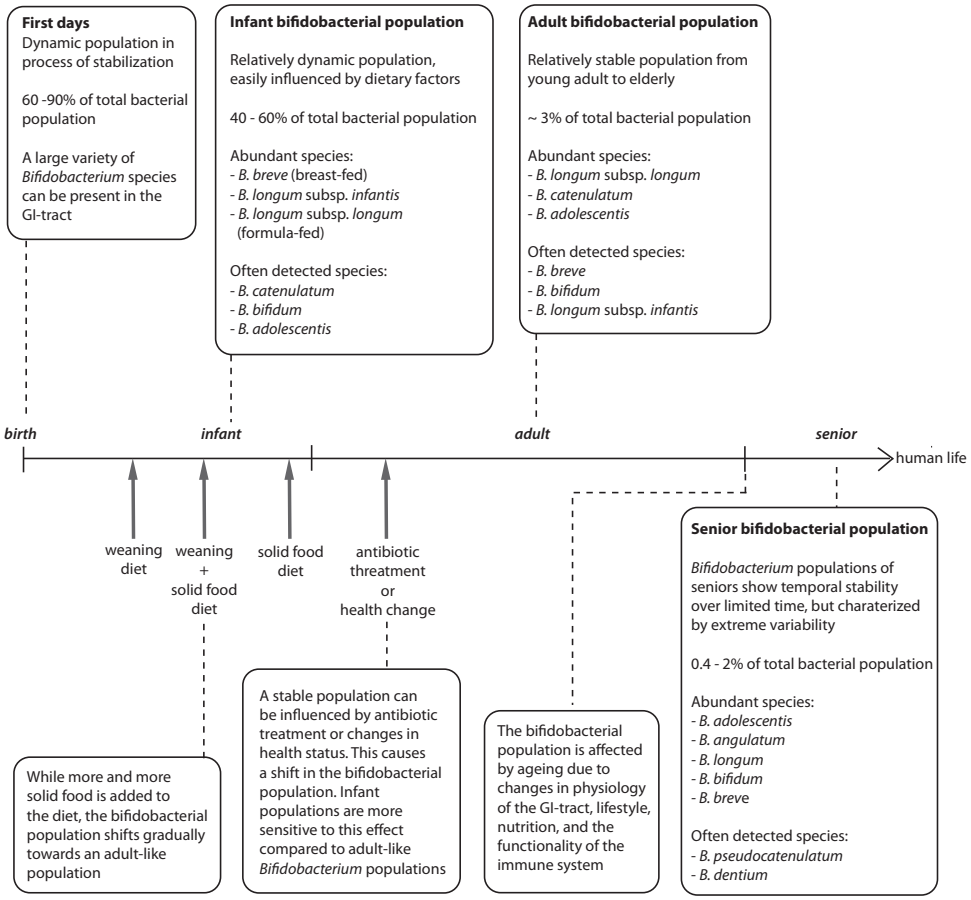

Figure 3. Schematic representation of the dynamics of the intestinal bifidobacterial population during human life

babies for up to a year in the first molecular studies on the infant microbiota (Favier et al., 2002; Favier et al., 2003). Similar observations have been described in **Chapter 4**, where a more systematic analysis of six infants for up to a year were monitored and correlations between diet and the composition of the intestinal bifidobacterial population were observed. In this study, direct mapping of microarray hybridization patterns enabled detailed analysis of the bifidobacterial population in the infant gut. Obtained findings consolidating with other studies, indicate that diet is an important factor with respect to the development, composition, and stability of our microbiome. Furthermore, the unexpected hybridization pattern of a sample obtained in week 16 of infant B, that showed an abrupt drop in *Bifidobacterium* counts, might be also explained by a fever or antibiotic treatment, in line with observations of others

Table 1. Bifidobacterial population composition - the output of various studies . High (H), medium (M), low (L) relative % of bifidobacterial population (Favier et al., 2002; Magne et al., 2006; Matsuki et al., 1999; Matsuki et al., 2004; Mitsou et al., 2008; Roger et al., 2010; Sakamoto et al., 2003; Sakata et al., 2005; Stsepetova et al., 2007; Woodmansey et al., 2004; Young et al., 2004). Geographical locations; The Netherlands (NL), United Kingdom (UK), Japan (JP), Algeria (ALG), New Zeeland (NZ), Ghana (GH), Estonia (EST). Generally abundant species in the infant or adult bifidobacterial population are indicated (dark and light grey shading, respectively).

	Infant								Adult							
Publications	Favier 2002	Magne 2006	Sakata 2005	Mitsou 2008	Matsuki 1999	Roger 2010	Young 2004	Young 2004	Woodmansey 2004	Stsepetova 2007	Sakamoto 2002	Matsuki 1999	Takahiro 1999	Mitsou 2008	Matsuki 2004	Publications
Geographical location	NL	ALG	JP	JP	JP	UK	UK + NZ	GH	UK	EST	JP	JP	JP	JP	JP	Geographical location
B. breve	H	H	L	H	H	H			M	M	L	L	L		L	*B. breve*
B. longum subsp *infantis*		H	H	H	H	H		H			H				L	*B. longum* subsp. *infantis*
B. longum subsp *longum*		H	H		H		H	L	M	H	L	H	H	H	H	*B. longum* subsp *longum*
B. adolescentis			L		L	H			H	H	H	H	H	H	H	*B. adolescentis*
B. catenulatum					L		L		H	H	H	H	H	H	H	*B. catenulatum*
B. bifidum			L	H	L	H	L		L	L	L		L		M	*B. bifidum*
B. pseudocatenulatum	H						L		L							*B. pseudocatenulatum*
B. angulatum					L				H			L			L	*B. angulatum*
B. dentium					L				M	L		L	L		L	*B. dentium*

(Koenig et al., 2010). Unfortunately, we did not have access to detailed information on the infant's conditions but it is tempting to speculate that a change in health status may have caused the drop of bifidobacterial abundance in this particular sample. When the infants changed to diet of solid foods we observed a 90% drop of *Bifidobacterium*-specific hybridization signals (**Chapter 4**). This corresponds to the change in abundance of *Bacteroidetes* following the same event (Koenig et al., 2010). Additionally, direct mapping of hybridization patterns, as described in **Chapter 4**, provided highly detailed information on genomic variation within species which has not been described in other studies.

Interestingly, in none of the six infant fecal samples that were analyzed in **Chapter 4**, we could observe any *B. longum* subsp. *infantis* above the detection level of around 2 ng DNA. This is remarkable, as this subspecies has been described as specialized in human milk utilization (Sela et al., 2008). Several chromosomal loci of *B. longum* subsp. *infantis* reflect the potential adaption to the infant host. And example is the presence of a 43 kbp cluster encoding catabolic genes, extracellular

solute binding proteins and permeases predicted to be active on HMOs which are abundant in breast-milk (Sela et al., 2008). A phenotypic study underlines these findings and illustrates the ability to consume HMOs as a sole carbon source, which is a unique property of *Bifidobacterium* and *Bacteroides* species (Zivkovic et al., 2010). However, this ability does not extend to all bifidobacterial isolates. The ability to grow vigorously in HMOs is most common in *B. bifidum* and *B. longum* subsp. *infantis* strains. Moderate growth has been observed for *B. longum* subsp. longum and *B. breve* strains and *B. adolescentis* and *B. animalis* seem to lack this ability. Moreover, these common infant-borne bifidobacteria possess different modes for consumption of HMOs. *Bifidobacterium longum* subsp. *infantis* strains likely imports the lower weight oligosaccharides while *B. bifidum* exports fucosidases and lacto-N-biosidase for extracellular hydrolysis to remove lacto-N-biose (LNB) from the HMO structure. *Bifidobacterium breve* on the other hand is suggested to be dependent on possible cross-feeding, while it has the ability to consume various liberated monomer constituents of HMO and imports them as mono-saccharides, followed by intracellular catabolism. These observations are in concordance with the data presented in **Chapter 4** showing the presence of *B. bifidum, B. longum* subsp. *longum, B. breve*, and *B. adolescentis* in the infant intestine. Based on their HMO degrading abilities, all of these species showed competence for the occupation of the unique niche found within the intestinal tract of a breast-fed infant (Zivkovic et al., 2010). In all infants, at least *B. breve* or *B. longum* was present, although *B. longum* subsp. *infantis* was not detected. The sole presence of *B. adolescentis* was never observed, which can be explained by its lacking ability to grow on HMOs. The presence of *B. animalis* correlated with the supplementation of living *B. animalis* subsp. *lactis* Bb12 cells to the formula diet. Overall, it has been postulated that there are different strategies for the use of HMOs suggesting possible niche-specific mechanisms divided among the different bifidobacterial species within the developing infant microbiome.

The findings presented in **Chapter 5** are the first to show that it is feasible to analyze and compare global transcript profiles of bifidobacterial populations isolated from infants that received different diets. However, exact quantification can be complicated as it is not known how many active bifidobacteria are present in the samples. It is evident from the results of this study that carbohydrate metabolism is really operating in bifidobacteria derived from the baby GIT. A putative operon for galactose metabolism was found to be expressed in both breast-fed and formula-fed infants. Products of this operon could be involved in the breakdown of LNB structures. The fact that formula-fed infants received an additional mixture of GOS and FOS supplements can be the explanation of the activation of galactose metabolism in this group. Furthermore, the expression of genes predicted to be involved in folate

production was observed, indicating that bifidobacterial cells are actively producing folate while colonizing our gut. Overall, it revealed that bifidobacterial species undergo dynamic changes in infant faeces at the level of persistence as well as their functional complements.

UNRAVELING THE BIFIDO-EFFECT

Many health benefits of treatment with probiotic bacteria have been described (Fig. 1). Numerous studies have been performed to confer these beneficial effects. Although some have come with underlining proof, most studies had to cope with small sample sizes, non-significant results, or have failed to clarify the underlying mechanisms (Lee and O'Sullivan, 2010). Overall, proposed immune-stimulatory effects of *Bifidobacterium* strains are most significant and thus most convincing. In comparison to other proposed effects, the molecular mechanism involved in immune modulation of bifidobacteria is relatively well studied and characterized. For example, a large human study showed reduction of symptom scores and extracellular IL-10/IL-12 ratios which indicates up-regulation of anti-inflammatory cytokines in human subject suffering from irritable bowel syndrome after supplementation with *B. longum* subsp. *infantis* (O'Mahony et al., 2005). A smaller human study showed some increase in the extracellular levels of IFN-γ in their peripheral blood mononuclear cells following consumption of milk supplemented with *B. animalis* subsp. *lactis* NH019 (Arunachalam, 1999). Additionally, A mice study showed the anti-inflammatory effect of administration of *B. animalis* subsp. *lactis* DN173010. This strain showed an indirect anti-inflammatory effect on mice suffering from inflammatory bowel disease (IBD) by altering a niche for colitogenic microbes (Veiga et al., 2010). Another small human study showed no significant change in symptom scores but some decrease in expression of genes encoding human pro-inflammatory cytokines, such as TNF-α and IL-1α, in ulcerative colitis (UC) patients who were supplemented with *B. longum*, inulin, and fructooligosaccharides (Furrie et al., 2005). Also mRNA levels for human beta defensins 2, 3, and 4 which are strongly up-regulated in active UC, were significantly reduced in the test group after treatment (Furrie et al., 2005). A small mouse study showed some reduction in the pro-inflammatory cytokines IFN-γ, TNF-α, and IL-12 from supplementation with *B. longum* subsp. *infants* (McCarthy et al., 2003). Other data obtained from a mouse study described a reduction in CD4+ T cells in the spleen and colon following supplementation with *B. bifidum* (Kim et al., 2007). Furthermore, *in vitro* co-culture incubation experiments with *B.*

bifidum performed in this same study showed suppression of MCP-1 and IFN-γ , and induction of IL-6 and IL-10 (Kim et al., 2007). All these findings indicate for the existence of immune-modulating abilities of some *Bifidobacterium* strains.

In **Chapter 6**, the responsiveness of the different HIECs, Caco-2, T84, and HT-29, for TNF-α was tested. Subsequently, the most response cell line, HT-29, was used in an co-incubation study with *B. breve* cells. The goal of the co-incubation experiment described in **Chapter 6** was to expand the information on the molecular mechanism of immune modulation by bifidobacteria. One general conclusion, extracted from the co-incubation experiments was that the effects of *B. breve* strains on HIECs are strain-specific. Furthermore, we detected partial suppression of TNF-α induced expression in HT-29 cells by *B. breve* M-16V. Other *B. breve* strains tested did not or on a very low level show the ability to suppress TNF-α induced inflammation under the conditions tested.

A recent study has shed new light on the *Bifidobacterium*–host interaction (Maslowski et al., 2009). It describes the signalling mechanism between short-chain fatty acids (SCFAs) and the G-protein-couplet receptor 43 (GPR43). SCFAs, such as acetate, are produced by commensal bifidobacteria after utilization of specific oligosaccharides (**Chapter 1**). Particularly acetate and propionate have recently been found to bind and activate GPR43. Interestingly, GPR43 gene expression showed close regulation with receptors important for innate immunity, such as Toll-like receptors (TLS2 and TLR4). Furthermore, stimulation of GPR43 by SCFAs was necessary for the normal resolution of certain inflammatory responses. Both GPR43-deficient and germ-free mice showed dysregulation of certain inflammatory responses. In summary, SCFA-GPR43 signalling is one of the molecular pathways whereby commensal bacteria regulate immune and inflammatory responses. The question rises, whether the strain-specific *B. breve* effects observed in **Chapter 6** will be overruled by a more general *Bifidobacterium*-effect *in vivo*, based on mechanisms like SCFA-GPR43 signalling. In order to answer this question dietary compounds should be integrated in future co-incubation experiments. The interaction experiment presented in **Chapter 6** illustrates the complexity of the dynamic interplay between bacteria and human cells. We want to emphasize here that the final outcome of a host cell response against a microorganism depends on the combination of distinct MAMPs that can interact with the various PRRs and associated co-receptors that fine-tune signaling, as well as on the concentration of the MAMPs, their accessibility for the PRRs and the presence of other microbial effector molecules that can modulate host responses (Lebeer et al., 2007).

THE FUTURE EXTENSION OF THE BMS-MICROARRAY

The role of the microarray platform described in **Chapters 2**, **3**, and **4** was to circumvent high-cost and time-consuming whole genome sequencing. Arraying thousands of DNA fragments enabled high resolution screening for *Bifidobacterium* species unique genes. These were subsequently used for studying the diversity of the genus by analyzing pure bifidobacterial cultures. The functionalities of the proteins encoded by the marker genes could predict species-specific biochemical characteristics. Furthermore, this approach was also suitable for the screening complex microbial communities, such as human faecal samples. The observed compositions of fecal bifidobacterial populations and genomic shifts within these populations in time revealed the flexibility of the population. This indicated that diet affects the population. A future alternative of the microarray-based comparative hybridizations could be *in silico* comparison of whole genomes. This will enable fast and accurate selection of marker DNA fragments. However the described microarray platform represents as a good alternative. It should be stressed that in all cases the experiments could be realized without the need for PCR amplification. In view of the series of biases such as preferential and non-linear amplification and formation of chimers, that can originate from PCR, this is an important and possibly decisive advantage. On the other hand, the era of next-generation sequencing (NGS) has begun. To date several commercial platforms have been developed, each of which is linked to a unique combination of protocols determining the type of data output. A technical review, describing the differences with respect to template preparation, sequencing and imaging, genome alignment and assembly approaches, and recent advantages in current and near-term commercially available NGS instruments, has recently been published and include half a dozen different technologies (Metzker, 2010). NGS technologies clearly have an impressive range of applications and its field is still a fast-moving area of research. Commercially available NGS-platforms have caused an explosion of sequenced genomes (Chain et al., 2009). Using the newest technologies, researchers can process hundreds of millions of base pairs in just a few hours (Table 2 and 3). In a short period of time numerous *Bifidobacterium* genomes have been sequenced and many more are in progress or in the planning phase (Table 4). Multiple genome sequences of species and notably strains of *Bifidobacterium* will be generated and NGS also is a powerful tool for genome resequencing of mutant or transformed strains. This enables the screening for highly discriminatory and unique marker gene sequences. Finally, it contributes to the definition of the *Bifidobacterium* pan-, core-, and species-specific genome and allows to link the functionalities to their survival strategies. Another application of

NGS can be mRNA detection and sequencing in complex microbial samples, as an extension of the microarray-based transcriptome analysis presented in **Chapter 5**. Possible problematic features could be abundance of highly expressed genes. High abundant mRNA molecules will prevent the detection of low level transcripts. Furthermore, in order to obtain a dynamic range of one microarray spot, more than 10^5 sequencing reads are needed.

Table 2. Completed bifidobacterial genome sequencing projects (modified after Lee and O'Sullivan, 2010) and (Fanning, 2010)

Organism	Size (Mb)	GenBank	Sequencing Center
Bifidobacterium adolescentis ATCC 15703	2.1	AP009256	Gifu University, Life Science Research Center, Japan
Bifidobacterium animalis subsp. lactis AD011	1.9	CP001213	Korea Research Institute of Bioscience and Biotechnology
Bifidobacterium animalis subsp. lactis BB-12	1.9	CP001853	Chr. Hansen A/S
Bifidobacterium animalis subsp. lactis Bl-04	1.9	CP001515	Danisco USA Inc.
Bifidobacterium animalis subsp. lactis DSM10140	1.9	CP001606	Danisco USA Inc.
Bifidobacterium animalis subsp. lactis V9	1.9	CP001892	Southwest Agricultural University, China
Bifidobacterium bifidum S17	2.2	CP002220	Institute of Microbiology and Biotechnology, University of Ulm
Bifidobacterium dentium Bd1	2.6	CP001750.1	University of Parma, Italy [more]
Bifidobacterium longum DJO10A	2.39	CP000605	University of Minnesota [more]
Bifidobacterium longum NCC2705	2.3	AE014295	Nestle Research Center, Switzerland
Bifidobacterium longum subsp. infantis ATCC15697	2.8	CP001095	DOE Joint Genome Institute [more]
Bifidobacterium longum subsp. longum JDM301	2.5	CP002010.1	Genome Sequencing Center (GSC) at Washington University (WashU) School of Medicine
Bifidobacterium adolescentis L2-32	2.4	AAXD00000000	Washington Univ, USA
Bifidobacterium angulatum DSM 20098	2	ABYS00000000	Washington Univ, USA
Bifidobacterium bifidum JCM 1255	2		Japan
Bifidobacterium bifidum NCIMB 41171	2.2	ABQP00000000	Broad Institute, USA
Bifidobacterium breve DSM 20213	2.3	ACCG00000000	Washington Univ, USA
Bifidobacterium catenulatum DSM 16992	2.1	ABXY00000000	Washington Univ, USA
Bifidobacterium dentium ATCC 27678	2.6	ABIX00000000	Washington Univ, USA
Bifidobacterium dentium ATCC 27679		AEEQ00000000	BCM-HGSC, USA
Bifidobacterium dentium JCVIHMP022		AEHJ00000000	J. Craig Venter Institute, USA
Bifidobacterium gallicum DSM 20093	2	ABXB00000000	Washington Univ, USA
Bifidobacterium longum infantis ATCC 55813	2.4	ACHI00000000	BCM-HGSC, USA
Bifidobacterium longum infantis CCUG 52486	2.5	ABQQ00000000	Broad Institute, USA
Bifidobacterium pseudocatenulatum DSM 20438	2.3	ABXX00000000	Washington Univ, USA

Table 3. Ongoing or targeted bifidobacterial genome projects(modified after (Lee and O'Sullivan, 2010) and (Fanning, 2010)

Organism	Project status	Sequencing Center
Bifidobacterium animalis subsp. lactis HN019	In progress	Fonterra Research Center, NZ
Bifidobacterium angulatum JCM7096	In Progress	Japan
Bifidobacterium animalis ACS-248-V-Col6	Awaiting DNA	J. Craig Venter Institute, USA
Bifidobacterium animalis DN-173010	Awaiting DNA	Broad Institute, USA
Bifidobacterium bifidum DSM 20456	In Progress	Washington Univ, USA
Bifidobacterium bifidum PB2003/035-T3-1	Awaiting DNA	J. Craig Venter Institute, USA
Bifidobacterium breve ACS-071-V-Sch8b	In Progress	J. Craig Venter Institute, USA
Bifidobacterium breve EX336960VC18	In Progress	Virginia Commonwealth Univ, USA
Bifidobacterium breve EX336960VC19	In Progress	Virginia Commonwealth Univ, USA
Bifidobacterium breve EX336960VC21	In Progress	Virginia Commonwealth Univ, USA
Bifidobacterium breve EX533959VC13	In Progress	Virginia Commonwealth Univ, USA
Bifidobacterium breve JCM1192	In Progress	Japan
Bifidobacterium catenulatum JCM1194	In Progress	Japan
Bifidobacterium dentium	Targeted	USA
Bifidobacterium dentium	Targeted	USA
Bifidobacterium dentium	Targeted	USA
Bifidobacterium dentium JCM1195	In Progress	Japan
Bifidobacterium lactis Bb-12	Awaiting DNA	Broad Institute, USA
Bifidobacterium lactis Bi-07	Awaiting DNA	Broad Institute, USA
Bifidobacterium longum 3_1_37DFAAB	Awaiting DNA	Broad Institute, USA
Bifidobacterium longum FB030-04AN	Awaiting DNA	J. Craig Venter Institute, USA
Bifidobacterium longum infantis 157F-NC	In Progress	Japan
Bifidobacterium longum infantis JCM 1217	In Progress	Japan
Bifidobacterium longum infantis JCM 1222	In Progress	Japan
Bifidobacterium longum longum F8	In Progress	Sanger Institute, United Kingdom
Bifidobacterium pseudocatenulatum D2CA	In Progress	Sanger Institute, United Kingdom
Bifidobacterium scardovii JCM12489	In Progress	Japan
Bifidobacterium sp. 12_1_47BFAA	In Progress	Broad Institute, USA
Bifidobacterium sp. FB030-04AN	Awaiting DNA	J. Craig Venter Institute, USA
Bifidobacterium sp. HM5	In Progress	Japan
Bifidobacterium sp. JCM15439	In Progress	Japan

When considering other future applications, it can be envisaged that the identified species-unique gene sequences form the basis for the design and *in vitro* synthesis of marker oligos. Arrayed, these oligos could form a tool for studying the genomic diversity in highly complex microbial populations. Notably, to be successful this future platform will not only require complete and reliable whole genome sequence data from multiple strains per species but also a well-designed bioinformatics tool together with high capacity hardware. Currently, an ongoing initiative of the European Commision, called Metagenomics of the Human Intestinal Tract (MetaHIT, www.metahit.eu) focusing on defining the human metagenome, is applying this approach.

Table 4. Overview of currently available next-generation sequencing platforms. * Average read-lengths. ¥Fragment run. §Mate-pair run. Frag, fragment; GA, genome Analyzer; GS, Genome Sequenser; MP, mate-pair; N/A, not available; NGS, next-generation sequencing; PS, pyrosequencing; RT, reversible terminator; SBL, sequencing by ligation; SOLiD, support oligonucleotide ligation detection. (according to Metzker, 2010)

Platform	Library/template preparation	NGS chemistry	Read length (bases)	Run time (days)	Gb per run	Machine cost (US$)	Pros	Cons	Biological applications
Roche/454's GS FLX Titanium	Frag, MP/emPCR	PS	330*	0.35	0.45	500,000	Longer reads improve mapping in repetitive regions; fast run times	High reagent cost; high error rates in homo-polymer repeats	Bacterial and insect genome *de novo* assemblies; medium scale (<3 Mb) exome capture; 16S in metagenomics
Illumina/Solexa's GAII	Frag, MP/solid-phase	RTs	75 or 100	4¥, 9§	18¥, 35§	540,000	Currently the most widely used platform in the field	Low multiplexing capability of samples	Variant discovery by whole-genomeresequencing or whole-exome capture; gene discovery in metagenomics
Life/APG's SOLiD3	Frag, MP/emPCR	Cleavable probe SBL	50	7¥, 14	30¥, 50§	595,000	Two-base encoding provides inherent error correction	Long run times	Variant discovery by whole-genomeresequencing or whole-exome capture; gene discovery in metagenomics
Polonator G.007	MP only/emPCR	Non-cleavable probe SBL	26	5§	12§	170,000	Least expensive platform; open source to adapt alternative NGS chemistries	Users are required to maintain and quality control reagents; shortest NGS read lengths	Bacterial genome resequencing for variant discovery
Helicos BioScience HeliScope	Frag, MP/single molecule	RTs	32*	8¥	37¥	999,000	Non-bias representation of templates for genome and seq-based applications	High error rates compared with other reversible terminator chemistries	Seq-based methods
PacificBiosciences (target release: 2010)	Frag only/single molecule	Real-time	964*	N/A	N/A	N/A	Has the greatest potential for reads exceeding 1 kb	Highest error rates compared with other NGS chemistries	Full-length transcriptome sequencing; complements other resequencing efforts in discovering large structural variants and haplotype blocks

TOWARDS DEFINING THE CORE-, PAN, AND SPECIES-SPECIFIC GENOME

Recently, a great variety of extended genome sequencing projects have been started (see for overview Table 1). A trial analysis was initiated by the Human Microbiome Jumpstart Reference Strains Consortium (Nelson et al., 2010). This initiative focuses on describing the biodiversity of species within the human microbiome. Hundreds of microbe genomes have been sequenced. Among others, genome sequences of five different *Bifidobacterium longum* strains were compared (Nelson et al., 2010). Preliminary results of this comparison of *B. longum* sequences, suggest a core genome

size of approximately 1430. Four of the five genomes contribute approximately equally to the pan-genome (~50 to 150 genes). The fifth strain (ATCC 15697) was special in contributing a much higher number of specific genes (~640). The genomic diversity of these strains belonging to the same species was measured by defining the average nucleotide identity (ANI), a measure of the evolutionary relatedness based on sequence similarity between the set of shared genes (Konstantinidis et al., 2006). Functional and ecological relatedness was studied by measuring the gene content similarity between to strains. Again four strains had pairwise ANI similarities at the higher end of the spectrum, ranging between 96 and 98%, but with relatively low gene-content similarity (that is, below 82%), indicating a broad range in gene complements. One additional strain (ATCC15697) showed an ANI below 95% and a gene content similarity below 65%, confirming its unique evolutionary and ecological position (Nelson et al., 2010). These findings are in line with the work presented in **Chapter 2**. A hybridization homology, which is comparable to a gene-content similarity, of approximately 72% was described for *B. longum* strains LMG13197 (ATCC15707) and LMG8811 (ATCC15697). As mentioned earlier, the core genome of *B. longum* species was predicted to contain around 1430 genes. The question rises whether the core genome of the genus *Bifidobacterium* is much smaller? Interestingly, **Chapter 2** showed a gene-content similarity of *B. longum* LMG13197 and *B. pseudolongum* LMG11571 of approximately 9%, based on hybridization experiments. Due to the relatively large phylogenetic distance of *B. longum* LMG13197 and *B. pseudolongum* LMG11571, these species might cover most of the genus and therefore this value could be an indication for its core genome size. This means that the *Bifidobacterium* core genome consists of roughly 200 genes, based on gene-content similarity. This clearly reveals the diversity of this genus.

Relatively high diversity rates were described within the species *B. breve* in **Chapter 3**, where 20 fecal isolates of *B. breve* were compared to the type strain. In this study the DNA-based microarray platform served as a comparative genome hybridization tool. This approach was fruitful and resulted in a high-resolution data set revealing detailed information on the genomic diversity of the different isolates. It displayed an overall *B. breve* isolate gene-content similarity of 60 – 90% in comparison to the *B. breve* LMG13208 type strain. These results testify for the existence of various subspecies within the species *B. breve*. Currently, taxonomic research on these strains is ongoing in order to clarify this matter.

Chapters 2, **3**, and **4** describe the selection of sets of DNA fragments, showing either species-unique, strain cluster-unique, or conserved hybridization characteristics on the microarray, for sequence analysis. In order to link these three experiments these sets were checked for overlapping DNA fragments or homologies (Table 5). We

Table 5. Comparison of the genes identified for their unique hybridization characteristics and selected for sequencing analysis in Chapters 2, 3, and 4 of this thesis

#	Chapter	Specificity	Best BLAST hit	Remarks
25	Chapter 2 - Fig 4	B. longum spp./INF/BRE	gi|23465396|ref|NP_695999.1| possible thioredoxin-dependent thiol peroxidase [Bifidobacterium longum NCC2705]	Two different sequences with the same 'best'-BLAST hit, but different e-value
47	Chapter 2 - Fig 4	CAT	gi|23465396|ref|NP_695999.1| possible thioredoxin-dependent thiol peroxidase [Bifidobacterium longum NCC2705]	
1	Chapter 3 - Fig 2	conserved	ref|ZP_06595093.1| beta-galactosidase [Bifidobacterium breve DSM 20213]	Refer to the same DNA fragment originating from one single spot on the
4	Chapter 4 - Fig 4	breve strain-specific	ref|ZP_06595093.1| beta-galactosidase [Bifidobacterium breve DSM 20213]	BMS-microarray. We have to note that the specificity, defined in Chapter 3, is based on a limited set of *B. breve* strain which could explain this observation.
42	Chapter 3 - Fig 2	specific	ref|ZP_06595986.1| putative bacterial regulatory protein [Bifidobacterium breve DSM 20213]	Sequences originate from different clo but show similar 'best'-BLAST hit
43	Chapter 3 - Fig 2	specific	ref|ZP_06595986.1| putative bacterial regulatory protein [Bifidobacterium breve DSM 20213]	results, which indicates high homology between the genes they represent.
26	Chapter 3 - Fig 2	specific	ref|ZP_06596330.1| conserved hypothetical protein [Bifidobacterium breve DSM 20213]	Sequences originate from different clo but show similar 'best'-BLAST hit
27	Chapter 3 - Fig 2	specific	ref|ZP_06596330.1| conserved hypothetical protein [Bifidobacterium breve DSM 20213]	results, which indicates high homology between the genes they represent.
28	Chapter 3 - Fig 2	specific	ref|ZP_06596330.1| conserved hypothetical protein [Bifidobacterium breve DSM 20213]	
31	Chapter 3 - Fig 2	specific	ref|ZP_06596397.1| glycosyl transferase, group 2 family [Bifidobacterium breve DSM 20213]	Refer to the same DNA fragment originating from one single spot on the
6	Chapter 4 - Fig 4	breve strain-specific	ref|ZP_06596397.1| glycosyl transferase, group 2 family [Bifidobacterium breve DSM 20213]	BMS-microarray, which confirms the specificity
29	Chapter 3 - Fig 2	specific	ref|ZP_06596398.1| phosphatidylglycerol--membrane-oligosaccharide glycerophosphotransferase [Bifidobacterium breve DSM 20213]	Refer to the same DNA fragment originating from one single spot on the BMS-microarray, which confirms the specificity
9	Chapter 4 - Fig 4	breve strain-specific	ref|ZP_06596398.1| phosphatidylglycerol--membrane-oligosaccharide glycerophosphotransferase [Bifidobacterium breve DSM 20213]	
6	Chapter 3 - Fig 2	conserved	ref|ZP_06596810.1| proteasome ATPase [Bifidobacterium breve DSM 20213]	Refer to the same DNA fragment originating from one single spot on the
3	Chapter 4 - Fig 4	breve strain-specific	ref|ZP_06596810.1| proteasome ATPase [Bifidobacterium breve DSM 20213]	BMS-microarray. We have to note that the specificity, defined in Chapter 3, is based on a limited set of *B. breve* strain which could explain this observation.
38	Chapter 3 - Fig 2	specific	ref|ZP_06597004.1| conserved hypothetical protein [Bifidobacterium breve DSM 20213]	Refer to the same DNA fragment originating from one single spot on the
10	Chapter 4 - Fig 4	breve strain-specific	ref|ZP_06597004.1| conserved hypothetical protein [Bifidobacterium breve DSM 20213]	BMS-microarray, which confirms the specificity
39	Chapter 3 - Fig 2	specific	ref|ZP_06597149.1| conserved hypothetical protein [Bifidobacterium breve DSM 20213]	Refer to the same DNA fragment originating from one single spot on the
8	Chapter 4 - Fig 4	breve strain-specific	ref|ZP_06597149.1| conserved hypothetical protein [Bifidobacterium breve DSM 20213]	BMS-microarray, which confirms the specificity

observed that two DNA fragments, one described to be specific for *B. catenulatum* (#25) and the other for the *B. longum group* (#47), showed high, but different, homologies to an identical gene based on their 'best'-BLAST hit (highest homology % in combination with the lowest E-value of all BLAST hit results). Could they have similar 'best'-BLAST hit results and also represent species-specific sequences? To answer this question we analyzed both sequences in more detail. We concluded that they had little overlap with each other and that the *B. longum* specific fragment was the only one identical to *B. longum* NCC2705 genome, which gave the 'best'-BLAST hit. Therefore, we suggest that the difference between the sequences is large enough

for the fragments to have species-unique features. Sequenced fragments #42 and #43 originated from different clones but represented the same gene. This explains their similar hybridization characteristics. The same is true for fragments #26, #27, and #, 28. Several sequences DNA fragments were described to be specific for a cluster of strains within the *B. breve* species in **Chapter 3**, and showed *B. breve* strain-specific characteristics in **Chapter 4** (#31 and #6; #29 and #9; #38 and #10; #39 and #8) . These results confirm the uniqueness of the DNA sequences. DNA fragments #1 and #4, as well as #6 and #3, are described as conserved in *B. breve* in **Chapter 3**, but show strain-specific hybridization patterns in **Chapter 4**. This could be explained by the limited number of strains in the set of *B. breve* isolates analyzed in **Chapter 3**.

GENERAL CONCLUSION

In conclusion, the work presented in the thesis, which formed part of a larger IOP Genomics project, contributed to an advanced insight in the interaction between bifidobacteria and the human host. Furthermore, it resulted in the development of genome-based molecular platforms suite for analyzing genomic diversity between and within species, as well as population dynamics in complex microbial communities. We anticipate that the molecular approaches pioneered in this thesis will be instrumental in the further elucidation of the host–microbe interactions in the GIT of human an other animals.

Chapter 8

Nederlandse Samenvatting

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NEDERLANDSE SAMENVATTING

Dit proefschrift beschrijft een verzameling van, op microarray-technieken gebaseerde studies, die bijdragen tot een beter inzicht in de rol van bifidobacteriën in relatie tot hun menselijke gastheer. Het behandelt onder andere recent beschreven interactiemechanismen tussen bifidobacteriën en humane gastrointestinale cellen. Ook belicht het de unieke eigenschappen van het genus *Bifidobacterium*, die essentieel zijn voor hun voortbestaan in het ecosysteem van onze darm. Met behulp van een '*Bifidobacterium* mixed-species (BMS)' microarray-platform is er een middel gecreëerd waarmee het genetisch materiaal van verschillende bifidobacteriesoorten, die voorkomen in het spijsverteringskanaal van de mens, vergeleken kunnen worden. Gebaseerd op gedetailleerde microarraydata, konden soortspecifieke genoomfragmenten geïdentificeerd worden. Een groot gedeelte van deze voorspelde genen codeerden voor eiwitten die betrokken zijn bij het suikermetabolisme van de bifidobacteriën. Een unieke eigenschap van het ontwikkelde microarray-platform is dat het de mogelijkheid biedt om tot op stamniveau verschillen in genetische samenstelling te meten. Het microarray-platform werd toegepast om de genetische variatie tussen verschillende *B. breve* isolaten in kaart te brengen door de hybridisatiepatronen, van de uit de desbetreffende stammen geïsoleerde DNA monsters, te vergelijken. Dit onthulde een relatief grote genomische variatie binnen de *B. breve* soort en leverde zelfs bewijs voor het bestaan van verscheidene subsoorten binnen de soort *B. breve*. Uit het clusteren van de hybridisatiepatronen kwam een duidelijk verband naar voren tussen het patroon en de baby waar de bacteriën uit geïsoleerd waren. Dit was een aanwijzing voor niche-specifieke aanpassing van verschillende stammen binnen dezelfde soort. Verder werden in een studie, beschreven in dit proefschrift, ook bifidobacteriepopulaties in fecale monsters van verschillende zuigelingen geanalyseerd. Totaal DNA extracten werden gehybridiseerd op de ontwikkelde BMS microarray. Deze analyses fungeerden als test voor de toepasbaarheid van het microarray platform voor onderzoek naar de dynamiek van bifidobacteriële populaties in borst- en flesgevoede baby's. Het microarray-platform bewees zijn bruikbaarheid voor het monitoren van de ontwikkeling in de tijd en de effecten van de bijbehorende voedingspatronen. De waargenomen verschillen in de bifidobacteriële populaties, zowel in de tijd als tussen de individuen, vertoonden een correlatie met het dieet van de gastheer. Analyse van de hybridisatiepatronen maakte het in de tijd monitoren van de veranderingen in de genetische compositie van het genoom, binnen een bifidobacterie soort, mogelijk. Sequentie-analyse van DNA fragmenten, die onderscheidende hybridisatiekarakteristieken vertoonde, leverde selecties van voorspelde genen op, die of geconserveerd of stamspecifiek zijn binnen de soort *B.*

breve. Naast onderzoek naar genomische variatie, zijn ook transcriptiepatronen van zowel bifidobacteriën als humane darmepitheel cellen bestudeerd. Transcriptiegericht onderzoek bij bifidobacteriën leverde duidelijk bewijs voor transcriptionele activiteit in bifidobacteriecellen door RNA te isoleren uit fecale monsters van baby's. Zover wij weten is dit het eerst beschreven voorbeeld van *in situ* activiteit van bifidobacteriën in het menselijk spijsverteringskanaal. Daarbij geven onze resultaten een indicatie voor een verband tussen de genexpressie en het dieet van het kind waar het fecale monster vandaan komt. Bifidobacteriën geïsoleerd uit borstgevoede kinderen vertonen een ander transcriptionele reactie in vergelijking tot kinderen die flesvoeding hebben ontvangen. Sequentie-analyse van de transcripten onthulde de expressie van voorspelde genen, die homologie vertonen met genen die betrokken zijn bij de productie van folaat. Dit getuigt voor de productie van deze belangrijke vitamine door bifidobacteriën, die aanwezig zijn in onze darmen tijdens onze jonge jaren. Als laatste laten we, door middel van de analyse van het transcriptome van HT-29 cellen (een cellijn van humaan darmepitheel), het soortspecifieke effect van de aanwezigheid van de *B. breve* M-16V stam zien op de expressie van genen die in afwezigheid van deze bifidobacterie door TNF-α verhoogd tot expressie werden gebracht. Twee andere, parallel geteste, bifidobacteriestammen lieten een extreem mild of zelfs onmeetbaar effect zien op TNF-α-gestimuleerde HT-29 cellen. Hoewel we geen complete onderdrukking van het TNF-effect waargenomen hebben, konden we aantonen dat apoptotische en immunologische regulatoire cascades beïnvloed werden door de aanwezigheid van de *B. breve* M-16V cellen. Samengevat draagt dit proefschrift, wat onderdeel was van een overkoepelend IOP Genomics project genaamd 'A genomics approach towards gut health', bij aan een verbeterd inzicht in de interactie tussen bifidobacteriën en de menselijke gastheer. Het heeft geresulteerd in de ontwikkeling van een moleculair platform wat toepasbaar is voor het onderzoeken van de genomische diversiteit tussen en binnen soorten, naast het bestuderen van de populatiedynamiek van complexe microbiële gemeenschappen. We verwachten dat de moleculaire aanpakken, aangedragen in dit proefschrift, bij zullen dragen aan het in kaart brengen van de interacties tussen de gastheer en het micro-organisme in het spijsverteringskanaal van de mens en dier.

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ABOUT THE AUTHOR

Rudolf (Rolf) Johan Boesten was born in Eindhoven on the 29th of June, 1977, in the Netherlands. He grew up in the beautiful village of Waalre. He graduated twice from high-school: the first time in 1994 when he obtained his HAVO diploma and secondly when he graduated from VWO in 1996 both at the Were Di College of Valkenswaard. In September of that same year he moved to Utrecht to start a study in Biology, at the University of Utrecht. During this study, the major subjects were animal behavior, developmental biology, cell biology, and microbiology. In the course of his traineeship at the Microbiology group of Prof. Jan Tommassen he has contributed to the understanding of the molecular mechanism of the *Escherichia coli* page shock protein (PSP) operon. Subsequently, during his second traineeship, at TNO Food & Nutrition in Zeist, he studied the gene-expression profiles of *Aspergillus niger*, while supervised by Dr. Frank H.J. Schuren. He concluded his MSc with a thesis on biofilm formation under the supervision of both Dr. Fank H.J. Schuren and Prof. Jan Tommassen. He formally graduated at the University of Utrecht on August 2002, just after he started his PhD at the Laboratory of Microbiology at Wageningen University, under supervision of Prof. Willem M. de Vos and Dr. Frank H.J. Schuren. His PhD-research was part of the IOP Genomics Project “A genomics approach towards gut health”, which was conducted at the Microbiology Department of TNO Quality of Life in Zeist. His scientific output with respect to this project is presented in this thesis. In 2008, he started his first post-doctorate at the CBS-KNAW Fungal Biodiversity Centre in Utrecht, working on a project for improving molecular diagnostics of clinically important *Candida* yeasts.

PUBLICATIONS

- Rob te Biesebeke, **Rolf Boesten**, Eline S. Klaassens, Carien C.G.M. Booijink, Maaike C. de Vries, Muriel Derrien, David P.A. Cohen, Frank Schuren, Elaine E. Vaughan, Michiel Kleerebezem and Willem M. de Vos, 2004, Microbial functionality in the human gastrointestinal tract. Microbes and Environments, Vol. 19, No. 4, 276-280.

- **Boesten RJ**, de Vos WM, 2008, Interactomics in the human intestine – lactobacilli and bifidobacteria make a difference. J Clin Gastroenterol,42, Suppl 3, Pt 2:S163-7.

- **Boesten RJ**, Schuren FH, de Vos WM, 2009, A *Bifidobacterium* mixed-species microarray for high resolution discrimination between intestinal bifidobacteria. J Microbiol Methods. 2009 Mar;76(3):269-77.

- Klaassens ES, **Boesten RJ**, Haarman M, Knol J, Schuren FH, Vaughan EE, de Vos WM, 2009, Mixed-species genomic microarray analysis of fecal samples reveals differential transcriptional responses of bifidobacteria in breast- and formula-fed infants. Appl Environ Microbiol, 75(9):2668-76.

- **Rolf Boesten**, Frank Schuren, Kaouther Ben Amor, Monique Haarman, Jan Knol, and Willem M. de Vos, 2010, *Bifidobacterium* population analysis in the infant gut by direct mapping of genomic hybridization patterns: potential for monitoring temporal development and effects of dietary regimens. Microbial Biotechnol, early online, DOI: 10.1111/j.1751-7915.2010.00216.x

- **Rolf Boesten**, Richèle Wind, Frank Schuren, Jan Knol, and Willem M. de Vos, Analysis of infant isolates of *Bifidobacterium breve* by comparative genome hybridization indicates the existence of new subspecies with market infant-specificity. Submitted to Res Microbiol (2010)

- **Rolf Boesten**, Frank Schuren, Linette Willemsen, Aldwin Vriesema, Jan Knol, and Willem M. de Vos, *Bifidobacterium breve* – HT-29 cell line interaction: modulation of TNF-α induced gene expression. 2011; Manuscript in preparation.

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Discipline specific activities

• Symposium LAB-7, LAB-8 (2002; 2005)

• 2nd Thematic Day, Centre of Human Nutrigenomics Intestinal Function (2002)

• Genomics Momentum (2002)

• TNO Intestinal Research Platform (TNO, 2003)

• Ecophysiology of the gastrointestinal tract (VLAG, 2003)

• Towards Comprehending Scanned Arrays; expression profiling (MGC, 2003)

• Darmendag (2003; 2004; 2005)

• Bioinformation Technology 1 (VLAG/ WU, 2004)

• 10th Netherlands Biotechnology Conference (NBV, 2004)

• Symposium Networks in Bio-informatics (2004)

• UVA Seminar (2004)

• Systems Biology course (VLAG, 2005)

• Yakult Symposium (Ghent, 2005)

• SSA GutImpact 2nd Platform Meeting on Foods for Intestinal Health (Tallinn, Estonia, 2006)

General courses

• Radiation Hygiene course (WU, 2002)

• Patent Workshop (SenterNovem, 2002)

• Scientific Writing in English (James Boswell Institute, UU, 2005)

• Communication Course (TNO, 2006)

Optionals

• Microbiology PhD students trip (Japan, 2004)

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