Exploring the Functionality of Intestinal Bifidobacteria:

A Post-Genomics Approach

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Contents

Abstract

Following birth, the human gastrointestinal tract is rapidly colonized by microorganisms that profoundly impact the physiology and health of the host by contributing to host nutrition and natural defense, amongst other activities. In the large intestine, bifidobacteria are especially predominant in infants, where they can comprise up to 90% of the total microbiota, and in adults still account for a significant several percent. In this thesis, we applied post-genomic technologies, including metaproteomics and bifidobacterial community transcript profiling, to deepen our understanding of the activity of commensal bifidobacteria in the human intestine. Proteins were extracted from total infant fecal microbiota that was dominated by bifidobacteria, and two-dimensional gel electrophoresis was used to visualize the metaproteomes. The succession of the proteins was studied in fecal microbiota of two infants during 40 days. This revealed that the number and intensity of protein spots changed in time, but the patterns remained similar and specific for each infant. In-gel digestion of protein-spots and sequencing of the peptides, revealed the presence of a bifidobacterial transaldolase supporting the future application of this approach. Global transcript profiling of the infant fecal bifidobacteria was also performed. Total RNA from the fecal microbiota of infants that were solely breast-fed or formula-fed was hybridized to a DNA microarray comprising clones covering the genomes of several bifidobacterial species. Significantly hybridizing clones were sequenced and compared with those in the public databases. While some sequences were found to be bifidobacterial ribosomal RNA, the majority showed similarity to protein-encoding genes predicted to be involved in carbohydrate metabolism, processing of information and housekeeping functions. Remarkably, significant similarity was observed to an operon involved in the utilization of specifically human milk oligosaccharides and mucin sugars, supporting the functionality of bifidobacteria in the infant intestine. Overall, transcript profiling revealed significant differences between breast-fed and formula-fed infants, which was also reflected in the diversity of fecal bifidobacterial species measured with quantitative real time PCR. In another study, a specific prebiotic mixture showed a shift in the dominant adult fecal microbiota as well as abundance of the different bifidobacterial species. Subsequent transcriptomics using the same bifidobacterial-targeted microarrays showed the activity of genes in a wide range of functional groups, the majority being involved in metabolism of carbohydrates of plant origin, house keeping functions, and membrane transport of a wide variety of substrates including sugars and metals. Furthermore, the transcriptome of *Bifidobacterium longum* was studied *in vitro* in human and formula milk and semi-synthetic medium with galactooligosaccharides which showed quite some differentially expressed genes for sugar utilization. Overall, the transcription of genes involved in carbohydrate metabolism and uptake were specifically induced. In conclusion, post-genomics studies of fecal bifidobacteria and batch cultures of *B. longum* resulted in enhanced understanding of the life style and generated important leads for further investigation of genes for metabolism and colonization of intestinal bifidobacteria within the human host.

Preface

Bifidobacteria were first isolated and described over a century ago from human infant feces and were quickly associated with a healthy intestinal tract due to their numerical dominance in breast milk-fed infants compared to formula-fed infants. Also adults contain intestinal bifidobacteria, and various foods containing living cells of *Bifidobacterium* spp. are marketed as probiotics while also prebiotic foods are available that aim to increase the numbers of intestinal bifidobacteria. In spite of the wide consumption of these functional foods, there is only fragmentary information about the physiology, ecology, and genetics of bifidobacteria. Moreover, the functionality of bifidobacteria in the intestinal tract has not been addressed at all. Hence, this thesis is focusing on the activity of *Bifidobacterium* spp. in milk-based delivery systems as well as in the intestinal tract of infants and adults. As the complete genome sequences of several bifidobacteria have become available, significant attention will be given to the potential of genomics information and tools for the analysis of the complex intestinal microbiota, and especially the genus *Bifidobacterium.*

Chapter 1 reviews the potential of the current molecular technologies to study the diversity and functionality of intestinal bifidobacterial species. It summarizes the studies performed with molecular tools on the *in situ* diversity and dynamics of the intestinal bifidobacterial population.

In **Chapter 2**, the metaproteome of the infant fecal microbiota was determined using two-dimensional gel electrophoresis. The temporal development of specific proteins in fecal samples was followed to get insight in the functional complement of the indigenous microbiota of the human infant gastrointestinal tract.

Chapter 3 describes the method and potential for the isolation of high quality messenger and ribosomal RNA from intestinal samples, which is applied in Chapters 4, 5 and 6.

Chapter 4 describes the application of clone-library based microarrays, generated from DNA of a mixture of bifidobacterial species, to reveal gene expression profiles of infant fecal bifidobacteria. The succession of bifidobacterial activity and function was followed in breast-fed or formula-fed infants to study dietary influence. Simultaneously, the diversity of the total microbiota and bifidobacterial community was studied using 16S ribosomal RNA gene-targeted molecular techniques.

Chapter 5 describes the effect of a prebiotic on the presence of bifidobacterial species in adult feces using qualitative and quantitative 16S ribosomal RNA gene-based techniques, denaturing gradient gel electrophoresis, and quantitative real time-PCR. The effect on transcriptome of the adult bifidobacterial population was studied using the mixed bifidobacterial species microarrays.

Chapter 6 presents the results of the study of the kinetics and metabolic activity of *B. longum* under several *in vitro* conditions relevant for the *in vivo* situation. The transcriptome of *B. longum* was studied upon growth in batch cultures with human milk, formula milk or prebiotic galactooligosaccharides using a clone library-based microarray.

Finally, in **Chapter 7** (in English) and **Chapter 8** (in Dutch) the main findings presented in this thesis are summarized and discussed with respect to the implications of the work on the basic understanding of the diversity and functionality of the intestinal microbiota and especially the genus *Bifidobacterium*.

1

Molecular Approaches to Assess Activity and Functionality of Commensal and Ingested Bifidobacteria in the Human Intestinal Tract

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Introduction

Bifidobacteria were first isolated and described over a century ago from human infant feces and were quickly associated with a healthy intestinal tract due to their numerical dominance in breast-fed infants compared to bottle-fed infants (218, 219). The human gastrointestinal tract is essentially sterile at birth but is rapidly colonized during the first few days of life by microbes acquired from the mother and the surrounding environment. Bacteria capable of aerobic growth, such as enterobacteriaceae, streptococci and staphylococci, initially colonize the gastrointestinal tract and create reducing conditions favourable to the proliferation of anaerobic bacteria, including bifidobacteria, clostridia, ruminococci and bacteriodes (57, 122). Bifidobacteria generally thrive and dominate in the infant intestine due to their selection by the breast milk. Bifidobacteria still remain a relatively dominant group in the intestine of adults (255). Based on initial studies on breast-fed infants that harboured high numbers of bifidobacteria and which resisted intestinal infections better than their formula-fed counterparts, bifidobacteria have been incorporated into probiotic functional foods. Health benefits of bifidobacteria to the host as supported by clinical trials have led to their wide application as probiotic components of health-promoting foods, especially in fermented dairy products in the form of yoghurts and daily shots. Although foods containing probiotic bifidobacteria are widely consumed, there is only fragmentary information about their physiology, ecology, and genetics. Various species originating from the human intestinal tract including *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium longum* bv. *infantis*, *Bifidobacterium longum* bv. *longum*, and *Bifidobacterium animalis* ssp. *lactis* (see Table 1) have been applied during or after the manufacture of fermented milks together with 'traditional' starter cultures to obtain products with satisfactory quality and added health-promoting value (213). Bifidobacteria are also available as probiotic components in microcapsules following fermentation by 'traditional' starter cultures which enhances survival (92, 211).

Besides being added to functional foods, indigenous commensal bifidobacterial species are also common targets for prebiotic action. These prebiotics are oligosaccharides that are selectively fermented by bifidobacterial species aiming at improving the host's health (40, 67, 157). Thus there is considerable need to understand functional activity of bifidobacteria and their interactions with the host. The conventional way to study the intestinal ecosystem is by focusing on its structure and specifying the number and variety of inhabitants in different sites. Classical culturing methods are used to define the total number of microbes and species-specific numbers of bacteria. Over the last decade it has been established that only a minority of the gastrointestinal tract microbes have been enumerated and isolated in pure cultures owing to insufficient suitable conditions and challenges in cultivating anaerobic microbes (5, 255). Similarly, for bifidobacterial species the selective media used today are not sufficient to enumerate and isolate all bifidobacterial species

present in gastrointestinal samples (190, 216, 227). Consequently, molecular approaches are necessary to reveal the complete architecture of the intestinal microbial ecosystem. The majority of culture-independent methods are based on targeting the 16S ribosomal (rRNA) genes (13). A wealth of technologies to study and track the bifidobacteria including fingerprinting techniques such as 16S rRNA PCR-DGGE up to genome-wide targeting DNA microarrays are under development. In addition to revealing the identity and diversity of the intestinal inhabitants, functional genomics approaches are now also providing insight in the activity of the bifidobacteria population within the human intestine. Such a functional analysis of the intestinal ecosystem is developing rapidly and is considered to be essential for the further development of functional foods that impact health.

In this chapter we review molecular and new genomics-based technologies that may be applied in the absence of culturing to gain more insight into the presence and diversity of bifidobacteria in the human gastrointestinal tract, as well as recent advances of these techniques that allow us to study functionality and activity *in situ*. Molecular techniques will provide data that can help explain the physiology of the bifidobacteria and their adaptation to their ecological niche, and health-promoting effects in the host.

Table 1 References and habitats of species of the genus *Bifidobacterium*

Species	Type strain*	Source	Reference
Found in human GIT			
B. adolescentis	ATCC 15703T	Infant and adult feces, appendix, dental caries and vagina	(177)
B. angulatum		Adult feces	(192)
B. bifidum	DSM 20215 JCM 1254 NCIMB 41171	Infant and adult feces and vagina	(155)
B. breve	UCC2003	Infant feces and vagina	(177)
B. catenulatum		Infant and adult feces and vagina	(192)
B. dentium	Bd1	Human dental caries, oral cavity and adult feces	(192)
B. gallicum		Adult feces	(111)
B. longum bv. *infantis*	ATCC 15697	Infant feces and vagina	(177) (186)
B. longum bv. *longum*	DJO10A	Infant and adult feces and vagina	(177, 186)
	NCC2705	Human intestine	DOE JGI
		Infant feces	(197)
B. pseudocatenulatum		Infant feces	(194)
B. scardovii		Adult urine and blood	(88)
B. thermophilum		Piglet, chicken and calf feces and rumen infant	(138)
Found in animal GIT			
B. animalis ssp. *animalis*		Rat, chicken, rabbit and calf feces	(129, 192)
B. asteroides		Honeybee	(193)

B. boum		Rumen and piglet feces	(194)
B. choerinum		Piglet feces	(194)
B. coryneforme		Honeybee	(19)
B. cuniculi		Rabbit feces	(194)
B. gallinarium		Chicken feces	(238)
B. indicum		Honeybee	(193)
B. longum bv. *suis*		Piglet feces	(131)
B. magnum		Rabbit feces	(195)
B. merycicum		Rumen	(17)
B. pseudolongum ssp. *globosum*		Piglet, calf, rat, rabbit, lamb feces and rumen	(19)
B. pseudolongum ssp. *pseudolongum*		Piglet, rat, chicken, calf feces and rumen	(193, 245)
B. psychraerophilum		Pig caecum	(202)
B. pullorum	ATCC 49618	Chicken feces	(220)
B. ruminantium		Rumen	(17)
B. saeculare		Rabbit feces	(17)
B. simiae		Monkey abdomen	(54)
B. thermacidophilum ssp. *porcinum*		Piglet feces	(46, 248)
Other origins			
B. animalis ssp. *lactis*		Fermented milk	(133) (129)
B. minimum		Sewage	(19)
B. subtile		Sewage	(19)
B. urinalis		Human urine	(87)

Adapted from (20, 113, 227). * Characterized type strain with 16S rRNA gene sequence available in the database.

Development of bifidobacteria in the intestine and beneficial effects

Bifidobacteria form part of the normal intestinal microbiota of human infants and adults and are believed to play a beneficial role in maintaining the health of the host. During birth and the first few days of life, bifidobacteria, amongst other intestinal microbes acquired from the mother and the surrounding environment, colonize the infant intestine (58). A succession of microbes occurs commencing with aerobes such as enterobacteriaceae, that create reducing conditions favourable to more strict anaerobic bacteria including the bifidobacteria (62, 122). The changing microbial ecology is coincident with a marked functional and morphological maturation of the infant gut barrier functions and immune system development (30, 85). The microbiota in breast-fed infants is usually dominated by bifidobacteria within a few days, but following a short delay they also reach high numbers in formula-fed infants (57, 78). Apparently lower numbers of bifidobacteria are found in feces of pre-term infants (reviewed

by Westerbeek et al., 2006 (239)). A combination of sequence analysis of 16S rRNA gene clone libraries (51, 208, 249) and the fluorescent *in situ* hybridization (FISH) approach targeting the 16S rRNA (80, 112) has shown that the most abundant bacterial groups in the human intestine belong to, in order of numerical importance, the phyla of the Firmicutes (including the large class of Clostridia and the lactic acid bacteria), Bacteroidetes, Actinobacteria (including Colinsella and *Bifidobacterium* species) and Proteobacteria. Thus, the adult intestinal microbiota comprises a majority of low and high G+C content Gram-positive bacteria.

The association of bifidobacteria with a healthy infant intestinal tract has promoted the addition of bifidobacteria to dairy products as probiotics. Probiotics are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (56). Survival of the probiotic upon gastrointestinal transit, adhesion to the intestinal mucosa, and stability in the product are considered desirable properties for a probiotic, although not necessarily a good indicator for health-improving properties of the probiotic (215). Ultimately, the efficacy of these probiotic strains in the products for a certain health benefit must be proven in clinical trials. The viability of the ingested probiotic as well as the effect on the autochthonous microbiota has been reported in numerous studies. The therapeutic efficacy of these probiotic strains (and combination species and strains) has been demonstrated in various clinical trials usually involving prevention or alleviation of symptoms of gastrointestinal disorders. The various benefits included alleviation of symptoms of diarrhea, irritable bowel syndrome, inflammatory bowel disease, and improvement in immunity. Some studies are described in more detail below.

The importance of the microbiota composition may initiate in early infancy when maturation of the gut barrier functions and immune development occurs. A few studies suggest that infants that do not harbour bifidobacterial species or harbour decreased numbers may be more prone to rectal bleeding (4, 64), allergy (64) and eczema (123). Very early administration of *B. breve* to low birth weight infants was shown to be useful in promoting the colonization of bifidobacterial and the formation of a normal intestinal microbiota (199). In several other studies bifidobacterial supplementation modified the infant gut microbiota in a manner that appeared to alleviate allergic inflammation (96) and reduced the incidence of acute diarrhea and rotavirus infections (182). Also combinations of bifidobacterial species with *Streptococcus thermophilus* strains reduced the incidence of rotavirus associated diarrhea (182) and antibiotic-associated diarrhea in children (38, 217). A mix of *Bifidobacterium* species and *Lactobacillus acidophilus* increased energy intake and promoted weight gain in acutely ill children receiving antibiotics (199). The effects of supplementation with probiotic bifidobacteria have also been studied in adults, especially in relation to alleviating symptoms of gastrointestinal disorders. For example, a recent report indicated that oral therapy with a fixed combination of *B. longum* and *Lactobacillus gasseri* shortened the duration and decreased the severity of acute diarrhea in adults (126). Other studies showed

beneficial effects of bifidobacterial species mixed with lactobacilli and/or *S. thermophilus* strains in the maintenance of antibiotic-induced remission in chronic pouchitis (70, 134). However, in another acute pouchitis trial, supplements of *L. acidophilus* and *B. lactis* Bb 12 did not lead to an improvement in symptoms (107). In collagenous colitis patients, a preliminary probiotic trial involving treatment with *B. animalis* ssp. *lactis* Bb 12 demonstrated amelioration of clinical symptoms (242). In a trial, intake of capsules of live combined *Bifidobacterium*, *Lactobacillus* and *Enterococcus*, significantly decreased relapse and inflammatory activity in the colon of patients with ulcerative colitis (42). The probiotic strain *B. infantis* 35624 was able to alleviate inflammation symptoms in patients suffering from irritable bowel syndrome (152) suggesting an immune-modulating role for *B. infantis*. Supplements of lactobacilli and bifidobacteria effectively suppressed *Helicobacter pylori* infection in adults (236). Different probiotic preparations, including a combination of bifidobacteria and *Lactobacillus* spp. or *Lactobacillus rhamnosus*, *Saccharomyces boulardii*), were found to be superior to the placebo for side effect prevention but had no effect on *H. pylori* eradication (39). Although recovery of *B. animalis* ssp. *lactis* Bb 12 from feces was observed in healthy young adults, the supplementation had only a slight effect on the immune function (32). Several other feeding trials reported in healthy individuals to study persistence of the ingested bifidobacteria mainly using fecal samples will be discussed in the section below on 'Tracking bifidobacteria in human intestinal samples'.

Bifidobacteria are not only used as probiotic ingredients but the indigenous bifidobacteria are also targets of prebiotics. A prebiotic is a nondigestible food ingredient that beneficially affects the host by selectively stimulating growth and/or modifying the metabolic activity of one or a limited number of bacterial species in the colon that have the potential to improve host health (40, 65). A formula supplemented with a prebiotic mixture (galacto- and fructooligosaccharides) showed consistently higher fecal SIgA levels which suggested possible stimulation of the mucosal immune response. SIgA antibodies are associated with increased neutralization and clearance of viruses and this effect may be transferred via breast milk to support maturation of humoral immunity (7). In a previous study the prebiotic supplemented formula resulted in a similar effect on metabolic activity of the microbiota as in breast-fed infants (6). The increase of bifidobacterial numbers by dietary oligosaccharides (90% galacto-oligosaccharides and 10% fructooligosaccharides) reduced the presence of clinically relevant pathogens in preterm formula-fed infant fecal microbiota, indicating protective capacity of prebiotics against enteral infections (102).

Despite the variable results obtained in the numerous clinical trials, many scientists argue in favour of bifidobacteria for adjunct therapy. The main reason is the observation that bifidobacteria are the dominant commensal microbiota of most breast-fed baby's, and the negative effects seen upon delayed colonization of bifidobacteria in pre-term baby's (239). The underlying assumption is that at least part of the protective effect of breast-feeding is linked to the presence of bifidobacteria.

Taxonomy and typing of bifidobacteria colonizing the human intestine

Bifidobacteria were first isolated from the feces of breast-fed infants by Tissier (219) and since then it has been established that their predominant habitat is the human gastrointestinal tract. Although the *Bifidobacterium* genus shares phenotypic features typical of lactic acid bacteria, such as acid production, they belong to the Actinomycetales branch of the high–G+C Gram-positive bacteria that also includes the corynebacteria, mycobacteria, and streptomycetes (see Figure 1: Phylogenetic tree obtained using the 16S rRNA gene).

Today, 37 bifidobacterial species have been characterized in the literature available in the public database, Pubmed. Many species have been isolated from human as well as animal feces, and sewage, but they have also been isolated from the human oral cavity and the intestinal tracts of insects such as bees. Species of the genus *Bifidobacterium* and the habitat from which they were isolated are presented in Table 1. The *Bifidobacterium* branch forms a coherent phylogenetic unit as their 16S rRNA sequences share over 93% similarity (139). Phylogenetic classification of all 16S rRNA gene sequences of bifidobacterial species originating from the human gastrointestinal tract resulted in a selection of 48 sequences. Twelve of these species were already fully characterized and are part of Table 1 (20). The remaining, uncharacterized or uncultured, 36 sequences contain five new unique OTUs (operational taxonomic units), and share less than 97% similarity to the identified 16S rRNA genes of other bifidobacterial species. These five sequences were all acquired from adult fecal samples using culture-independent techniques (11, 188). Thus, the latter study indicates that culture-independent approaches are a valuable tool for the identification of the total community of microbes in the gastrointestinal tract including not yet identified new bifidobacterial species. As most studies on diversity and identification of microbes are based on 16S rRNA gene sequences, the database of bacterial 16S rRNA genes is expanding daily. Therefore this gene is the general target for bifidobacterial strain and species identification in mixed microbial samples. Nevertheless, characterization of bifidobacterial species remains difficult as the 16S rRNA genes show more than 93–99.5% interspecies similarity (226, 237).

For differentiation between closely related bifidobacterial species other genes including the elongation factor Tu (*tuf*) gene (227), recombinase A (*recA*) (228), chaperone GroEL gene (225), ATP synthase subunit B (*atpD*) gene (129, 224), pyruvate kinase (223), and, xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (*xfp*) (246) have been introduced as phylogenetic markers besides the 16S rRNA gene. Multiparametric PCR based methods support and complement the 16S rRNA based division of bifidobacterial species and provide a finer taxonomic differentiation between the closely related species such as *B. animalis* ssp. *lactis* (also called *B. lactis*) and *B. animalis* ssp. *animalis*, and *B. longum* bv. *infantis* and *B. longum* bv. *longum*.

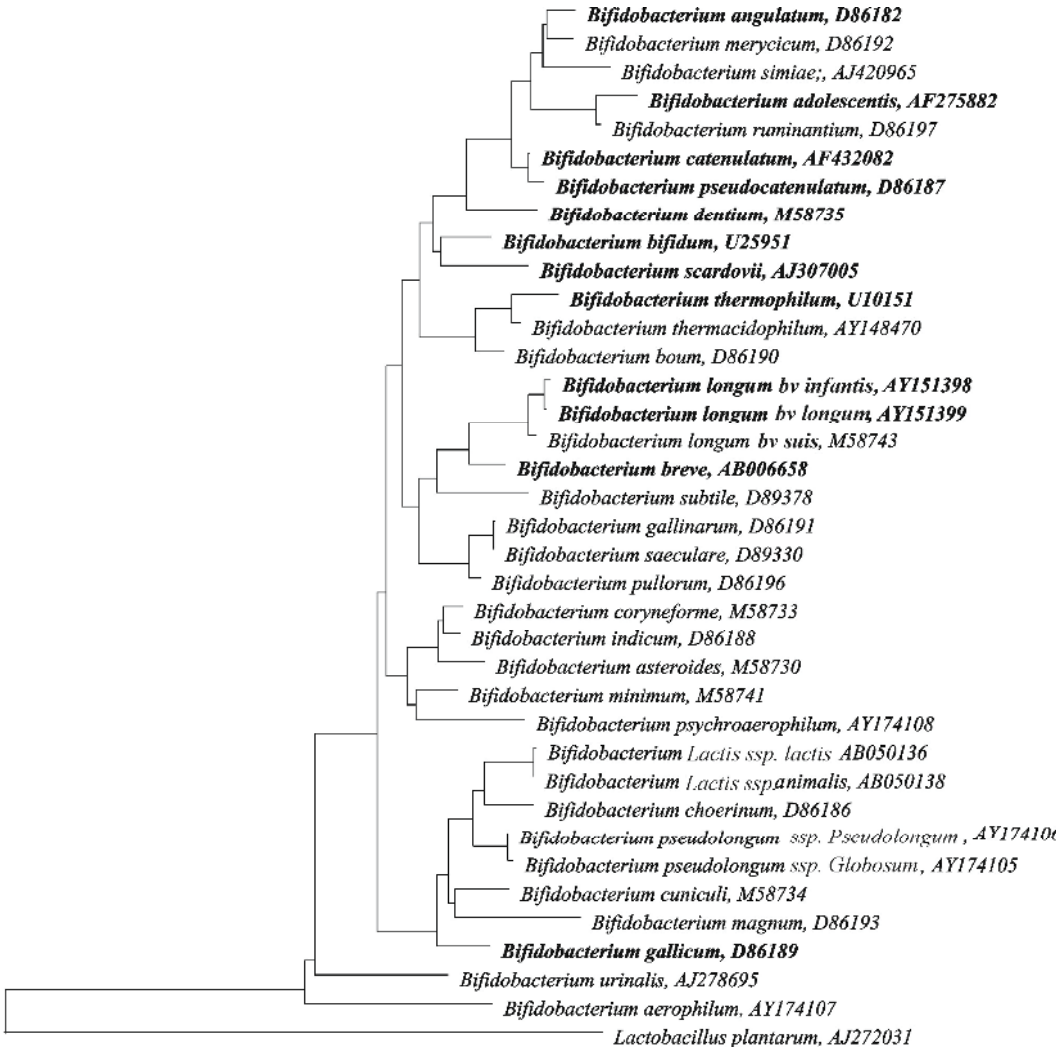

Figure 1 Phylogenetic tree based on 16S rDNA sequences of bifidobacteria rooted with *L. plantarum*. The tree was generated from the tree-of-life ARB software package. Accession numbers of the 16S rDNA sequences are indicated. Species isolated from human intestine are indicated in bold.

The release of the complete genomes of *B. longum* NCC2705 (Nestle Research Centre, Switzerland) and *B. adolescentis* (Gifu University, Life Science Research Centre, Japan/National Center for Biotechnology Information, NIH, USA, Bethesda), two draft assemblies of *B. longum* DJO10A (DOE Joint Genome Institute) and *B. adolescentis* L2-32 project (Washington University Genome Sequencing Center) as well as the initiatives to sequence *B. breve* UCC2003 (University College Cork), *B. dentium* Bd1 (University of Parma, Italy/National University of Ireland, Ireland), and *B. longum* bv. *infantis* ATCC 15697 (DOE Joint Genome Institute/JGI-PGF) are expected to open up a new era of comparative genomics in bifidobacterial biology. The genomes of *B. longum* strains DJO10A and NCC2705 possess a wide range of catabolic pathways which confer a growth advantage where readily fermentable carbohydrates are in short supply, like the competitive ecosystem in the gut depending on the dietary intake of the host. The ability of *Bifidobacterium* species

to survive and persist in this competitive environment is due to the use of negative transcriptional regulation as a flexible control mechanism in response to nutrient availability and diversity, as well as predicted genetic features such as exo- and endo-glycosyl hydrolases and high-affinity oligosaccharide transporters. These features likely help to compete for uptake of structurally diverse oligosaccharides released from digestion of plant fibres (101, 197).

The availability of genome sequences has enabled large scale and high throughput analysis using DNA microarrays. Genome-wide expression profiling using DNA array technology can be used to determine changes in transcription levels of literally every gene in the genome in response to environmental changes. This will be discussed in the section 'Functional genomics' below. Moreover, the development of microarray technology is being used for the global comparative analysis of gene content between different isolates of a given species without the necessity of sequencing many strains, and may provide information about the degree of relatedness among various strains. This has been performed based on the complete genome sequence for *Lactobacillus plantarum* WCFS1 which showed a high degree of gene content variation among *L. plantarum* strains in genes related to lifestyle adaptation. The *L. plantarum* strains clustered into two clearly distinguishable groups, which coincided with an earlier proposed subdivision of this species based on conventional methods (140). The comparison of genome sequences provides insight in speciation and evolution. This approach, often termed genomotyping, can provide useful information about the degree of relatedness among various species and strains of the genus *Bifidobacterium*.

For the majority of microorganisms for which the complete genome sequence is not yet available, including most bifidobacterial species, clone-based microarrays, on which each spot represents a random genomic fragment, are a valuable alternative to open reading frame-based microarrays. In this approach, a chromosomal DNA library is constructed from the strain of interest, and the inserts of the DNA library, are amplified from the clones by PCR with generic primers and spotted on array-slides, the so-called smartmans arrays (167). Thus, as an alternative to the complete sequencing of genomes, smartman and oligonucleotide microarrays of the sequenced microorganisms can be used to obtain a highly detailed view of the gene content of related organisms, especially of strains of closely related species or of the same species (140). Large-scale mutation events can often, with a high degree of certainty, be reconstructed from a comparison of related strains. The results obtained with microarray-based genotyping support a model of high evolutionary plasticity of bacterial genomes. It has become clear that horizontal gene transfer is an important mechanism for generating genotypic and phenotypic diversity in bacteria. The phenomenon has been studied in particular in relation to niche adaptations like the emergence of virulence, antibiotic resistance and symbiosis or fitness. A recently constructed clone based mixed-species genomic DNA microarray showed great potential for identification and characterization of bifidobacterial strains and species and appears suitable for biomarker screening (26).

Tracking bifidobacteria in human intestinal samples

Bifidobacteria represent one of the most important bacterial groups of the human gastrointestinal tract. Their significant numerical dominance was demonstrated by culturing from fecal samples on selective medium which indicated levels of between one and ten percent in adults (79), and even up to 90% in infants (78). However, these conventional plate counts overestimated bifidobacteria counts by 2 to 10% as a result of underestimation of the total microbial counts by culturing (137). In addition, it is very challenging to obtain pure cultures of the majority of species in the intestinal microbiota due to the largely anaerobic nature of this community and the paucity of suitable enrichment strategies to stimulate intestinal conditions. Less is known about bifidobacterial numbers in other gastrointestinal sites such as the ileum. Studies are limited to ileostomy patients in which the microbiota differs from those of the healthy distal ileum and these indicated that bifidobacterial counts varied between 0.1 and 10% of the total microbiota. The number of bacteria in the terminal ileum of ileostomy subjects has been estimated to be 10^7–10^8 per gram compared with 10^5–10^6 per gram in the normal ileum (60, 148).

Molecular techniques that bypass culturing allow a more complete assessment of the bifidobacterial strains present among the complex intestinal microbiota by unraveling the extent of the diversity, abundance and population dynamics of the bifidobacterial population. Molecular tools will greatly expand the capacity to differentiate between bifidobacterial species and evaluate the evolutionary relationship between isolates. Molecular tools can also monitor the functional activity of the bifidobacterial cells and whether they play a role or not at a particular time or at a given site of the intestinal tract. In the following section, various qualitative and quantitative methods are described for this purpose. Figure 2 shows the links between the molecular and new genomics-based technologies assessing the diversity and functionality of the intestinal microbiota.

Qualitative fingerprinting techniques

The most widely applied fingerprinting methods are the PCR-denaturing and temperature gradient gel electrophoresis (DGGE and TGGE, respectively) of PCR amplified fragments of 16S rRNA genes (145). When PCR-DGGE of the 16S ribosomal DNA (rDNA) of the gut microbiota is performed, due to the high GC content of bifidobacterial DNA, the bands corresponding to bifidobacterial amplicons usually migrate to the base of the DGGE gel and can be rather conveniently recognized in this manner (58, 204) (Fig. 3). Following the establishment of the microbiota in infant fecal samples over the first years of life, DGGE profiles showed the dominance of ruminococci and bifidobacterial species based on stability and intensity of the bands over time (57). Owing to the resolution of gels targeting the local community and the properties of the amplicons, different bifidobacterial amplicons do not separate into distinct bands at the base of the gel. Specific primers have been developed to

target the bifidobacterial population within fecal microbiota (189). These fingerprinting techniques have been invaluable in gaining more insight in the diversity and dynamics of bifidobacteria within individuals. Bifidobacterial-specific PCR-DGGE has shown bifidobacterial species three days after birth and remain constant in time, although the composition of bifidobacterial species was variable (58). DGGE profiles of adult fecal bifidobacterial communities were host-specific and stable in time (189). Comparison of DGGE profiles of specific bifidobacterial strains has suggested their vertical transmission from parents to offspring (58), confirming earlier studies based on a culturing approach and diagnostic PCR on colony DNA (78). These profiling techniques may also be used to monitor behaviour of probiotics or native bifidobacteria during dietary interventions, for example with prebiotics. PCR-DGGE was used to demonstrate that intake of a probiotic *B. lactis* Bb 12 transiently colonized the adult intestine although the indigenous population remained unaffected (189).

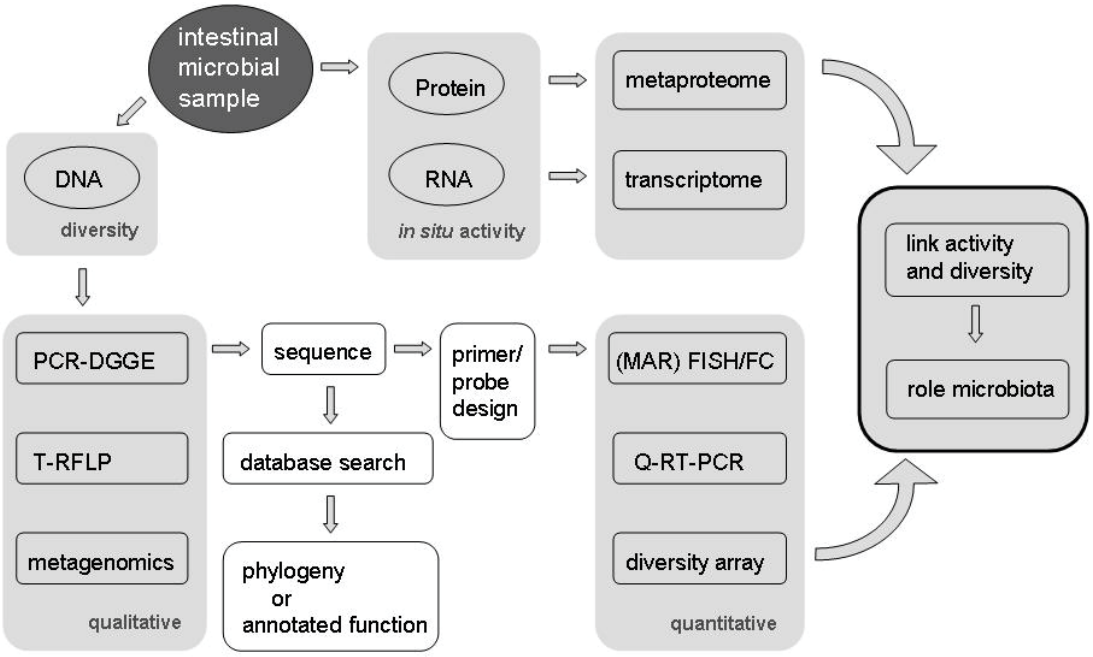

Figure 2 Overview of molecular and new genomics-based technologies to monitor intestinal microbiota at diversity and functionality. Abbreviations: DGGE, Denaturing Gradient Gel Electrophoresis; T-RFLP, Terminal-Restriction Fragment Length Polymorphism; (MAR) FISH/FC, (Microautoradiography) Fluorescent *in situ* Hybridization/ Flow Cytometry; qPCR, Quantitative Real Time PCR.

In the same study ingestion of prebiotic galactooligosaccharides showed no effect on the qualitative composition of the indigenous bifidobacterial community (189). Intake of a potential prebiotic lactulose intensified the bifidobacterial band on DGGEs of healthy adults especially in subjects with lower initial bifidobacterial counts. Quantitative real time PCR

confirmed increase of the total levels of bifidobacteria and *B. adolescentis* (221). Comparison of the ascending, transcending, and descending colon wall showed the presence of bifidobacterial species in most sampling sites, but with a very simple profile suggesting few mucosa-associated species (149).

Qualitative analysis of the bifidobacterial community in feces has also been studied using terminal restriction fragment length polymorphism (TRFLP). Comparison of breast-fed, formula-fed and mixed-fed baby's using T-RFLP combined with bifidobacterial species specific PCR revealed no significant differences in the distribution of different bifidobacterial species between the groups (187). Comparison of different locations in the human gastrointestinal tract using T-RFLP revealed bifidobacterial species in the colon and rectum of healthy adults (82). The inability to generate sequence information from T-RFLP peaks makes the identification of unknown species in a sample difficult. Ideally identification may be artificially determined by performing virtual restriction digests on public database sequences. However this approach may result in a misidentification as it assumes that only a single species or operational taxonomic unit (OTU) can have a peak of that size in a sample. It is further based upon the assumption that the database sequence is of good quality and accurately identified, which may or may not be the case.

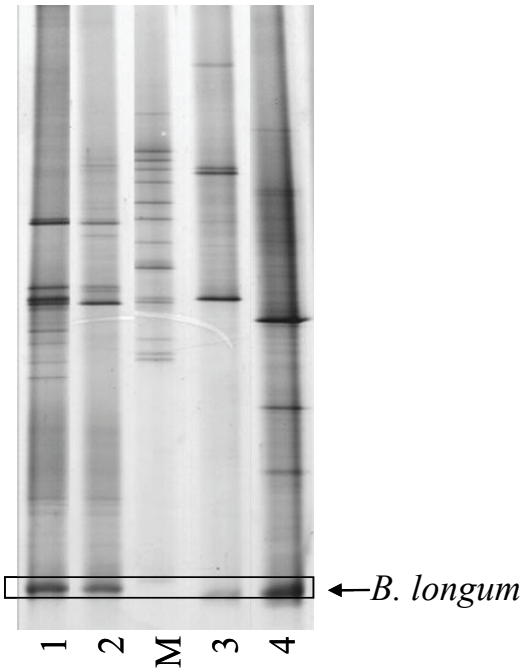

Figure 3 Separation of PCR amplicons obtained from infant fecal microbiota compared to *B. longum* with universal 16S rDNA targeting primers (968f-GC/ 1401r) on 30–60% DGGE gel. 1: infant 1, age 18 weeks; 2: infant1, age 26 weeks; M: marker; 3: infant 2, age 1 week; 4: infant 2, age 6 weeks; *B. longum* pure culture. Bifidobacterial amplicon in box.

The development of DNA oligonucleotide microarrays offers a fast, high throughput option for detection and estimation of the diversity of microbes in a complex ecosystem (120). Typical microarrays contain hundreds of probes, usually based on the 16S rRNA gene, specific for different strains, species or genera of microorganisms that are detected in one single experiment. Both labeled RNA and DNA can be hybridized to the slides with

immobilized probes, and following a washing step, be detected by a scanner. A microarray fully covering all species inhabiting the human gastrointestinal known at present is designed and being validated currently with promising reproducible results (171). More studies are underway to develop and apply this microarray technology to unravel the diversity of the human intestinal microbiota in a wide range of intestinal samples. Palmer *et. al.* (159) describe development and validation of diversity arrays for gut microbes. Ultimately, this high throughput technique may be used for quantitative detection of all human gastrointestinal tract microbial inhabitants.

Quantitative detection

Alternative approaches are required in order to obtain culture-independent quantification of the numbers of microbes in human intestinal samples. Within the past decade the most common approach was based on hybridization of oligonucleotide probes to microbial cells in mixed samples (2). Just like most fingerprinting analysis the most commonly used target for hybridization, either by dot blot or fluorescent *in situ* hybridization (FISH), is the 16S rRNA gene. FISH is applied to morphologically intact cells and thus provides a quantitative measure of the target microbes. Following fixation, bacterial cells can be hybridized with species-, genus-, or group-specific probes. Prior to hybridization, cells can be either immobilized on glass slides or kept in suspension when analyzed by the flow cytometer (11). The flow cytometer has the possibility to offer a platform for high resolution, high throughput identification and enumeration of microorganisms using fluorescent rRNA-targeted oligonucleotide probes. Species and genus-specific probes have been designed and validated for the bifidobacterial population (see Table 2). Several studies using FISH have provided a more realistic estimation of the bifidobacterial numbers in the human intestine, and the current average for the autochthonous bifidobacterial community in human European adults is approximately 4% of the total bacterial count (144). Especially feces of breast-fed infants were demonstrated to be dominated by bifidobacteria, an estimated 40–90% of the total microbiota, but they also reached high numbers in formula-fed infants (78). Dot blot hybridization with rRNA-targeted probes was used to show that bifidobacteria constituted approximately 5% of the total community within the adult caecum (127).

Table 2 Major primers and probes used to study the bifidobacteria in fecal samples and products for PCR/DGGE, FISH and qPCR.

Oligo- nucleotide	Sequence (5'-3')	name	Reference
PCR			
	GGGTGGTAATGCCGGATG	Bif164-f	(103)
	CCACCGTTACACCGGGAA	Bif662-r	(103)
	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGC ACGGGGGG-CCACCGTTACACCGGGAA	Bif662-GC-r*	(189)
	GATTCTGGCTCAGGATGAACG	Im26-f	(94)
	CGGGTGCTICCCACTTTCATG	Im3-r	(94)
FISH			
	GGGTGGTAATGCCGGATG	Bif164-f	(10, 109)
	GATAGGACGCGACCCCAT	Bif228	(127)
qPCR			
Bifidobacterium spp.	GATTCTGGCTCAGGATGAACG	Im26-f	(94) (75)
	GATAGGACGCGACCCCAT	Bif228	(75, 127)
Bifidobacterium spp.	GGGTGGTAATGCCGGATG	Bif164-f	(109) (8)
	TAAGCCATGGACTTTCACACC	Bif601R	(16) (8)
Bifidobacterium spp.	CTCCTGGAAACGGGTGG	g-Bifid-F	(176)
	GGTGTTCTTCCCGATATCTACA	g-Bifid-R	(176)
B. adolescentis	CTCCAGTTGGATGCATGTC	BiADOg-1a	(130)
	TCCAGTTGACCGCATGGT	BiADOg-1b	(130)
	CGAAGGCTTGCTCCCAGT	BiADO-2	(130)
B. angulatum	CAGTCCATCGCATGGTGGT	BiANG-1	(130)
	GAAGGCTTGCTCCCCAAC	BiANG-2	(130)
B. bifidum	CCACATGATCGCATGTGATTG	BiBIF-1	(130)
	CCGAAGGCTTGCTCCCAAA	BiBIF-2	(130)
B. breve	CCGGATGCTCCATCACAC	BiBRE-1	(130)
	ACAAAGTGCCTTGCTCCCT	BiBRE-2	(130)
B. catenulatum group	CGGATGCTCCGACTCCT	BiCATg-1	(130)
	CGAAGGCTTGCTCCCGAT	BiCATg-2	(130)
B. longum bv. *longum*	TTCCAGTTGATCGCATGGTC	BiLON-1	(130)
	GGGAAGCCGTATCTCTACGA	BiLON-2	(130)
B. longum bv. *infantis*	TTCCAGTTGATCGCATGGTC	BiINF-1	(130)
	GGAAACCCCATCTCTGGGAT	BiINF-2	(130)
B. dentium	ATCCCGGGGGTTCGCCT	BiDEN-1	(130)
	GAAGGGCTTGCTCCCGA	BiDEN-2	(130)
B. infantis Y 1	GTCAAGTCATGAAAGTGGGTA	Y116Sl	(230)
B. infantis Y 1	GTCAAGTCATGAAAGTGGGTA	Y1ITSr	(230)
B. breve Y 8	TACAACGGGATGCGACAGC	Y816Sl	(230)
B. breve Y 8	GAACGAGGAATCAAACCCCGTCT	Y8ITSr	(230)
B. longum Y10	GCAAGGCACTTTGTGTTGAG	Y1016Sl	(230)

B. longum Y10	AAGAACGAGGAATCAAAGGAAACC	Y10ITSr	(230)
Primer-probe based qPCR			
Bifidobacterium spp.	GGGATGCTGGTGTGGAAGAGA	F_allbif_IS	(76)
	TGCTCGCGTCCACTATCCAGT	R_allbif_IS	(76)
	TCAAACCACCACGCGCCA	P_allbif_IS	(76)
Bifidobacterium spp.	GATTCTGGCTCAGGATGAACGC	F LM26	(94); (75)
	CTGATAGGACGCGACCCCAT	R Bif228	(127); (75)
	CATCCGGCATTACCACCCGTTT CCTC	P Bif164	(109); (75)
B. adolescentis	ATAGTGGACGCGAGCAAGAGA	F_adol_IS	(76)
	TTGAAGAGTTTGGCGAAATCG	R_adol_IS	(76)
	CTGAAAGAACGTTTCTTTTT[a]	P_adol_IS	(76)
B. angulatum	TGGTGGTTTGAGAACTGGATAGTG	F_angul_IS	(76)
	TCGACGAACAACAATAAACAAAACA	R_angul_IS	(76)
	AAGGCCAAAGCCTC	P_angul_IS	(76)
B. bifidum	GTTGATTTCGCCGGACTCTTC	F_bif_IS	(76)
	GCAAGCCTATCGCGCAAA	R_bif_IS	(76)
	AACTCCGCTGGCAACA	P_bif_IS	(76)
B. breve	GTGGTGGCTTGAGAACTGGATAG	F_breve_IS	(76)
	CAAAACGATCGAAACAAACACTAAA	R_breve_IS	(76)
	TGATTCCTCGTTCTTGCTGT	P_breve_IS	(76)
B. catenulatum	GTGGACGCGAGCAATGC	F_cate_IS	(76)
	AATAGAGCCTGGCGAAATCG	R_cate_IS	(76)
	AAGCAAACGATGACATCA	P_cate_IS	(76)
B. dentium	CCGCCACCCACAGTCT	F_dent_IS	(76)
	AGCAAAGGGAAACACCATGTTT	R_dent_IS	(76)
	ACGCGTCCAACGGA	P_dent_IS	(76)
B. infantis	CGCGAGCAAAACAATGGTT[a]	F_inf_IS	(76)
	AACGATCGAAACGAACAATAGAGTT	R_inf_IS	(76)
	TTCGAAATCAACAGCAAA	P_inf_IS	(76)
B. lactis	CCCTTTCCACGGGTCCC	F	(75)
	AAGGGAAACCGTGTCTCCAC	R	(75)
	AAATTGACGGGGGCCCGCACAAGC	P	(75)
B. longum	TGGAAGACGTCGTTGGCTTT	F_long_IS	(76)
	ATCGCGCCAGGCAAAA[a]	R_long_IS	(76)
	CGCACCCACCGCA	P_long_IS	(76)
B. longum	CAGTTGATCGCATGGTCTT-	F	(108)
	TACCCGTCGAAGCCAC	R	(108)
	TGGGATGGGGTCGCGTCCTATCAG	P	(108)

* Primer with GC clamp for DGGE, F: forward primer, R: reverse primer, P: probe.

Interestingly the latter technique of targeting of rRNA from complex microbial ecosystems gives more information about the activity of the microbes rather than FISH which mainly indicated presence. FISH has been employed in many studies successfully to determine especially the bifidobacterial numbers as well as other microbiota groups following dietary interventions with functional foods. For example in one such study, following a week of daily ingestion of 5×10^9 colony forming units (cfu) of the probiotic *B. lactis* Bb 12, the *B. lactis* cells could still be detected in the feces of five of the nine subjects one week after cessation which indicated at least temporary colonization by the probiotic (158). Similarly a feeding trial with the probiotic *B. lactis* LAFTI®B94 indicated survival of the bacterial cells following intestinal transit, detected using a specific probe, and presence of the probiotic in fecal samples for up to a maximum of four weeks after intake, but no permanent colonization (207).

Quantitative real time PCR (qPCR) assays are currently being developed for detection and quantitation of human intestinal microbiota, which has the advantages of being high throughput as well as having a high dynamic range and high sensitivity. There are different approaches for qPCR. One involves using DNA binding dyes such as SYBR Green I which are easy to design and optimize in the assays, and cost effective (244). When free in solution, SYBR Green I displays relatively low fluorescence, but when bound to double-stranded DNA its fluorescence increases by over 1000-fold. The more double-stranded DNA that is present, the more binding sites there are for the dye, so fluorescence increases proportionately to DNA concentration. This property of the dye provides the mechanism that supports its application to track the accumulation of PCR product. As the target is amplified, the increasing concentration of double-stranded DNA in the solution can be directly measured by the increase in fluorescence signal. Compared to nonspecific chemistries for qPCR such as the latter SYBR Green I dye, a higher level of detection specificity is provided by using an internal probe together with a primer set to detect the qPCR product of interest. In the absence of a specific target sequence in the reaction, the fluorescent probe does not hybridize and therefore the fluorescence remains quenched. When the probe hybridizes to the target sequence of interest, the reporter dye is no longer quenched, and fluorescence will be detected. The level of fluorescence detected is directly related to the amount of amplified target in each PCR cycle. DNA binding dyes allow labeling of probes with different reporter dyes to detect more than one target in a single reaction (multiplex qPCR).

The value of qPCR has been demonstrated in several studies. A study in elderly subjects, using the SYBR green qPCR approach, showed decreased bifidobacterial numbers in patients treated with antibiotics compared to healthy and non-treated patients (8). The primer probe based technique has successfully been applied to fecal samples to measure bifidobacterial numbers and can also differentiate between several bifidobacterial species. *Bifidobacterium* genus specific primers targeting infant fecal samples showed decreased numbers in infants born through caesarean section as well as in infants using antibiotics, on

the contrary infants with older siblings had slightly higher numbers of bifidobacteria with a sensitivity of $10–10^6$ cfu/ml (164). Another study targeting the *Bifidobacterium* genus showed high specificity and reproducibility and a detection limit of 5×10^4 cfu/ml (75). Multiplex qPCR, targeting *B. adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. dentium*, *B. infantis*, and *B. longum* as well as the total number of bifidobacterial species, have been used in a study which compared infants that were breast-fed, formula-fed and a formula supplemented with galacto- and fructo-oligosaccharides. The amount and diversity of bifidobacteria in the infants' feces receiving the supplemented formula was higher than the standard formula-fed infants and mimicked that of the breast-fed infants. The dynamic range of the study was $10^4–10^9$ cfu/ml and all results were comparable to FISH (76). qPCR targeting the bifidobacterial transaldolase gene instead of the 16S rRNA gene did not always give reproducible results (176). Common primer and probe combinations are listed in Table 2. It should be the taken into account that bacterial quantification by qPCR can be influenced by differences in the number of rRNA operons between the quantified species or groups, sequence heterogeneity between different operons of the same species, and differential amplification of different DNA molecules. Nonetheless, this technique is likely to become more popular in quantifying microbiota numbers owing to its high throughput nature and relatively ease to perform and it is also less expensive compared to the purchase of, for example, a flow cytometer.

In situ activity of bifidobacteria

Molecular identification techniques such as PCR are based on DNA extractions which do not differentiate between live, dead or inactive (dormant or injured) microorganisms within the ecosystem. To study the activity and function of the members of the intestinal microbiota their identity needs to be linked to the role that the different organisms play in the host. Although a percentage of the gut microbes can be studied *in vitro*, physiological characterization cannot be directly linked to their function *in situ*. Laboratory conditions cannot represent the intestinal conditions, and organisms behave differently in pure culture compared to the complex ecosystem that resides inside the gut. There are several approaches being used to study the activity of the microbial gut inhabitants, and their potential for assessing bifidobacteria activity is discussed.

Quantitative hybridization with fluorescent rRNA probes (as in FISH) is a useful indicator of activity as there is a correlation between the growth rate, which is coupled to efficient protein synthesis, and the number of ribosomes. The FISH technique has been used to estimate growth rates of *E. coli* cells colonizing the intestinal tract of mice (174). *In situ* activity of pure cultures of the human commensal *L. plantarum* has been measured by correlating the rRNA, as determined by fluorescent intensity, with the cell growth rate (233). However, at the very high cell densities which are a typical property of *L. plantarum* at late

stages of growth, changes in the cell envelope appeared to prevent effective entry of the fluorescent probe into the cells. This has yet to be ascertained for bifidobacteria. Permeabilization issues may preclude application of this technique to certain microbes in complex environments like the intestine. Furthermore, recent data suggest that cellular ribosome content is not always an indicator of physiological activity. Apparently some bacterial cells might be highly active but possess a low ribosome content (165), while other bacterial types possess high RNA even after extended starvation periods (143).

The metabolic activity of microbes from complex systems like the intestine may be studied using a variety of physiological fluorescent probes together with fluorescent microscopy or flow cytometry. Together with cell sorting using the flow cytometer (FCM) viability of cells can be measured very rapidly in environmental samples (11). Ability to grow in medium is the current standard to recover viable cells, but in many instances suitable medium is not established for gut microbes, and it is further recognized that some cells enter a non-culturable state although still exhibit metabolic activity. The criteria by which viability can be evaluated by the physiological probes include membrane permeability or integrity, enzyme activity, and/or maintenance of a membrane-potential. The most common dye used for assessment of viability is carboxy-fluorescein diacetate, a non-fluorescent precursor that diffuses across the cell membrane, but is retained only in viable cells with intact membranes which convert it into a membrane impermeant fluorescent dye by non-specific esterases of active cells. Another probe is propidium iodide, a nucleic acid dye, which is excluded by viable cells, but enters cells with damage membranes and binds to their DNA and RNA. Simultaneous staining of fecal *Bifidobacterium* species with both probes was used to assess their viability during bile salt stress (10). Subsequent detection and cell sorting revealed three populations representing viable, injured and dead cells, whereby a significant portion (40%) of the injured cells could be cultured. This approach highlights the importance of multi-parametric FCM as a powerful technique to monitor physiological heterogeneity within populations at the single cell level. In a further study the probes were used to determine activity of human adult fecal microbes (11). Subsequent bifidobacterial-specific PCR-DGGE analysis of sorted fractions, and identification by cloning and sequencing of the 16S rRNA genes revealed bifidobacterial populations with sequences with low similarity to characterized species in the database. This suggests the potential of as yet uncultured novel bifidobacterial species inhabiting the human intestine (11, 222). Certain species such as *B. longum* and *B. infantis* were retrieved from all sorted fractions, while *B. adolescentis* was mostly recovered from the sorted dead fraction. Also quantitative hybridization with fluorescent rRNA probes combined with real time quantitative PCR targeting 16S ribosomal genes could provide insight in activity of bifidobacterial species in the human intestine by comparison of DNA and RNA patterns.

Another technique to link taxonomic identity to activity and function in microbial communities is microautoradiography (MAR) which determines the uptake of specific

radiochemicals by individual cells (114, 149). In combination with FISH, MAR-FISH allows monitoring of the radio-labeled substrate uptake patterns of the probe identified organisms under different environmental conditions (90). The drawback of this technique is that only seven different probes can be used in a single experiment, keeping in mind that natural microbial communities can comprise thousands of species. And, as mentioned above, not all species are accessible to FISH probes. Recently MAR-FISH was extended by rendering it quantitative and by combining it with other approaches including microelectrode measurements or stable isotope probing (Wagner et al., 2006). These approaches, when applied to the intestinal microbes, have the potential to identify those members involved in metabolism of specific dietary compounds. Upon addition of [13C] glucose to an *in vitro* model of the human colon, labeled RNA from glucose-consuming bacteria was detected after only 1 h of incubation, and molecular analysis of 16S rRNA genes indicated detectable differences in glucose use by the bacteria present (52).

Functional genomics

Upon arrival of the post-genomic era, the focus of studies on gut microbiota and its members has shifted from composition and activity to include the study of the functional products of gene expression. Using emerging techniques, such as transcriptomics and proteomics, the molecular activity of the microbiota can be studied *in situ* on different levels.

To capture the immediate, ongoing and genome-wide response of organisms to the environment, microarrays are the method of choice. A nice example is provided by microarray analysis of *B. longum* NCC2750 which revealed the mechanisms underlying preferential use of glucose over lactose as a carbon and energy source. This is achieved by down-regulation of glcP (putative glucose transporter gene) expression in a lactose-dependent manner, thereby shifting the balance of uptake and metabolism between glucose and lactose (162). The transcriptome of *B. longum* NCC2705 has also been studied in relation to stress response using microarrays and several protein coding sequences potentially involved in oxidative stress defence mechanisms were identified as well as several different regulatory mechanisms by which the cells protect themselves from various stresses (101). Such studies may be used to devise novel ways of protecting the bifidobacterial cultures during manufacture and storage and further upon gastrointestinal transit.

As explained in the section ‘Taxonomy and typing of bifidobacteria colonizing the human intestine’, even microbes for which no genome sequence is yet available can be studied using so called smartman arrays, although a complete genome sequence of a related microorganisms will substantially facilitate interpretation of the data. Thus it is now feasible to study the transcriptome of the bifidobacterial community within the intestinal microbiota using bifidobacterial microarrays (25). Using well-established methods good quality total RNA can be isolated from fecal samples (254). Total RNA extracted from the microbiota in

breast-fed infant fecal samples was hybridized to a microarray containing clone libraries of several bifidobacterial species spotted on glass slides (Chapter 4 and 5, this thesis). Positive hybridization was observed to clones with insert sequences that showed high similarity to genes with a range of functions. Particularly interesting functions were carbohydrate metabolism and transporters, and vitamin metabolism. The annotated functions of the genes on the inserts could be related to the influence of the diet on the activity of the bifidobacterial community within the fecal microbiota.

To complement transcriptomics the functional complement of the genome can be studied using a proteomics approach. Figure 4 shows a picture of the proteome of *B. breve* visualized using two dimensional gel electrophoresis. Large-scale characterization of the entire protein complement of environmental microbiota at a given point in time is designated metaproteomics (243). So far there are two reports which illustrate the feasibility of metaproteomics to characterize complex bacterial ecosystems. One was a model sludge ecosystem which revealed several protein sequences using two dimensional gel electrophoresis and quadrupole time of flight mass spectrometry (Q-ToF MS) (243). The second study applied a combination of community genomics and proteomics in a low complex natural microbial biofilm and disclosed a substantial percentage of all the proteins encoded by the genome of the five most abundant organisms (173). This example illustrates the feasibility of this approach in analyzing complex ecosystems. Metaproteomics can also be applied to the intestinal microbial ecosystem and the first characterization of the fecal metaproteome of newborns, with high fecal bifidobacterial content, indicated the presence of a bifidobacterial enzyme (99). This indicates *in situ* activity of bifidobacterial species. Although it is still very challenging to approach the enormous proteome, as we are limited by the resolution of the technical properties of the proteomics techniques, more studies are underway.

The number of microorganisms that colonize the human epidermal and mucosal surfaces is estimated at 10^{13}–10^{14}. This exceeds our population of human cells by a factor of 10 and in particular the colonic ecosystem is one of the most dense microbial communities on earth. This community is estimated to comprise 1000 species (172) and may contain more than hundred times as many genes as the human genome. Many of these microbes rely on the human host for survival which offers a relatively non-hostile environment and supply of nutrients that is produced and consumed by the host and the microbes. However, considering the size of the microbiome, i.e. the sum of the microbial genomes, the host also depends on this co-evolving microbial gut community to a certain extent. Sequencing of the microbiome is a logical extension of the human genome project. Currently, 2000 partial or complete microbial 16S rRNA gene sequences of intestinal source are present in the database which is very useful for identification of species. The next step is to link the intestinal microbiota to functional insight in the total microbial genome and encoded functional attributes.

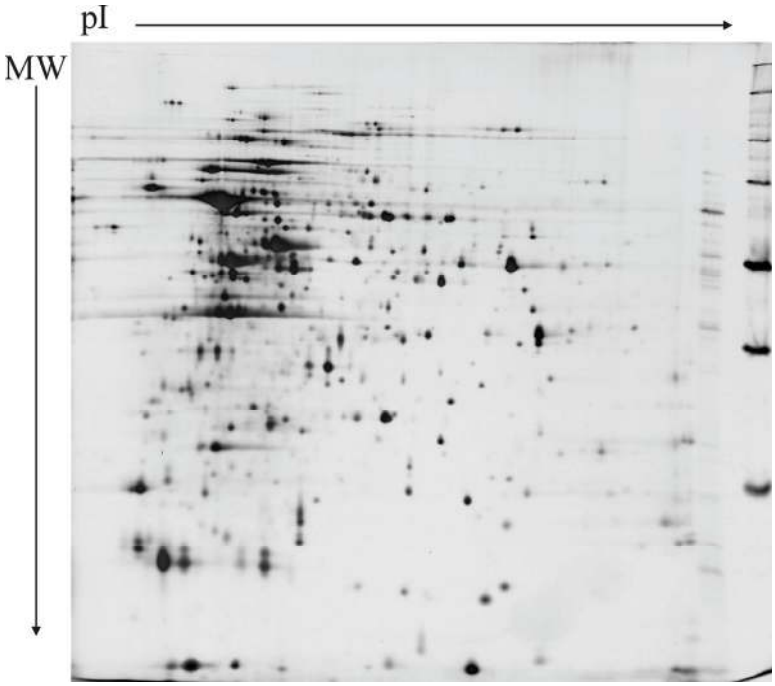

Figure 4 Silver stained 2D gel of *B. breve* grown in MRS using IPG pH 4- 7 and 12% acrylamide SDS PAGE gel.

Currently poor database matches due to insufficient genome sequence information of the human intestinal microbes confound identification of the proteins. However, the ongoing construction of metagenomic libraries as explained below and sequence analysis of the human intestinal microbiota will enable meaningful identification in time (69, 73, 115).

Recent studies showed the potential of functional screening of metagenomics of the human intestinal microbiota. In the majority of the studies clone libraries represented 16S rRNA genes to study the diversity of the target samples (28, 51, 63, 124). A BAC library was constructed from the mouse colonic microbiota, which was functionally screened for ß-endoglucanases (235). Clones with ß-glucanase activity as well as other putative genes were detected originating from probably uncultivated bacteria and *Bacteroides* species. Several efforts are underway to sequence the microbiome (47, 252). Sequencing of an extensive metagenomic library of the human distal gut revealed a high diversity in bifidobacterial genes when compared to the *B. longum* NCC2705 genome (69). This suggests that the sequences in the metagenomic library were not derived from a single discrete strain, but instead, reflect the presence of multiple strains, as well as other *Bifidobacterium* phyloptypes in the gut microbiota of the studied individuals. Although the metagenome is not directly linked to specific species but rather to the complete microbiome, it is a start to define the gene content and encoded functional attributes. Eventually, study of the actual activity of all annotated genes within the human microbial microbiome needs to be measured and linked to specific groups of healthy or diseased individuals or special diets.

Future trends

Understanding the mechanisms by which bifidobacteria affect the host is essential to support their possible role in functional foods. This requires more than the insight we have accumulated so far on the diversity, survival and general activity of bifidobacteria. At least three complementary lines of research need to be further developed. These include the development of (i) functional genomics to determine the global activity of bifidobacteria, (ii) genetic tools to allow for testing hypotheses of the bifidobacterial function, and (iii) models and real life studies that show the impact of bifidobacteria on the host and its intestinal microbiota.

Table 3 Plasmids found in bifidobacterial species.

strain	Accession number	plasmid	Size (nt)	reference
B. longum				
BK51	NC_006843	pTB6	3,624	(214)
RW048	NC_004770	pNAC1	3,538	(37)
RW041	NC_004769	PNAC2	3,684	(37)
RW041	NC_004768	pNAC3	10,224	(37)
DJO10A	NC_004253	pDOJH10S	3,661	University of Minnesota, USA
DJO10A	NC_004252	pDOJH10L	10,073	University of Minnesota, USA
KJ	NC_002635	pKJ36	3,625	Food Science and Technology, Korea
KJ	NC_004978	pKJ50	4,960	(163)
NCC2705	NC_004943	pBLO1	3,626	(197)
	NC_006997	pMG1	3,682	Research Center, BIFIDO Co., Korea
B2577	X84655	pMB1	1,847	(180)
B78	DQ452864	pBG2.2	2,197	Institute of Food Research, UK
B. breve				
NCFB 2258	NC_002133	pCIBb1	5,750	(153)
VMKB44	NC_004443	pB44	3,624	University of Minnesota, USA
B. pseudocatenulatum				
VMKB4M	NC_003527	p4M	4,488	University of Minnesota, USA
B. catenulatum				
L48	NC_007068	pBC1	2,540	IPLA-CSIC, Spain
B. asteroides				
DSM 20089	Y11549	pAP1	2,140	Swiss Federal Institute of Technology, Switserland

Genetic tools for bifidobacteria are scarce especially in comparison to many other microbes of food industry interest. While plasmids are found in some bifidobacteria they are usually small in size (200). Hence, a dozen have been sequenced (see Table 3) and some have been used to construct plasmid vectors such as pMB1 (180) which led to a first generation of cloning vectors (136). However, efficient gene transfer systems seem to be the bottleneck for further advancing the genetics of bifidobacteria and this is a serious limitation for further genetic approaches aiming at establishing cause-effect relations between genes and function.

Functional genomics approaches are developing rapidly as discussed above. However, insight into the global expression of bifidobacteria is only starting to develop. The public availability of complete *Bifidobacterium* genomes together with the metagenomic analysis of uncultured bifidobacteria will form the basis of advanced multi-species functional genomics approaches that address the transcriptome, proteome and metabolome of all members of this important group of intestinal bacteria. Included in this analysis is the use of Stable Isotope Probing (see above) that has recently been applied to notably the colonic microbiota and may establish the link between prebiotic substrates and bifidobacterial function.

Finally, the impact of bifidobacteria on the host and its associated microbes is an important area for future studies. Notably the presumed interactions with the immune and neuroimmune systems are highly relevant as are other epithelial interactions with the host. This should not only be studied in adults but especially in neonates and this requires either novel or non-invasive methods as well as useful animal and other alternative models. The use of cell line models to determine interactions between bifidobacteria and their host has recently started and may provide one of such alternative models.

In conclusion, a sound framework for understanding the role of bifidobacteria in health and development has been realized – however, a continuing interest of academic researchers and industry scientists is needed to further develop bifidobacteria beyond this level and harvest the fruits of these bacteria that have been close to our heart since the beginning of our life!

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2

Metaproteomics Approach to Study the Functionality of the Microbiota in the Human Infant Gastrointestinal Tract

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A metaproteomics approach comprising two-dimensional gel electrophoresis and matrix-assisted laserdesorption ionization–time of flight (mass spectrometry) was applied to the largely uncultured infant fecal microbiota for the first time. The fecal microbial metaproteome profiles changed over time, and one protein spot contained a peptide sequence that showed high similarity to those of bifidobacterial transaldolases.

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Introduction

The human gastrointestinal tract is rapidly colonized during the first days of life by microbes (57, 122) which ultimately contribute significantly to host nutrition and immunity among other beneficial effects (5) (86). Analysis of 16S ribosomal DNA clone libraries revealed numerous undescribed species in intestinal samples, emphasizing the importance of techniques that bypass cultivation of microbiota (51, 57, 190, 208, 249) such as proteomics. So far there have been two reports of the use of metaproteomics to characterize complex bacterial ecosystems, illustrating the feasibility of this approach in analyzing complex ecosystems (173, 243). In the present study we investigated the potential use of metaproteomics for characterization of the human fecal microbiota.

Dynamics of the intestinal microbiota

The relatively simple infant fecal ecosystem was chosen for this study as it is dominated by bifidobacteria, which were used in this study to optimize the protein isolation and two-dimensional (2D) gel procedures (data not shown). Informed consent was obtained from parents for use of the fecal samples of their infants. Infant fecal samples containing high bifidobacterial content were selected by *Bifidobacterium*-specific fluorescent in situ hybridization (62, 251). Infants A (8 days old) and B (117 days old) harbored 45% and 63% bifidobacteria in the total microbiota, respectively. PCR-denaturing gradient gel electrophoresis (DGGE) (189, 250, 253) of the 16S rRNA gene was performed to monitor the total bacterial community (Fig. 1). The profiles were relatively simple (Fig. 1) (57), as expected, and the predominance of bifidobacteria was supported by the presence of abundant 16S rRNA gene amplicons that comigrated with the control *Bifidobacterium longum* strain and was confirmed by sequencing (189).

Metaproteome production of infant fecal microbiota

Fecal samples were collected from the infants prior to weaning: for infant A, at days 8, 24, and 41, and for infant B, at days 103, 117, and 144. Microbial cells were released from the feces and washed as previously described (61) (251). The bead beating method was confirmed to be applicable for protein extraction of infant fecal microbiota containing bifidobacteria (data not shown), as expected from previous systematic studies (61, 189, 249, 253, 254). Total soluble protein was obtained by three treatments of 45 s of bead beating (FastPrep; Qbiogene), interspersed by 1 min on ice, in 500 μl isoelectric focusing (IEF) buffer (Fluka, Switzerland) (10 M urea) containing 2% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) (Roche, Switzerland), 0.65 mM dithiothreitol (DTT)

(Sigma, Switzerland), 0.2% Biolytes 3/10 (Bio-Rad), Pefabloc Sc (Fluka), and glass beads (Sigma) (≤0.1 mm).

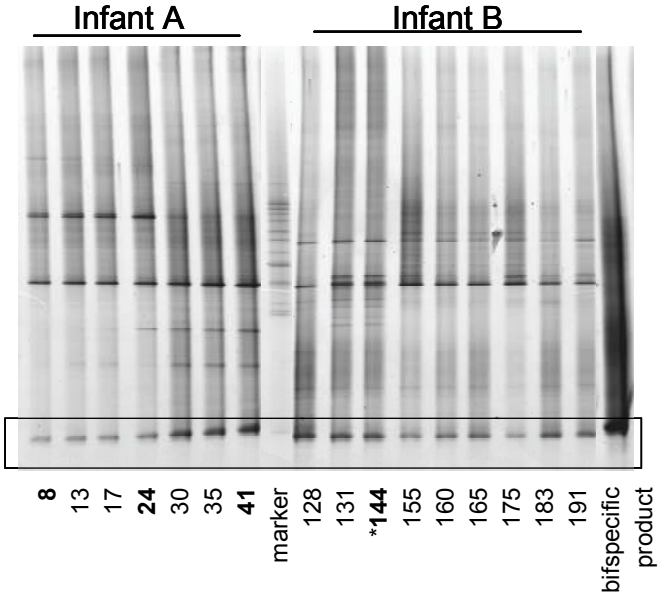

Figure 1 PCR-DGGE profiles of regions V8 to V6 of 16S rRNA genes of fecal samples from two infants at different ages (infant A, 8 to 41 days; infant B, 128 to 191 days), reflecting the predominant bacterial community. The bands in the box represent amplicons of bifidobacterial species, including *Bifidobacterium longum* DSM 20219. *, start of weaning period for infant A. Sampling days for protein extraction are shown in bold. marker, mix of DNA size standards; bifspecific product, amplicon of 16S rRNA gene of *B. longum* DSM 20219.

Protein concentrations were determined using a 2-D Quant kit (Amersham Biosciences). Each protein sample (100 µg) was loaded onto 11 cm immobilized pH gradient (IPG) ReadyStrips (Bio-Rad) (pH 4 to 7) and rehydrated for 12 h. IEF was carried out for 98,000 Vh (Protean IEF; Bio-Rad). Reduction and alkylation of proteins were performed prior to electrophoresis in the second dimension by incubating for 10 min in 6 M Urea–0.1 M Tris-HCl (pH 8.8) – 2% (wt/vol) sodium dodecyl sulfate (SDS) with 130 mM DTT followed by a 10 min incubation in the presence of 216 mM iodoacetamide (instead of DTT). The IPG strip was positioned on an SDS-polyacrylamide electrophoresis gel (Criterion gel, Bio-Rad) (12.5% polyacrylamide) with 1% low melting agarose in 40 mMTris-HCl (pH 6.8). Electrophoresis was run at 100 V and 30 W. Silver staining was performed as described previously (201). Protein maps were scanned with a GS-800 densitometer (Bio-Rad) and analyzed with PDQuest software (Bio-Rad). Triplicate 2D gels for each sample were grouped. For each infant, three groups, each representing one time point in life, were compared using the quantitative function within PDQuest software. The mean coefficient of variation (CV) (standard deviation/ mean x 100) is a quantitative index for variation of

quantities among matched spots and was computed for gel-to-gel variations within each replicate group. More than 200 protein spots were visualized on each gel. A comparison of gels of infant A revealed changes in number and intensities of protein spots during the 33 days, although the patterns remained similar (Fig. 2A to C). In order to clearly demonstrate the changes a section of the gel corresponding to each time point was enlarged (Fig. 3). Comparisons of differential protein production values with percent CV values for various time points for the marked spots are illustrated with bar graphs (Fig. 3C). The metaproteome profiles of infant A (Fig. 2A to C) were different from those of infant B (see, e.g., Fig. 2D) in accordance with the uniqueness of each individual's microbiota (20), as was also observed in the PCR-DGGE profiles obtained as described above.

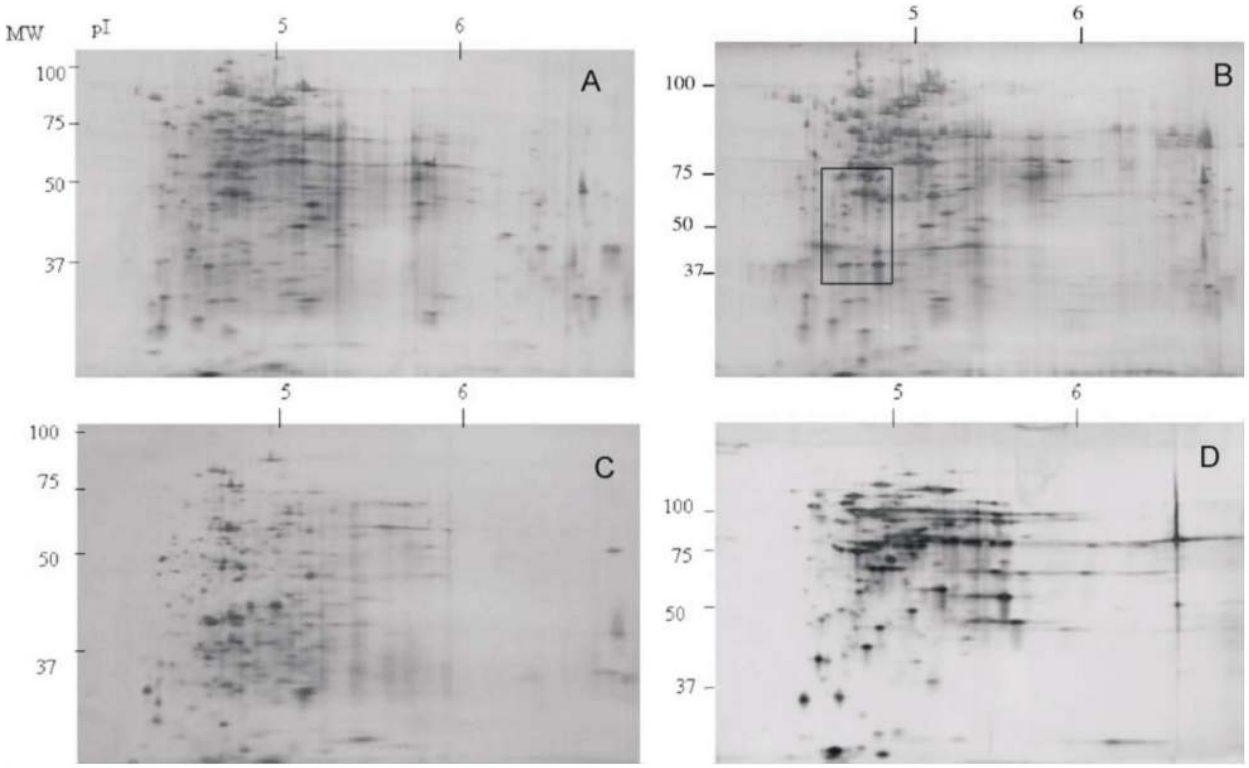

Figure 2 Silver-stained 2D gels of fecal samples from infant A at the ages of 8 days (A), 24 days (B), and 41 days (C) and from infant B at 117 days (D); data were obtained using IPG (pH 4 to 7). MW, molecular weight.

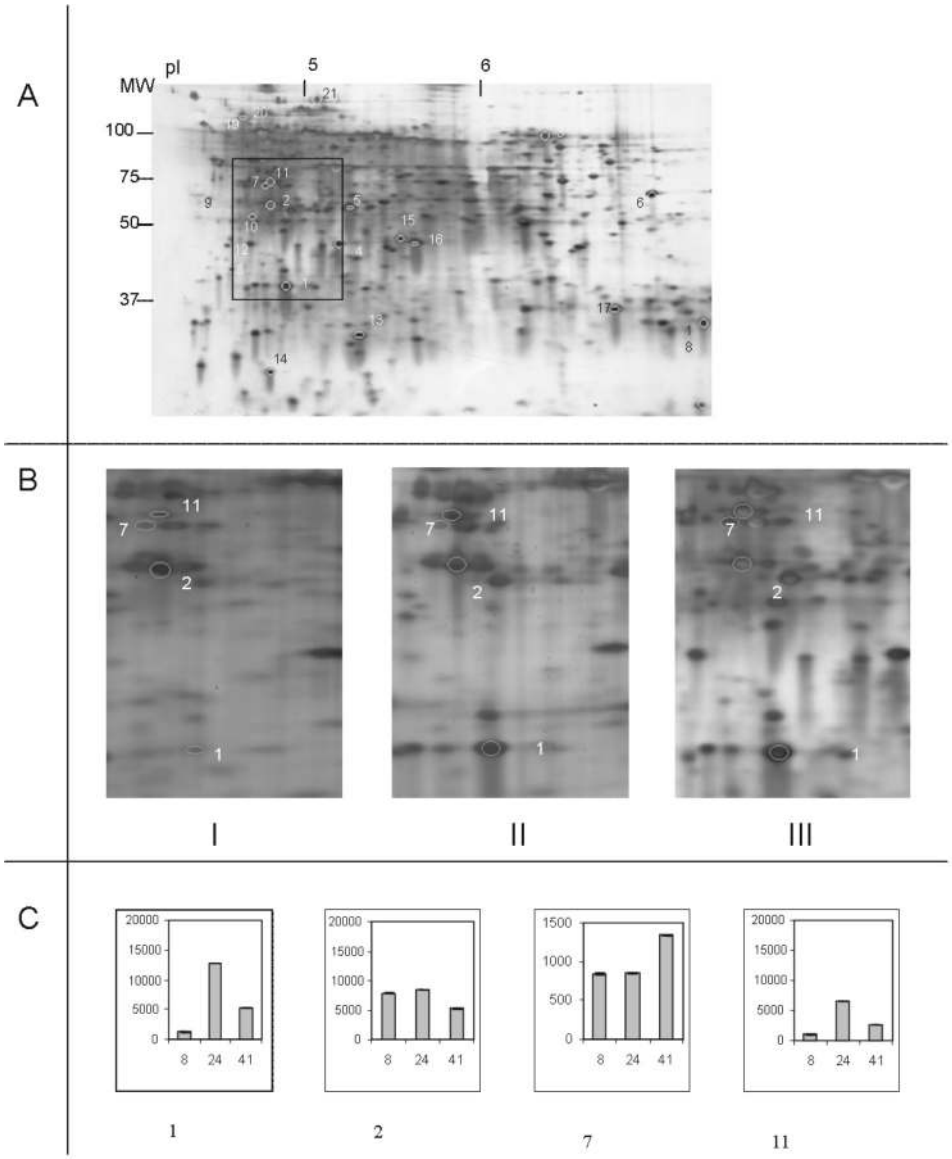

Figure 3 (A) Silver-stained 2D gel of a fecal sample from infant A at the age of 41 days obtained using IPG (pH 4 to 7). The circled protein spots were excised, and mass fingerprints were obtained by MALDI-TOF (MS). Table 1 shows the quantity of the selected protein spots. MW, molecular weight. (B) Areas of interest enclosed in the boxes are enlarged from gels of samples from infant A at the ages of 8 (I), 24 (II), and 41 (III) days. The circled protein spots (1, 2, 7, and 11) gave good peptide sequencing results with MALDI-TOF (MS). (C) The diagrams demonstrate changes in intensity of the protein spots (1, 2, 7, and 11) (see panel BI) with percent CV values at ages 8, 24, and 41 days.

Table 1 Mean quantity of selected protein spots over time with% CV and experimental pI and MW values

Protein quantity changecategory and protein spot no.	Mean protein quantity in ppm per spot area (% CV) for infant A at age:			Expt value	
	8 days	24 days	41 days	MW	pI
Increasing protein					
1a	1.263 (65)	12.780 (18)	6.988 (41)	39	4,8
2a	5.534 (141)	8.415 (0)	5.240 (52)	50	4,8
3	ND	8.221 (38)	3.521 (1)	88	6,0
4	2.344 (12)	5.135 (2)	4.060 (35)	45	5,2
5	11.283 (20)	8.266 (19)	3.480 (0)	50	5,2
6	S	10.547 (18)	7.755 (8)	55	6,5
Limited change					
7a	648 (55)	705 (37)	1.340 (3)	65	4,7
8	2.308 (21)	1.363 (25)	2.093 (37)	41	4,6
9	1.631 (15)	837 (22)	804 (17)	60	4,4
10	1.415 (28)	4.207 (59)	3.817 (1)	46	4,6
11a	1.044 (10)	786 (2)	1.007 (5)	70	4,8
12	387 (15)	290 (23)	351 (23)	42	4,6
Decreasing					
13	2.722 (1)	3.472 (0)	7.533 (14)	37	5,2
14	2.399 (21)	3.902 (10)	7.339 (41)	28	4,8
15	ND	1.571 (3)	4.974 (22)	47	5,3
16	2.148 (27)	2.899 (24)	6.472 (30)	46	5,4
17	ND	3.545 (23)	10.520 (3)	37	6,3
18	2.844 (10)	2.652 (23)	9.896 (13)	35	6,8
19	ND	ND	925 (23)	90	4,5
20	1.711 (6)	1.474 (1)	2.952 (15)	95	4,6
21	S	4.661 (18)	4.685 (3)	100	5,0

a Protein spots selected
ND, not detectable.
S, saturated.

Identification of proteins

A total of 55 protein spots were excised from the infant A 2D gel at day 41 (Fig. 3A). Peptides were extracted after tryptic digestion and analyzed by matrixassisted laser desorption ionization–time of flight (mass spectrometry) [MALDI-TOF (MS)] (43) (Applied

Biosystems 4700 proteomics analyzer; Technology Facility, Department of Biology, The University of York, York, United Kingdom), which provided a catalogue of 21 good mass peptide fingerprints. Peptide sequences were searched using blastp, with the default settings of the "Search for short, nearly exact matches" function, as well as MS-Pattern in Protein Prospector 4.0.5 (The University of California). As expected, the peptides' mass spectra showed low similarity to those of database entries and no similarities to those of human proteins. The mean differential protein amounts of the 21 spots with clean signals for days 8, 24, and 41 are indicated in Figure 3A and presented in Table 1. MALDI-TOF (MS) of spots 1, 2, 7, and 11 was performed for *de novo* peptide sequencing (Fig. 3B), which resulted in determination of 11 N-terminal sequences that shared similarity to those of bacterial proteins, a viral protein, and four eukaryal proteins, but the collective sequences were not significantly similar to any previously reported. One complete peptide fragment (ELAEATDFVDGR) of protein spot 4 gave a result showing high-level database matches of 91% identity (i.e., one mismatch) with the *Bifidobacterium infantis* and *B. longum* NCC2705 transaldolases and 83% identity with the DJO10A transaldolase. Production for this protein spot was relatively high at day 8 but increased approximately 10-fold and 6-fold at days 24 and 41, respectively, possibly due to the increase in numbers and activity of bifidobacteria in the infants' microbiota, based on PCR-DGGE (Table 1 and Fig. 1). The data obtained for the digestion sites of trypsin and the theoretical pI (4.87) and molecular mass (39.6 kDa) of the *B. infantis* transaldolase protein agreed with the position of the protein spot in the 2D gel (Fig. 3A). This transaldolase gene is a common target for PCR used to detect and enumerate bifidobacteria (176). Transaldolase was identified in a proteomic study by Vitali *et al.* (231) of *B. infantis* B107, where it represented, together with nine other proteins, the most abundant portion of the proteome. The dominance of the (tentatively identified) bifidobacterial transaldolase protein in the feces of a newborn infant may explain its detection by a metaproteomics approach.

Perspectives

For the first time, reproducible 2D gels, extraction of proteins, and tentative identification using MALDI-TOF (MS) demonstrated the applicability of the proteomics approach for the complex intestinal ecosystem. Currently insufficient microbiome sequence information confounds identification of the proteins, but ongoing metagenomic library analysis will enable meaningful identification in time (69, 115, 124). Furthermore, peptide mass fingerprints from sequence data will be sufficient to produce more statistically valid database matches, as was recently demonstrated by Ram et al. (173), who matched 6,000 peptide fragments to DNA sequences of an accompanying metagenomic library from a low-complexity natural microbial biofilm. Metaproteomics approaches may become a useful tool

to monitor the functional products of the microbiota in feces over time as affected by dietary intervention, length life, health, and disease.

Nucleotide sequence accession numbers and peptide sequences

The bifidobacterial nucleotide sequences of partial 16S rRNA genes have been deposited in the GenBank database under accession no. DQ323457, DQ323458, and DQ323459; the 11 peptide sequences are DLAVALSENKR, ATNSEL MHVGVSR, ELAEATDFVDGR, and PSSKVGSGSSGA GALK (for spot 1), DVAPDLALMHTKLSR (for spot 2), ADNFEGDDR and TAFTGYETLR (for spot 7), and KTGP KLFAADEALK, HYGLASDALANGGCVDSVSDSPA, AP MVALSELER, and TANSELLEAELAR (for spot 11).

Acknowledgements

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3

Isolation of RNA from Bacterial Samples of the Human Gastrointestinal Tract

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The human gastrointestinal tract contains a complex microbial community that develops in time and space. The most widely used approaches to study microbial diversity and activity are all based on the analysis of nucleic acids, DNA, rRNA and mRNA. Here we present an RNA isolation protocol which is suitable for a wide variety of gastrointestinal tract samples, including biopsies with minute amounts of material. We describe the protocols for isolating DNA from gastrointestinal tract samples in another Nature Protocols article. The RNA isolation protocol is set-up in such a way that sampling can be performed outside the laboratory which offers possibilities for implementation in large intervention studies. The RNA isolation is based on mechanical disruption followed by isolation of nucleic acids using phenol:chloroform:isoamylalcohol extraction and removal of DNA. This protocol has been successfully used in our laboratory resulting in rRNA and mRNA of sufficient quality for microbial diversity and activity studies. Depending on the number of samples, the sample type, and the quenching procedure chosen, the whole procedure can be performed in 2.5 – 4 hours.

Nature protocols, 2006, 1: 954 - 959.

Introduction

Microbial ecosystems are found all over the world ranging from the bottom of the ocean to the stratosphere. Without these microbial ecosystems life on earth is not possible since they are involved in recycling the elements that are crucial for all life forms. Our gastrointestinal tract is inhabited by large numbers of microbes which collectively outnumber host cells by a factor of ten (191). The main function of the gastrointestinal tract is the conversion of food into easily absorbable and digestible components in which the microbial community converts the indigestible components into nutrients that can be taken up by the epithelium. The microbial community in the gastrointestinal tract consists of different groups of microbes, from which anaerobic bacteria are the most prominent. This community structure is host-specific and affected by the genotyope, and its diversity varies in time and in space, which makes the gastrointestinal tract a very complex ecosystem to study (255). Currently, the estimated number of bacterial species in the human gastrointestinal tract is more than 1,000. It is generally accepted that we cannot cultivate all microbes from the human gastrointestinal tract and therefore, culture independent nucleic acids-based approaches have been introduced to study the microbial diversity and activity. The first and most critical step in these approaches is a reliable isolation of nucleic acids from gastrointestinal tract samples. Especially the isolation of RNA is complicated, since it is easily degraded. RNA is present in different forms in the cell, including ribosomal RNA (rRNA), messenger RNA (mRNA) and transfer RNA (tRNA). When total RNA is isolated, 95-99% of the RNA consists of rRNA. Ribosomal RNA is present as 16S, 23S and 5S rRNA and these molecules form together with the ribosomal proteins the ribosomes. Since rRNA is present in every cell and has a low mutation and horizontal transfer rate, it has been used as a phylogenetic marker for detection, identification and quantification of uncultured microbes from a variety of ecosystems including the human gastrointestinal tract. Especially 16S rRNA and its corresponding gene have been used, and at present more than 200,000 sequences are available in the databases (34). Compared to rRNA, bacterial mRNA is unstable with in many cases a half-life of not longer than a few minutes. Since mRNA is the result of transcription and therefore, reflects the activity of the microbes, quenching of the sample and high quality RNA isolation is of utmost importance.

An extra complication with studying the human gastrointestinal tract is the fact that intestinal samples cannot always be processed freshly since sampling often occurs at home of the volunteers or in hospitals, especially when it concerns babies, volunteers without a colon, such as ileostomy patients or biopsy specimens from routine clinical procedures. This argues for proper preparation and storing methodologies which are easy to implement. In our laboratory we have developed and validated protocols for the isolation of RNA from intestinal samples which are ready-to-use for RT-PCR-based diversity analysis or transcript analysis. The procedure is based on the quenching of the samples in either Methanol-HEPES

buffer (adapted from Pieterse *et al*. (166)) or RNAlater (Ambion, 7020) followed by the isolation of RNA (adapted from Fitzsimons *et al.* (61)) and the removal of DNA via DNAse digestion I (adapted from the RNeasy mini kit, Qiagen, 74104). This protocol has been used successfully for faeces, ileostomy effluent and tissue samples (232), and the nucleic acids have served as targets for 16S rRNA and mRNA-based approaches, which argues strongly for their use in functional analysis of gastrointestinal tract communities. We believe that the use of these protocols is not limited to samples from the human gastrointestinal tract but that they can also be applied to samples from other microbial ecosystems.

Procedural comments

RNA is very sensitive to degradation by RNase. Therefore, we recommend to perform all steps at 0˚C or on ice and to have all equipment and reagents free of RNase. In addition, wearing gloves and working in a hood is highly recommended. Moreover, we recommend to buy RNase-free microfuge tubes and pipet filter tips. Glassware should be baked at 180°C for 4 hours prior to use. Reagents should be prepared with DEPC treated water. Last but not least, we recommend cleaning of pipettes and benches using RNase AWAY (Invitrogen 10328-11, www.invitrogen.com) prior to use.

For sampling and storage of intestinal material suitable for RNA isolation there are two protocols successfully used in our laboratory. One is based on RNAlater, whilst the other on a methanol-HEPES buffer. Both protocols are suitable for sampling outside the laboratory. The advantage, however, of the methanol-HEPES buffer is that the sample can also be used for isolation of metabolites (166).

Materials

Equipment

Centrifuge bottle 3141 (Nalgene Labware, www.nalgenelabware.com)
Corning Lambda single channel pipettors 4958-4964 (Corning Inc., www.corning.com)
Corning Pipet tips 4809 and 4810 (Corning Inc., www.corning.com)
Eppendorf centrifuge 5415R (Eppendorf, www.eppendorf.com)
FastPrep® FP220A, 6001-220 (MP Biomedicals, www.mpbio.com)
Glass beads 3 mm 290004 (Omnilabo Int., www.omnilabo.nl)
Gloves TT Clear (Maxxim Medical Europe, www.maxxim-europe.nl)
Microfuge tubes 1.5 ml 04-210-1100 (Nerbe Plus, www.nerbe-plus.de)
2.0 ml 623 201 (Greiner Bio. www.gbo.com)
Probe-based sonifier B12 (Branson Sonic Power Company, www.bransonultrasonics.com)
RNeasy mini kit 74104 (Qiagen Inc., www.qiagen.com)
Water-based sonifier Bransonic 32 (Branson Sonic Power Company)

Zirconia beads 11079101Z (Biospec products, www.biospec.com)

Reagents

- Acetic acid (glacial) (Riedel-de Haën, 27225, www.riedeldehaen.com) CAUTION, corrosive. Handle using appropriate safety equipment
- Acid Phenol, pH 3.75 (Invitrogen, 15594-039, www.invitrogen.com) CAUTION, phenol is toxic. Handle using appropriate safety equipment and measures.
- Chloroform (Riedel-de Haën, 24216, www.riedeldehaen.com) CAUTION, harmful. Handle using appropriate safety equipment
- DEPC (Sigma, D5758, www.sigmaaldrich.com) CAUTION, harmful. Handle using appropriate safety equipment
- Dry ice CAUTION Dry ice can cause severe cold burns. Handle using appropriate safety equipment.
- EDTA (Sigma, E5134, www.sigmaaldrich.com)
- 95% Ethanol (VWR BDH Prolabo, 20 824.365, uk.vwr.com/app/Home) CAUTION, ethanol is flammable. Handle using appropriate safety equipment and measures.
- Glycogen 5 mg/ml (Ambion, 9510, www.ambion.com)
- HCl (Riedel-de Haën, 30721, www.riedeldehaen.com) CAUTION, corrosive. Handle using appropriate safety equipment
- HEPES (Agros Organics, 172572500) CAUTION, harmful. Handle using appropriate safety equipment
- Ice
- Isoamylalcohol (Riedel-de Haën, 59085, www.riedeldehaen.com) CAUTION, harmful. Handle using appropriate safety equipment
- KCl (Riedel-de Haën, 31248, www.riedeldehaen.com)
- KH_2PO_4 (Riedel-de Haën, 30407, www.riedeldehaen.com)
- Macaloid (Kronos Titan GmbH, www.kronostio2.com)
- milliQ water
- Methanol (Riedel-de Haën, 32213, www.riedeldehaen.com) CAUTION, Methanol is flammable. Handle using appropriate safety equipment
- NaAc (Sigma, S7545, www.sigmaaldrich.com)
- NaCl (Riedel-de Haën, 31434, www.riedeldehaen.com)
- $Na_2HPO_4 \cdot 2H_2O$ (Riedel-de Haën, 04272, www.riedeldehaen.com)
- RNAlater (Ambion, 7020, www.ambion.com)
- SDS (Sigma, L4390, www.sigmaaldrich.com) CAUTION, harmful. Handle using appropriate safety equipment
- Tris (Sigma, T1378, www.sigmaaldrich.com)

Reagent setup

Chloroform:isoamylalcohol.

Mix Chlororform and Isoamylalcohol in 24:1 volume ratio.

CAUTION, Both choloroform and isoamylalcohol are harmful. Handle using appropriate safety equipment and measures.

DEPC-treated water

1) Pipette 0,5 ml of DEPC (CAUTION, DEPC is harmful. Handle using appropriate safety equipment and measures.) to 500 ml of milliQ water
2) Incubate overnight at 37°C
3) Autoclave at 121°C for 20 minutes

This water can be used to prepare all water-based reagents.

Ethanol 70%.

Mix 95% Ethanol and milliQ water at 7:3 volume ratio and store at -20 ºC.

CAUTION, Ethanol is flammable. Handle using appropriate safety equipment and measures

Macaloid (modified from Shaffner (196))

1) Mix 5 g macaloid with 50 ml Tris-HCl (50 mM, pH 7.6)
2) Incubate at 100˚C for 5 minutes
3) Spin at 2,500 × g at room temp for 5 minutes
4) Remove supernatant and resuspend in 40 ml Tris-HCl
5) Repeat steps 3 and 4 two times
6) Treat sample with a probe-based sonifier for 1minute
7) Spin at 3,500 × g at room temp for15 minutes
8) Resuspend pellet in 30 ml Tris-HCl
9) Store at 4˚C

Result: ~17 mg/ml macaloid

Methanol-HEPES buffer (adapted from Pieterse *et al*. (166)

1) Dissolve 1.59 g HEPES (CAUTION, harmful) into 40 ml of water in a centrifuge bottle
2) Adjust pH to 6.5
3) Pipette 60 ml 100% methanol (CAUTION, flammable)
4) Store at -80°C or transport outside the laboratory on dry ice

3M NaAc

1) Dissolve 408.1 g of $NaAc \cdot 3H_2O$ in 800 ml milliQ water
2) Adjust pH to 5.2 with glacial acetic acid
3) Add milliQ water until total volume is 1.0 L

1 X PBS

1) Dissolve per liter milliQ water: 8.0 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4 \cdot 2H_2O$, and 0.24 g KH_2PO_4.
2) Adjust to pH 7.4

10%SDS

1) Dissolve 100 g SDS in 800 ml milliQ water CAUTION, SDS is harmful. Handle using appropriate safety equipment and measures.
2) Heat to 68 °C to enhance dissolving SDS
3) Adjust to pH 7.4
4) Add milliQ water until total volume reaches 1.0 L.

TE

Make solution consisting of 10 mM Tris-HCl pH 7.6 and 1 mM EDTA (pH 8.0)

Tris-HCL

1) Dissolve 121.1 g Tris in 800 ml milliQ water
2) Adjust to pH 7.6 with HCl CAUTION, corrosive. Handle using appropriate safety equipment and measures
3) Add milliQ water until a volume of 1.0 L is reached.
4) Use this solution for preparation of all Tris-HCl based solutions

Procedure

1) Obtain appropriate samples as detailed below

Sample	**Amount required**
Faeces from adults	15 g
Faeces from babies	as much as possible
Ileostomy effluent	150 g
Biopsy specimens	as much as possible

CRITICAL STEP These sample sizes are minimum sample sizes required.

Troubleshooting

2) As stated above, two protocols are described here, one that uses RNALater and one that uses Methnol-HEPES solution.

a. RNALater
   i. Add to each gram of intestinal material 1 gram of RNAlater to tube
   ii. Shake thoroughly to get a suspension

   CRITICAL STEP

   This is very important to ensure that all cellular processes are stopped. Especially fecal samples may remain in clumps which should be avoided

   iii. Store overnight at 4°C
   iv. Transport sample on ice to the laboratory in cool box

PAUSE POINT

Samples can be stored at -20°C for several weeks.

CRITICAL STEP

Although samples can be stored for a long time at -20°C, we recommend processing the samples as fast as possible. Longer storage may result in differential lysis of microbial cells.

v. Pipette 1 ml ice-cold 1 × PBS to each gram of RNAlater used
vi. Centrifuge at 700× g for 1 minute.
vii. Remove supernatant

b. Methanol and HEPES

i. Add one volume of intestinal sample to 4 volumes of methanol-HEPES buffer

CRITICAL STEP.

Samples added to methanol-HEPES buffer should become and kept below -15 degrees as fast as possible to prevent cellular processes to occur after sampling.

ii. Shake thoroughly until sample is homogenised
iii. Keep sample on dry ice

CAUTION Dry ice can cause severe cold burns. Handle using appropriate safety equipment.

PAUSE POINT

Samples can be stored for 1-2 days on dry ice.

iv. Transport sample to the laboratory on dry ice

PAUSE POINT

Samples can be stored at -80°C for several days

CRITICAL STEP

Although samples can be stored for a long time at -80°C, we recommend processing the samples as fast as possible. Longer storage may result in differential lysis of microbial cells.

v. Add 10-20 glass beads to sample
vi. Treat sample in a water-based sonifier at 40 kHz for 10 minutes

CRITICAL STEP

Water in the sonifier should be ice-cold during the treatment.

vii. Centrifuge at 300 × g for 5 minutes
viii. Remove supernatant

TROUBLE SHOOTING

RNA isolation

1) Resuspend the pellet into 0.5 ml ice-cold TE
2) Pipette suspension to a microfuge tube containing 0.18 g macaloid suspension, 0.8 g zirconia beads, and 50 µl 10% SDS
3) Pipette 500 µl acid phenol (cold 4°C; pH 3.75) (CAUTION, phenol is toxic. Handle using appropriate safety equipment and measures)
4) Treat sample in a FastPrep at speed 5.5 for 45 seconds (for biopsy specimen 1.5 minutes)
5) Cool on ice for 90 seconds
6) Repeat steps 6 and 7 two times (for biopsy specimen five times)
7) Centrifuge sample at 13,400 × g in a microcentrifuge at 4ºC for 15 minutes
8) Transfer the aqueous phase into a new microfuge tube
9) Pipette 250 µl acid phenol and 250 µl chloroform:isoamylalcohol (24:1) CAUTION both chloroform and isoamylalcohol are harmful. Handle using appropriate safety equipment and measures.
10) Shake vigorously for a few seconds
11) Centrifuge sample at 13,400 × g in a microcentrifuge at 4ºC for 5 minutes
12) Transfer the aqueous phase into a microfuge tube
13) Repeat steps 11 to 14 until a clear interface is obtained
14) Pipette 500 µl chloroform: isoamylalcohol (24:1)
15) Shake vigorously and centrifuge sample at 13,400 xg in a microcentrifuge at 4ºC for 5 minutes
16) a) Transfer upper layer into a 2 ml microfuge tube and continue with step 19)
    b) Alternatively (for optional PAUSE POINT)
        i. Transfer upper layer into a 2 ml microfuge tube and pipette 1/10 volume 3M NaAc (pH 5.2), 1/100 volume of glycogen (optional) and 3 volumes of 95% ethanol of -20ºC (CAUTION, flammable) to sample
    ii. Store at –80ºC for at least 1 hour
    PAUSE POINT
    iii. Centrifuge at 13,400 × g for 15 minutes
    iv. Discard supernatant and wash pellet with 70% ethanol of -20ºC (CAUTION, flammable)
    v. Centrifuge at 13,400 × g for 5 minutes
    vi. Dry pellet at room temperature
    vii. Resuspend pellet in 100 µl TE

DNAse digestion I

1) Pipette to each 100 µl of sample 350µl RLT buffer and mix by pipetting 5 times up and down
2) Add 250 µl 95% ethanol and mix by pipetting
3) Apply sample in 500 µl portions to RNAeasy minicolumn, centrifuge at 9,300 × g for 15 seconds and repeat this until all portions are applied
4) Replace the collection tube
5) Pipette 350 µl RW1 buffer to column and centrifuge at 9,300 × g for 15 seconds
6) Discard flow through
7) Pipette 10 µl DNAse I solution to 10 µl DNAse buffer and 60 µl RNAse free water
8) Mix gently and pipette this solution to middle of column
9) Incubate at room temperature for 15 minutes
10) Pipette 350 µl RW1 buffer to column
11) Centrifuge at 9,300 × g for 15 seconds
12) Discard flow through
13) Pipette 500 µl RPE to colum
14) Centrifuge at 9,300 × g for 15 seconds
15) Discard flow through
16) Pipette 500 µl RPE to column
17) Centrifuge at 9,300 × g for 2 minutes
18) Discard flow through. Centrifuge at 9,300 × g for 1 minute
19) Place column onto a 1.5 ml microfuge tube
20) Pipette 30 µl of RNase-free milliQ to column
21) Incubate at room temperature for 1 minute
22) Centrifuge at 9,300 × g for 1 minute
23) Repeat steps 38 to 40
24) a) For short-term storage, sample can be stored at –80ºC.

PAUSE POINT

b) For long-term storage of RNA

i) Pipette 1/10 volume 3M NaAc (4 ºC, pH 5.2) and 3 volumes of 95% ethanol of -20ºC to sample.

CRITICAL STEP

If a low concentration of RNA is expected we recommend adding 1/100 volume of glycogen to enhance RNA precipitation.

ii) Store sample at –80ºC

P AUSE POINT

Sample can be stored for several months at –80ºC. To use sample for analysis apply the following steps:

iii) Centrifuge at 13,400 × g for 15 minutes
iv) Discard supernatant and wash pellet with 70% ethanol of -20ºC
v) Centrifuge at 13,400 × g for 5 minutes
vi) Dry pellet at room temperature
vii) Resuspend pellet in 100 µl TE
viii) Incubate sample for 1-2 hours at 4ºC TROUBLESHOOTING

25) Analyse isolated RNA using appropriate procedures

TIME LINE

RNA isolation from 10 gastrointestinal tract samples can be performed in approximately 2.5 hours if the RNA isolation and DNase treatment are performed without ethanol precipitations.

Anticipated results

In our laboratory the RNA isolation procedure has been used successfully for gastrointestinal tract samples. Typical concentrations of RNA isolated from these samples are listed in Table 1.

Table 1 Range of the RNA concentration obtained during the isolation procedures.

Sample	RNA (µg/g sample)
Faeces	2.0-20.0*[1]
Ileostomy effluent	0.1-0.2*[2]
Biopsy specimen	60-200*[3]

*[1]Data from Klaassens unpublished results.
*[2]Data from Booijink unpublished results.
*[3]Data from (232). N.B The RNA is of microbial and human origin.

The RNA isolation method, preceded by either of the quenching buffers, resulted in high quality RNA from intestinal samples with 23S/16S rRNA ratios of approximately 2 (Fig. 1). This indicated that the RNA degradation was limited during the isolation. The RNA isolation can be used for 16S rRNA based approaches but also for mRNA-based approaches.

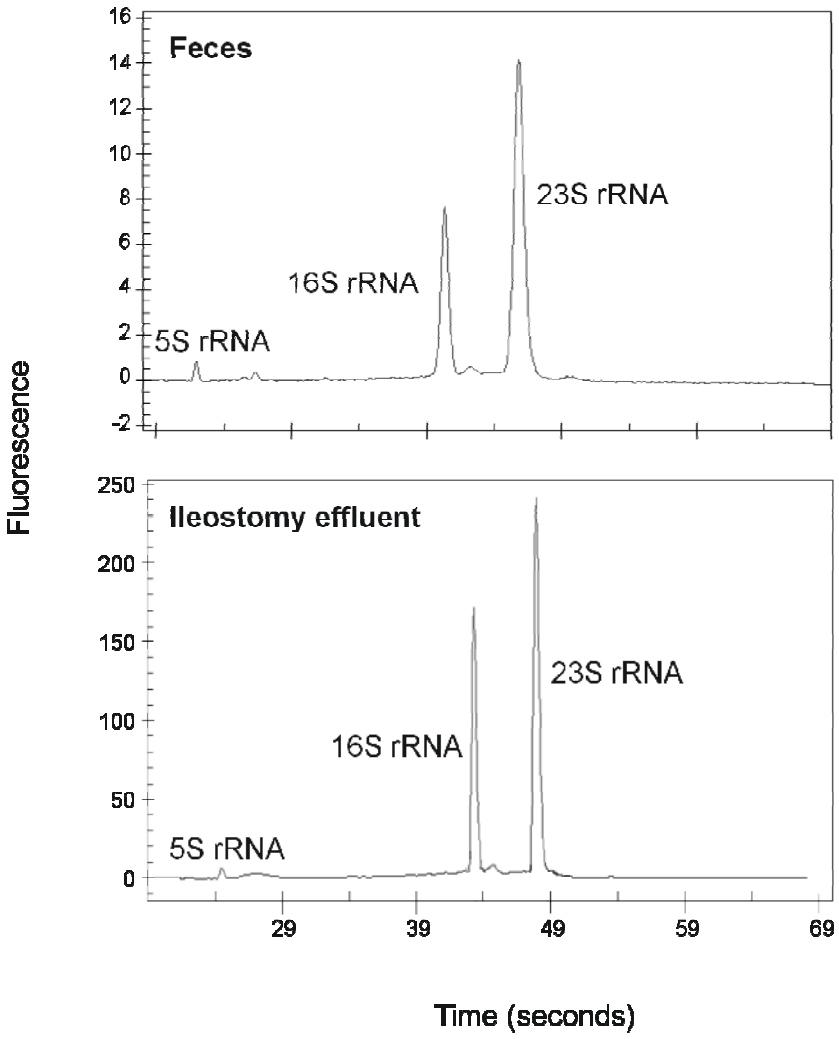

Figure 1: RNA plots (2100 Bioanalyzer, Agilent Technologies, www.agilent.com) showing examples of RNA isolated from feces and ileostomy effluent. The peaks corresponding to 23S, 16S, 5S rRNA (and tRNA) are indicated.

Troubleshooting

Problem	Possible Reason	Solution
No RNA isolated	RNA degradation took place	Repeat RNA isolation with freshly prepared RNase-free material
No RNA isolated	Sample not quenched	Repeat sampling and quenching of samples
23S/16S rRNA ratio <2	RNA degradation took place	Repeat RNA isolation with freshly prepared RNase-free material
RNA not suitable for RT-PCR	Impurities in template RNA	Dilute RNA 10 or 100 times*(191) or add BSA to RT-PCR mix
DNA present in RNA	DNase treatment not optimal	Repeat DNase treatment

*N.B. Diluting nucleic acids for (RT-) PCR purposes may require additional amplification cycles. However, this may result into a more biased representation of the initial diversity.

4

Mixed-Species Genomic Microarray Analysis of Fecal Samples Reveals Differential Transcriptional Response of Bifidobacteria in Breast- and Formula-Fed Infants

Eline S. Klaassens, Rolf Boesten, Monique Haarman, Jan Knol, Frank Schuren, Elaine E. Vaughan and Willem M. de Vos

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. Hence, the transcriptional response of bifidobacteria was studied in rapidly processed fecal samples of breast- and formula-fed infants that were also analyzed for the diversity of the total bifidobacteria-specific communities. The bifidobacterial fecal community was found to be stable and not very diverse over time in all infants up to 10 months of age, but the total numbers of bifidobacteria increased around 2.5 times in formula-fed infants during the time studied. Specific bifidobacterial species were identified by qPCR and changes were observed within a several weeks period indicating activity of bifidobacteria and possible functional changes. To obtain insight in the functional activity of infant fecal bifidobacteria a microarray was used that consisted of approximately 6000 clones of most major bifidobacterial species of the human intestinal tract. Total RNA of the fecal microbial community of infants, either breast- or formula-fed, was isolated, labeled and hybridized to the microarray. Approximately 270 clones that showed the most prominent hybridization with the samples were selected for sequencing to reveal the genes encoded on the cloned fragment. Less than 10% of the hybridizing clones contained rRNA genes and the vast majority of the inserts matched to protein-encoding genes from bifidobacteria or related species. A wide range of functional groups was covered by the obtained sequences although most were found to be involved in carbohydrate metabolism or processing of information. Several of these were selected for qPCR and sequence analysis confirming expression of the corresponding transcript with the expected nucleotide sequence. Thus, analysis of the bifidobacterial transcriptome using a mixed-species microarray is a generic approach to provide insight in the life style of bifidobacteria that colonize the infant intestine.

Introduction

At the start of life the sterile neonates gastro-intestinal tract becomes rapidly colonized by in complexity rapidly increasing microbial communities, collectively know as microbiota (57). It has been shown that various environmental factors affect the microbiota development, including the feeding regime of the infant (78).Within a few days infants are colonized by a more diverse microbiota, including bifidobacteria, bacteroides, clostridia, ruminococci and streptococci amongst others (57, 78). Bifidobacteria are heterofermentative, non-motile, non-spore forming rods and representatives of Gram-positive bacteria with a high G + C content in their genomic DNA that belong to the *Actinobacteria* phylum, within which they form a distinct order (18). At present, the genus *Bifidobacterium* includes 31 species and 8 subspecies (100), many of which have been isolated from fecal sources. Commonly isolated species detected in breast-fed or formula-fed infants include *B. breve*, followed by *B. infantis*, *B. longum* and *B. bifidum* (227). In addition, *B. catenulatum*, *B. adolescentis*, *B. pseudolongum*, *B. dentium* are also detected but less frequently (76). It has been reported that the postnatal maturation of a balanced immune system requires a constant microbial stimulation from the developing intestinal microbiota (30, 85). Many beneficial effects have been claimed for the intestinal microbiota and specifically for the bifidobacteria that have been implicated in protection against pathogens (68), normal development and maintenance of a balanced immune system (30, 85, 198), and positive nutritional effects for the intestinal cells and the host (154). However, 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 gastro-intestinal tract remains very sparse. Most studies have capitalized on molecular techniques targeting the 16S rRNA genes, such as PCR-DGGE (denaturing gradient gel electrophoresis) (189), FISH (Fluorescent *in situ* Hybridization) (80) and quantitative real-time PCR (76) to identify and quantify the different intestinal inhabitants of the gut. However, a new era has started with the sequence characterization of bifidobacterial genomes. *In silico* analysis of the total genome sequence of *B. longum* NCC2705 predicted this bacterium to be adapted to a special colonic niche (197). Several genes are predicted to encode for transcriptional regulators, which allow a quick and stringent response 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 (197). Unfortunately, only few complete bifidobacterial genomes have been reported, and only those of *B. longum* NCC2705 (197) and *B. adolescentis* ATCC15703 (212) have been made publicly available. A significant portion of the *B. adolescentis* genome differed from the *B. longum* genome reflecting their evolutionary difference based on 16S ribosomal genes. Genes coding for lacto-N-biose 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.

Genomics 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 mixed species microarray containing cloned inserts of bifidobacterial species. This mixed species microarray was recently developed and used to evaluate the genome relatedness between bifidobacterial species and strains (26). 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 prebiotics were studied at the transcriptional and diversity level. The results reveal expression of genes related to the adaptive response of these species to the physiological gastrointestinal conditions.

Materials and methods

Subjects

In a preliminary study the transcriptome of fecal microbiota of four infants ranging from 1 to 20 months was determined to test the feasibility of the clone-based microarray for the detection of protein encoded messenger RNA. Subsequently, two breast-fed infants (infants 1 and 2) and three formula-fed infants (infants 3-5) were followed over time for their microbiota analysis. The formula containing galactooligosaccharides (GOS) and long chain fructooligosaccharides (lcFOS) (9:1 w/w) was provided by Numico Research B. V. (The Netherlands). The breast-fed infants 1 and 2 were 6 and 7 months old, respectively. The age of both of the formula-fed infants 3 and 4 was 10 months, and that of infant 5 was 6 months. The infants were followed for up to 2-7 weeks and a total of 6 fecal samples were taken in this period as described below. Written consent was obtained from the parents of each infant.

PCR-DGGE analysis and qPCR

DNA was isolated using the QIAamp DNA stool mini kit (Qiagen Sciences, Maryland, USA) from samples stored at -20°C. PCR and denaturing gradient gel electrophoresis (DGGE) of the V6-V8 regions of the 16S rDNA of the total microbial community was performed as described previously using 16S rDNA-targeted primers 968-f/1401-r. The V6-V8 region of the 16S rDNA of the bifidobacterial population was targeted using Im26-f/Im3-r followed by Bif164-f/Bif662-rGC (189, 250). DGGE analysis of PCR amplicons was performed using the D-GENE System apparatus (Bio-Rad, Hercules, USA). Polyacrylamide gels (8% (w/v) acrylamide-bisacrylamide (37.5:1) in 0.5 x Tris-acetic acid-EDTA buffer with a denaturing gradient were prepared with a gradient mixer and Econo-pump (Bio-Rad), using solutions

containing 45% and 55% denaturant. A 100% denaturant corresponds to 7 M urea and 40% (v/v) formamide. PCR amplicons were separated by electrophoresis at a constant voltage of 85 V and a temperature of 60°C for 16 h. The DNA fragments were visualized by $AgNO_3$ staining and developing as described previously (146). DGGE profiles were normalized and subjected to computer assisted DNA fingerprint analysis using the BioNumerics software (GelCompar®II, Applied Maths, Kortrijk, Belgium), which measured band position differences against DNA size standards.

Quantitative real-time PCR was performed on DNA extracts in triplicate as described previously (76) to obtain percentages of 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 from two healthy breast-fed and three formula-fed infants between 5 and 20 months old. Samples were immediately stored in RNAlater (Ambion Inc., Austin, TX, USA) in a ratio of 1: 2, mixed, incubated overnight at 4°C and stored at -20°C until further processing as described in previously (254). Bacterial cells from fecal samples in RNAlater were diluted twice in ice cold PBS. After centrifugation at 900 x *g* for 1 min, 3 ml of supernatant was centrifuged for 15 min at 6000 x g to pellet bacterial cells at 4ºC. RNA was further isolated from the bacterial cells according to the Macaloid/phenol-based protocol, as previously described (105). The cells were resuspended in 0.5 ml TE (1 mM Tris- HCl and 0.1 mM EDTA) and added to ice-cold tubes containing 0.8 g glass beads (Sigma, St. Louis, MO, USA, ≤ (0.1 mm), 50 µl 10% SDS, 0.18 g 2% macaloid in TE, and 0.5 ml phenol. Cells were disrupted using 3 treatments of 45 sec in a Fastprep (Qbiogene, USA) interspaced by 1 minute one ice. Following centrifugation the aqueous phase was used for RNA isolation by extraction using chloroform: phenol: isoamylalcohol (1:1:1) twice and chlorophorm: isoamylalcohol (1: 24) once. This was followed by a cleaning step using RNeasy columns (Quiagen Sciences) according to the RNeasy cleaning protocol and treatment with DNAse (Roche Diagnostics, Mannheim, Germany) on the column. After elution from the column RNA concentrations were measured using the Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE, USA). Additionally, quality and concentration of the RNA was measured using the Experion Automated Electrophoresis System (Bio-Rad) with RNA StdSens microfluidic chips.

Design and fabrication of a clone library based DNA microarray

Three genomics libraries were constructed comprising *B. pseudolongum ssp. pseudolongum* LMG 11571 (245), *B. longum* LMG 13197 (177) and a third library of a mix of *B. adolescentis* LMG 10502 (177)*, B. animalis ssp. animalis* LMG 10508 (138), *B. bifidum* LMG 11041 (155), *B. catenulatum* LMG 11043 (192), *B. longum* LMG 13197, and *B.*

pseudolongum ssp. pseudolongum LMG 11571. Genomic DNA was isolated by resuspending bacterial pellet in Tris/EDTA saturated phenol and bead beating followed by DNA purification by chloroform extraction and ethanol precipitation. Equal amounts of DNA were pooled for the construction of the mixed library. Genomic DNA (10 μg total genomic DNA for each library in 100 μl) was fragmented using sonication (Branson 250/450 Sonifier, Best Lab Deals Inc, USA). Fragments of 1.3 to 1.9 kb were isolated from agarose gel using the glass milk method. The DNAterminator end repair kit (Lucigen Corporation, United Kingdom) was used to create blunt ends. Fragments were purified by phenol/chloroform precipitation. The DNA fragments were ligated in pSmartHCKan (Blunt smart cloning kit, Lucigen Corporation, United Kingdom) and transformed to *E. coli* ElectroMAX DH10B (Invitrogen Life Technologies, the Netherlands) by electroporation. Several plasmids were sequenced and checked for bifidobacterial specific genes. Inserts were replicated by PCR using primers SL1/SR2 annealing to pSmartHCKan. PCR products were checked by electrophoresis on a 1% agarose gel and subsequently purified by isopropanol/ sodium acetate precipitation followed by a washing step in 70% ethanol, and then dissolved in 3 × SSC. From each library 2000 clones were spotted by a robot (Engineering Services Inc.) on CSS-100 silylated aldehyde glass slides (TeleChem, Sunnyvale, CA, USA) in a controlled atmosphere. To reduce free aldehyde residues, slides were blocked with borohydride after spotting and drying. Slides were stored in the dark and dust-free until further use. Before adding the hybridization mix, the slides were pre-hybridized for 45 minutes at 42°C in 5 × SSC containing 0.1% SDS and 10 mg/ml bovine serum albumin, washed in milliQ water and isopropanol and then dried.

Fluorescent labeling and hybridization

Five μg of RNA was mixed with 2.5 μl random p6 primer (2 μg/μl, Roche Diagnostics) and 2 U RNasin (Promega, Madison, WI, USA). The mixture was incubated first on ice for 10 min following and then at 70 °C for 5 min and subsequently cooled to room temperature. Five U RNAsin, 0.1 μM DTT, aa-dNTP (Life Technologies S.A., Merelbeke, Belgium), and 200 U Superscript II RT enzyme (Life Technologies S.A.) were added to the mixture on ice and then incubated for 3 h at 42°C. RNA was hydrolyzed by adding 6.25 μmol NaOH and incubated at 37°C for 30 min. To neutralize the pH, 6.25 μmol HAc was added. Unincorporated aa-dUTP and free amines were removed using QIAquick columns (Qiagen Sciences) according to the supplier's protocol. Cy5 esters were coupled to the aa-cDNA by adding sodium carbonate buffer pH 9 to the sample and incubation of 10 min at room temperature. After adding CyDye (AmershamPharmacia, Freiburg, Germany) the mixture was incubated for 1 h in the dark at room temperature. After incubating the reaction was incubated for 1.5 h at 42°C, RNA was hydrolysed by adding NaOH. Free nucleotides were removed from the solution using AutoSeq G50 columns (AmershamPharmacia). DNA was

labeled directly during reverse transcriptase. Five μg of RNA was mixed with 5 μg random p6 primer (Roche Diagnostics) and 10 U RNasin and incubated on ice for 15 min following an incubation of 5 min at 70 °C. To anneal, the primer was cooled to room temperature. On ice, 5 U RNasin, 2.5 μl first strand buffer (5 x), 0.1 μM DTT, 2.5 μl lowT-nucleotide mix (2.5 mM dATP, dGTP, dCTP, 1.0 mM dTTP in H_2O, 1 μl Cy3-labelled dUTP (1mM) and 200 U Superscript II RT enzyme (Life Technologies S.A.) was added and incubated for 2 h at 42°C. Uncorporated dye and free amines were removed using AutoSeq G50 columns (AmershamPharmacia). Labeled cDNA was mixed with labeled DNA (control) and 100 μg yeast tRNA (Life Technologies S.A.) and vacuum dried. Samples were dissolved in 45 μl Easyhyb hybridization buffer (Roche Diagnostics), and incubated for 15 min at room temperature. The mixture was denatured for 2 min at 100°C. The samples were added to prehybridized microarray slides and incubated overnight at 42°C.

Microarray analysis

After several washing steps and drying, the microarrays were scanned with a ScanArray Express 4000 scanner (Perkin-Elmer). Fluorescent images were captured as multi-image-tagged image file format and analyzed with Imagene software (Axon, BioDiscovery, Marina del Rey, USA). Labeled chromosomal DNA of all bifidobacterial species on the microarray showed hybridization to virtually all spots on the array, as expected.

Spots that were flagged by the Imagene software (BioDiscovery) were not included in the data analysis. In total, 5314 spots met the set quality criteria and were included in the analysis. For each spot, the ratio of the mean signal intensity was divided by the local background intensity and considered as positive hybridization when it was at least 1.25 times higher than the background. The total hybridization intensity values were normalized to the total intensity to be able to compare different microarrays. Spots that did not show positive hybridization to any of the cDNA samples were removed for further analysis. Approximately 500 bp of both the 3'- and the 5'-end of the inserts in selected clones was subsequently sequenced using primers SL1 and SR2 based on pSMART. DNA sequences were identified by similarity searches against the bacterial and complete Bifidobacterial genomes at NCBI (www.ncbi.nlm.nih.gov) database libraries using BLASTn and BLASTx. Gene numbers used in this study (BL numbers) are based on the gene numbering of the *B. longum* NCC2705 genome.

Principal Component Analysis

Principal Component Analysis (PCA) 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 RNA spotted amplicons of the different bifidobacterial clone libraries on the microarray indication the activity of the bifidobacterial species among the

fecal microbiota. Redundancy analysis (RA) was chosen as it explains the structure of the "species" data table (in this case the ratio hybridization intensity divided by the local background intensity for each clone) by the effect of the diet (either breast-fed or formula-fed). 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 (diet). To test significance of the relationship of the hybridizations with the dietary group, unrestricted Monte Carlo permutation tests were performed with 499 random permutations and significance level (p) of 0.05.

Quantitative real time PCR

Primers were designed using geneID software which also takes the presence of secondary structures, including possible primer-dimers, 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) (132). *In silico* comparisons and PCR amplification products confirmed that primer sets were specific for both *B. longum* NCC2705 and *B. longum* LMG 13197 but non-targeting other organisms, including *L. plantarum WCFS1* 1 *and E. coli* (data not shown). Target genes were lacto-N-biose phosphorylase (BL1641) with primerset AAC CGT ACA AGG ACG GAT TCG/ CGG AAT ATC GGC GAT CAT GC, α-L-arabinosidase (BL1665) with primer set TAC ACG CAA CGG CCA AGG/ CCA GCA GGA CCA TCT GAC C, and thymidylate synthase (BL0544) with primer set CAC GTG CAT ATT TGG GAT GAG TG/ CCA GGA ACG CCA CTG CAC. iQ SYBR Green Supermix (Bio-Rad) was used in all reactions. iQ5 real-time PCR detection system (Bio-Rad) was used for all real-time Q-PCR. 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 s 95°C denaturation step, a 30 s annealing step (temperature defined for each primerset) 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°C 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 by PCR-DGGE

The fecal microbiota of infants is influenced by transitions between breast milk, formula and solid foods (14, 57, 78). Hence, we compared infants that were breast-fed (infants 1 and 2) with those that were subject to a diet with a specific formula containing prebiotic oligosaccharides (infants 3, 4 and 5). Both the expression of bifidobacterial genes (see below) and the bacterial diversity was analyzed in a period of 2-6 weeks during which periodic samples were taken.

The diversity of the total fecal microbiota and the bifidobacterial population was determined using PCR-based molecular techniques targeting 16S rRNA genes followed by DGGE analysis of the amplicons. Analysis of the diversity of the bifidobacterial community revealed relatively simple and stable patterns both for breast- and formula-fed infants as is illustrated for the representative infants 2 and 5, respectively (Fig. 1). This underlines the stability of the bifidobacterial microbiota over time, as well as host-specific bacterial profiles for each individual, as previously described (57). In contrast, The DGGE profiles representing the diversity of the total bacterial community of the breast-fed infants was more stable than for the formula-fed infants (Fig. 2). The lower bands in the gel represent the bifidobacterial population and support the predominance of bifidobacteria in the fecal microbiota of the breast-fed infant. Comparison of the profile of the breast- and formula-fed infants clearly showed that the one from the breast-fed infant is much simpler and stable, within the 43 days followed, compared to that of the formula-fed infant where the total bacterial profile reveals significant shifts within only 16 days. Similar observations were made with the fecal samples of the other infants (data not shown).

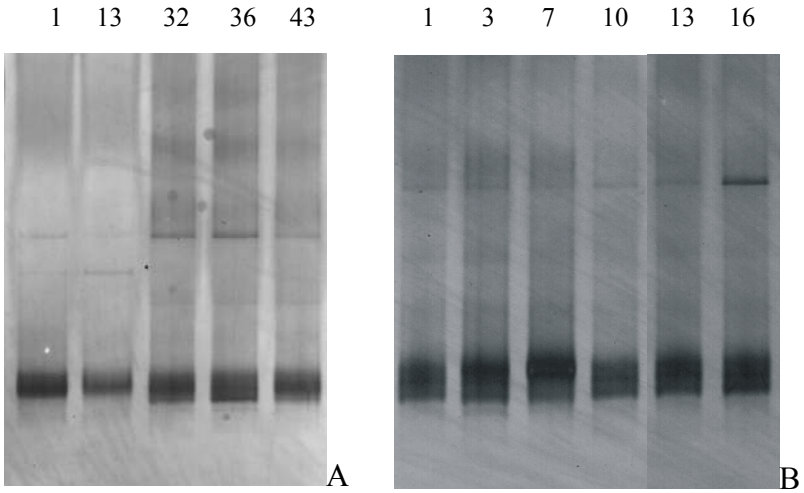

Figure 1 DGGE profiles of the fecal bifidobacteria of breast-fed infants 2 (A) and formula-fed infant 5 (B) at different time points (days).

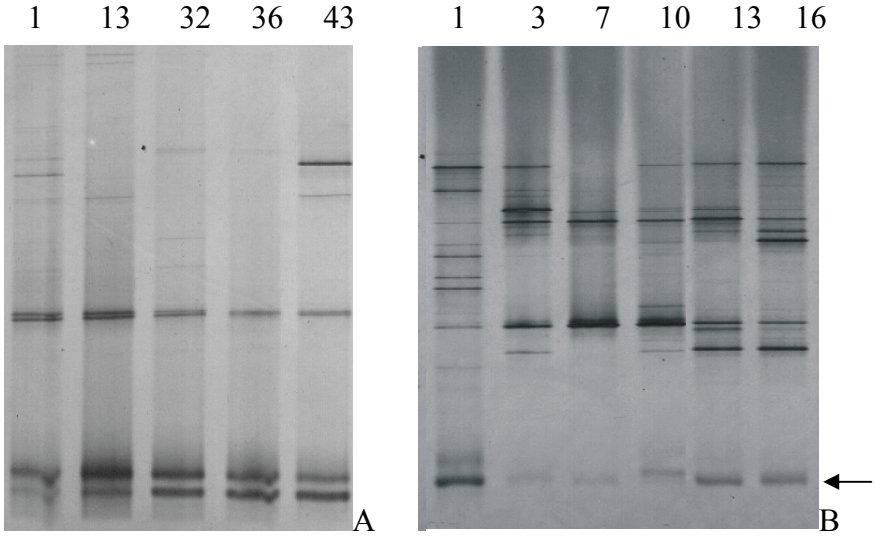

Figure 2 DGGE profiles of the fecal microbiota of breast-fed infants 2 (A) and formula-fed infant 5 (B) at different sample points (days). The arrow points out the place in the gel with bands that represent the bifidobacterial

Bifidobacteria species-specific quantitative real-time PCR

Analysis of the specific and total bifidobacteria using a qPCR method showed that the total bifidobacterial numbers were higher in the breast-fed infants at start of the trial but increased in the formula-fed infants over the time, possibly due to the intake of the prebiotic formula milk (Fig. 3). In both groups, *B. animalis* and *B. dentium* were not detectable and *B. angulatum* was present in very low numbers. *B. adolescentis*, more commonly found in adults, was detected in a very low proportion in two 10-month old infants that were formula-fed while *B. infantis*, *B. breve*, *B. bifidum* and *B. longum* were detected in all infants with *B. infantis* being the major species found in all infants (data not shown). The differences indicate a higher diversity for bifidobacterial species but a lower total number for formula-fed infants compared to breast-fed infants. During the study period, the formula-fed infants' fecal bifidobacterial diversity and quantity changed and approached that of the breast-fed infants.

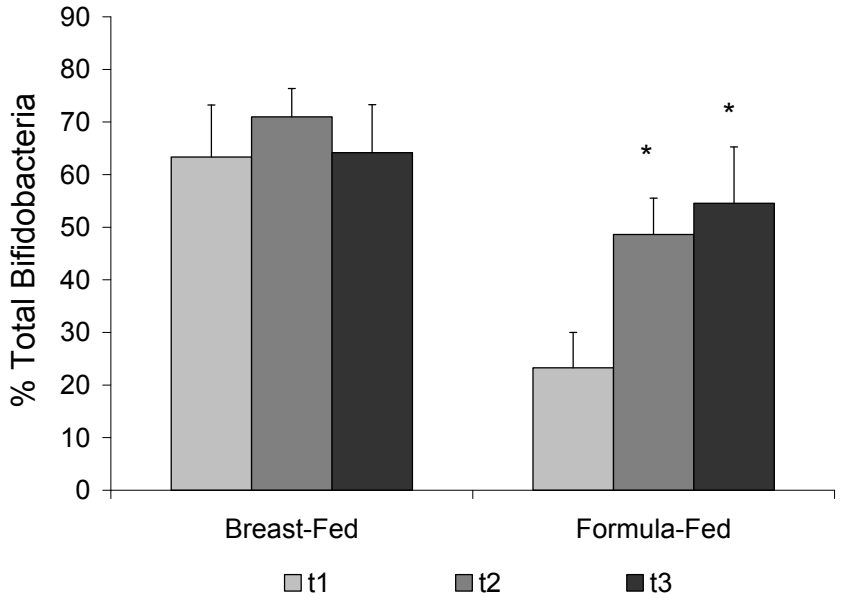

Figure 3 Total percentage of bifidobacteria in fecal samples of infants who were breast-fed (infants 1 and 2) or fed a standard formula supplemented with GOS/ lcFOS (infants 3, 4 and 5) as determined with qPCR. The bars represent the SEM. The percentage of bifidobacteria in the formula-fed but not in 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 for formula-fed infants, and t3 after an additional 1-2 weeks) as determined by student's t-Test (* $p<0.05$).

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. To investigate the potential of this microarray pilot experiments were performed in which total cDNA of fecal samples of 4 infants between 1 week and 20 months old was labeled and hybridized to the array. A number of 4524 clones showed positive hybridization of cDNA of at least one of the samples to the microarray whereby the total sum of signals per micro array was comparable (data not shown). A selection of the positively hybridized clones was sequenced showing a wide range of protein encoding genes indicating metabolic activity of the fecal bifidobacterial community and the power of this mixed species clone library based microarray.

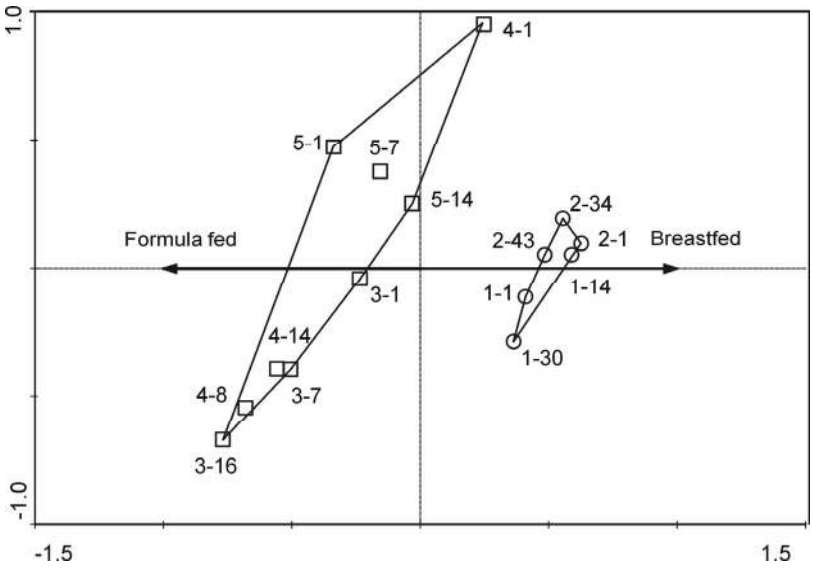

Figure 3 Redundancy analysis (RDA) ordination plot comparing the total hybridization of fecal bifidobacterial transcriptomes from infants at different time points, for breast- (○) (infant 1 and 2) and formula- (□) fed (infants 3, 4 and 5). Each sample is labeled with the number of the infant followed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

To study in more detail, five partly age-matched infants (infants 1-5; see above) with specified diets (breast-fed or formula-fed) were compared using the microarray at three time points in a 2-7 weeks period. This was done parallel to fecal microbial diversity analysis as described above. It is important to note here that the microarray consists of cloned genomic fragments derived from a mixture of six *Bifidobacterium* spp. and the hybridization takes place under stringent conditions - hence, only the expression can be monitored of 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.

Statistical analysis, performed with Canoco software package, was performed to identify whether host, environment or stochastic effects explained grouping of the studied samples. The RDA (redundancy analysis) showed that 44% of the difference could be explained significantly (p-value of 0.004) by the difference in feeding, either breast or formula. Figure 3 shows the RDA ordination plot of the hybridizations of the three samples for infant 2 to 5 and visualizes the effect of the different diets. The formula-fed infants were differed more from each other but all individuals within one diet group did not differ significantly from each other (P values above 0.05).

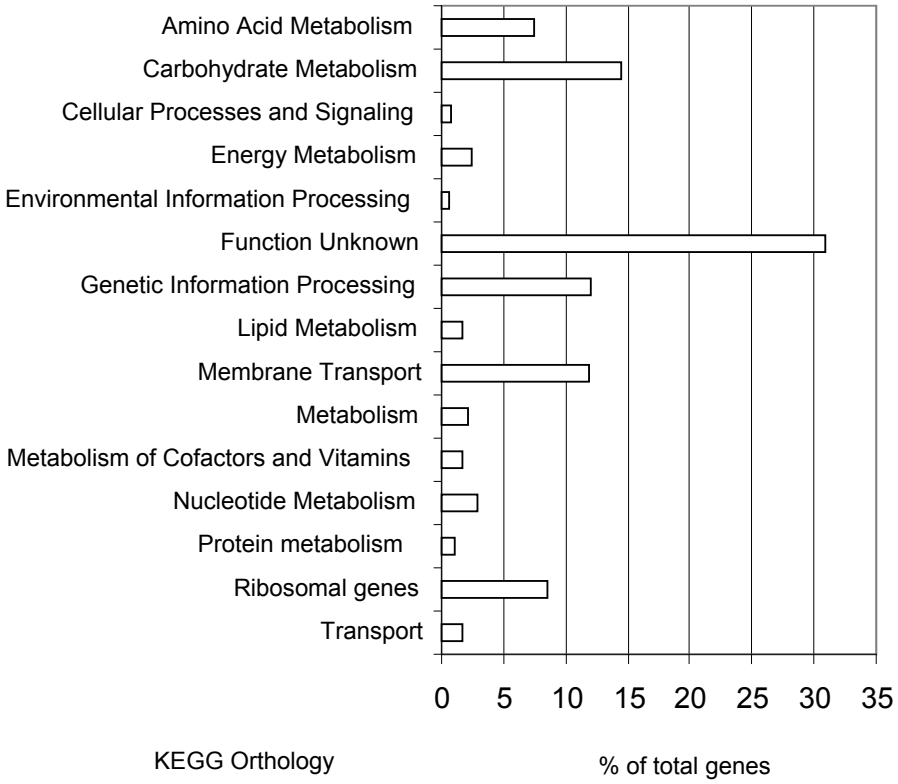

Figure 4 Classification of the predicted function of the 250 sequenced bifidobacterial inserts present on the microarray and showing significant hybridization to the labeled total RNA extracted from infant fecal samples.

A set of approximately 250 significantly hybridizing clones was selected for sequencing to predict the functional identity of the genes encoded on the inserts. Most of the sequences obtained were not full-length transcripts (500-800 bp). Almost all the inserts (90%) matched closely to describe bifidobacterial genes and all ribosomal genes matched closely to bifidobacterial rRNA genes. Less than 1% of the sequences were predicted be non-coding or could not be identified (data not shown). The sequences appeared to encode for a wide range of functionalities such as components of transport systems, genes for energy metabolism, and carbohydrate metabolism (Fig. 4). 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 was categorized as hypothetical (31%), and were partly "unclassified" (typically of known function but not readily placed in a role category during annotation).

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 RDA, as a measure for the impact of each gene on the difference between the dietary groups (Appendix 1- Supplementary Material). The clones are listed in order of importance for the clustering of the infants' transcriptome by the diet (formula-fed or breast-fed), using the hybridization signals of infants 1 to 5. This means, the list starts with genes that have a large influence on the deviation of the infants by dietary group. The hybridization signals to the clones at the end of the list do not have any impact on the difference between both diet groups.

Active carbohydrate metabolism. The genome of *Bifidobacterium longum* NCC2705 encodes numerous genes for carbohydrate utilization (197). 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 with transporters for a variety of disaccharides and oligosaccharides (161). The importance of carbohydrate metabolism for the activity of bifidobacteria in the intestinal tract is also evident from this study, as this is the most important category of predicted functions (Fig. 4). 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, impact the RDA analysis, or are organized into co-expressed operons (see Table 1).

Taking the RDA analysis into account, the DNA fragment that impacts most the difference between both diet groups included the *B. longum lacZ* gene (BL0978) that shows significant homology to the β-galactosidases I gene from *B. infantis* HL96, which degrades lactose and other sugars containing a β-D-anomer-linked galactoside (89). Recent growth studies in laboratory media showed induction of the *B. longum lacZ* gene by lactose, maltose and fructooligosaccharides (161). In the present study, the *lacZ*-like gene shows more expression in breast-fed infants though a clone with a very similar gene, BAD_1605, shows much lower hybridization signals, indicating the significance of the encoded □-galactosidase in intestinal sugar degradation. Similarly, the RDA value of the fragment including BL0597 (coding for a glycogen phosphorylase) is high and this gene is involved in the breakdown of starch in glucose units. The α-1,6 bonds in amylopectin and pullulan can be hydrolyzed by so-called pullulanases (BAD_0708), which were previously shown to be expressed in several bifidobacterial species growing on these sugar polymers in laboratory media (181). These latter genes show higher hybridization in breast-fed infants than in formula-fed infants.

A bifidobacterial α-L-arabinosidase-like gene (BL0544, *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 (184). It is known that α-arabinofuranosidases are required together with other hydrolases with endo- and exo-activities 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 (197) 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 bifidobacteria. The expression of the *abfB*-like gene and the influence of inducers and repressors have been studied in *B. longum* NIZO B667 indicating transcriptional regulation. Degenerate primers revealed widespread presence of this enzyme family (51) in *B. longum*, *B. infantis*, *B. animalis* and *B. bifidum* (74). A different gene, possibly coding for an arabinosidase (BL0146), was found with similar hybridization levels, and could 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 only received a breast milk-based diet also showed expression of an *abfB*-like gene, it is either possible that breast milk contains arabinose-like sugars or arabinose-decorated glycoproteins or that it contains related sugars that act as inducers of its expression. An alternative explanation is that milk serves a substrate for growth of microbes that produce arabinose-like compounds.

A novel putative operon for galactose metabolism (BL138-44) was detected in the *B. longum* clone library by hybridization of RNA from both breast-fed and formula-fed infants. This operon is involved in the breakdown of structures present in mucin sugars (44) indicating a way to colonize the intestine for bifidobacteria. Additionally the complex mixture of human milk oligosaccharides contains lacto-N-biose (LNB) structures (98) and could be broken down by this operon. BL1638-1640 genes are annotated as component proteins of the ABC-type sugar transporter and BL1641 as LNB phosphorylase. The cluster of genes BL1643, and BL1644 which were annotated as 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 (GNB) is the core structure of mucin type sugars (44). In this pathway, galactose 1-P formed by the phosphorolysis of LNB/GNB is converted to UDP-glucose. Other genes involved in galactose metabolism are probable α-1,4-glucosidase (BL0529) and α-galactosidase (BL1518), both higher present in formula-fed infants. Melibiose, raffinose, sucrose can also be degraded by α-1,4-glucosidase (BL0529), which hydrolyses terminal non-reducing 1,4- linked α-D-glucose. The enzyme α-galactosidase can hydrolyze terminal α-D-galactose residues from α-D-galactosides that include melibiose and raffinose. Finally, lactate dehydrogenase (BL1308) involved in the final step in anaerobic glycolysis and formation of lactate was found to hybridize to RNA

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) could be involved in breakdown of xylans by hydrolysis of 1,4-β-D-xylosidic linkages in xylans. Both genes are detected among all infants and show similar hybridization signals. BAD_1412, a probably sugar kinase has high similarities to xylose kinases and might be involved in breakdown of xylans as well. Sucrose phosphorylase (BL0536) breaks down sucrose and is involved in energy metabolism.

Overall, the expression of the glycobiome (Table 1), and especially the pullulanes, α-1,4-glucosidase and the glycogen phosphorylase, indicate a higher potential of carbohydrate metabolism in breast-fed infants. This might very well be explained by the high diversity of complex oligosaccharides in human milk (24) 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 to be the major contributor to adherence (241). In this study the expression of 2 putative glycosyltransferases, BL1104 and 1674, was detected in most of the samples, suggesting the in vivo production of polysaccharides that may also play a role in biofilm formation and colonization of bifidobacteria within the hosts' intestine. 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 (119), and could be involved in the recognition by the host of the bacteria. Only samples from formula-fed babies show hybridization to the clone containing BAS_1336 (see Appendix 1).

Activity of bifidobacteria within the human intestine. Several other genes and operons are worth mentioning as these are biologically relevant and might explain functions of the intestinal bifidobacteria related to host interaction and colonization and competition within the intestinal microbiota. The gene for transaldolase (BL0715), which takes part of 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 (99) and the proteome of *B. infantis* BI07 grown in a laboratory medium (231). The gene for transketolase (BL0716), also involved in the pentose phosphate route characteristic for the bifidobacterial metabolism, was expressed at lower levels.

Hybridization to the clone containing BTH_II0919, coding for a glutamine-dependent NAD+ synthetase (6.3.5.10), indicates presence of glutamine-rich substrate. A previous study showed increased intestinal bifidobacterial numbers upon intake of prebiotics containing glutamine rich protein in healthy adults (93). 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 of formula-

fed infants might be due to lower glutamine levels in the formula compared to breast-milk (1).

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 (185). Moreover, several bifidobacterial species have found to produce folate in laboratory media (168). 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 (168). 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 copper-transporting ATPase (BL0409) was expressed mostly in formula-fed infants, and its translation product was also found in the proteome of *B. infantis* (231). Recent, transcriptome analysis of *L. plantarum* indicated significant expression of a gene for a orthologous copper transporting protein in intestinal samples of human (232) and mice (29). This gene might be involved in copper acquisition or tolerance.

Quantitative Real Time PCR and sequencing analysis

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

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

Table 1 Hybridization values of glycobiome related genes with significant hybridization signals (signal/ background> 1.25), for infant 1 to 5. Infant 1 and 2, in bold, were breast-fed.

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

nd no significant hybridization signal detected. HP hypothetical protein.

Conclusions

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 major impact of diet on the transcriptional response of 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 studies reporting full-genome transcriptome profiles of *Bacteroides thetaiotaomicron* and *B. longum* residing in the caeca of germ-free mice (206) (205), as well as for *L. plantarum* in conventional mice (125) and even human (47). In the present study, the hybridization profile could be used to assess the influence of the diet on the transcriptome of fecal bifidobacterial community of infants. Particularly, the use of a mixed-species microarray made it possible to target several bifidobacterial species simultaneously in the complex fecal microbial ecosystem. Using this array it was possible to compare fecal bifidobacterial gene expression in individuals who all carry unique fecal microbial composition.

Breast-fed infants with stable total microbiota and bifidobacterial population show variety in transcripts at different time points indicating an active bifidobacterial population. As expected, the transcriptome of the formula-fed infants with a more unstable microbial diversity also shows difference in time. The observed differences can be caused by the activity of individual bifidobacterial species that varied and/ or environmental factors such as the diet. Possibly host factors are also of 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 encodes numerous genes for carbohydrate utilization especially more than 40 glycosyl hydrolases that are predicted to be involved in the degradation of higher order oligosaccharides (197) and 19 carbohydrate transport systems were proven to be active in laboratory media (161). *B. adolescentis* MB 239 preferred lactose, fructooligosaccharides and raffinose over glucose and fructose in laboratory media, which was explained by α-galactosidase, β-galactosidase, and β-fructofuranosidase activities (3). This study showed that at least a part of these genes are being transcribed *in vivo* in the infant intestinal tract. This might give these bifidobacteria the benefit to colonize the infants gut and explains the growth promoting effect of oligosaccharides which are described as prebiotics.

Sequencing of an extensive metagenomic library of the human distal gut revealed a high diversity in bifidobacterial genes when compared to the *B. longum* NCC2705 genome

(69). This suggests the presence of multiple and related bifidobacterial strains. The present and expected genome 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 will improve the molecular studies of the functional complement with tools such as transcriptomics, describes here, as well as metaproteomics (99) and metabolomics (72). Ultimately this will lead to a more detailed understanding of the nutritional lifestyle of bifidobacteria and its impact on the host. Linking functional activity 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% RDA. RDA could explain the grouping of the infants by the difference in diet, being either breast- or formula-fed.

% RDA	Library	locustag	function	KO
90.90	M	BL0978	glycosyl hydrolase (LacZ)	C
86.10	M	BL0597	glycogen phosphorylase	C, PS
81.20	P	BAD_0376	lysyl-tRNA synthetase 1	A
78.20	M	BAD_1319-20	DNA-directed RNA polymerase β chain; HP	GI, FU
76.30	P	BAD_0470	probable solute binding protein of ABC transporter system	MT
75.90	P	BAD_1130	DNA polymerase III α subunit	GI
72.90	L	BL0307-08	possible Rim-like protein involved in efficient processing of 16S rRNA; inosine-uridine preferring nucleoside hydrolase	GI, FU
69.60	L	BL0694-95	possible ABC transporter permease for cobalt; narrowly conserved protein with unknown function	T, FU
64.70	L	LSA0594	hypothetical prophage lsa1protein (*Lactobacillus sakei ssp. Sakei)*	FU
64.30	P	BTH_II0919	glutamine-dependent NAD+ synthetase	A, CV
62.60	P	BAD_1423-24	possible secreted peptidyl-prolyl cis-trans isomerase protein; HP	FU
62.20	P	BAD_0708	pullulanase	M
52.70	L	BL1197	large HP	FU
51.90	M	BL0337	HP possibly in TetR transcriptional regulator family	FU
51.70	M	BL0440-41	glucose-6-phosphate 1-dehydrogenase; narrowly conserved HP	C, FU
50.10	P	BL1057	argininosuccinate lyase	A
49.30	L	BL0356-57	ATP synthase epsilon chain; ATP synthase subunit B	EM
49.20	M	BL0395	valyl-tRNA synthetase	GI
48.20	L	BL1275-76	possible homoserine kinase; HP with MutT domain	A, FU
48.00	M	BAD_0914	tyrosyl-tRNA synthetase	A, GI
46.10	P	BAD_1575-76	possible NagC/XylR-type transcriptional regulator; α-galactosidase	C, GB
44.10	L	BL1450/ BL0527	leucyl-tRNA synthetase; 4-α-glucanotransferase	A, C
43.50	M	BL1429	DNA gyrase subunit B	GI
43.50	P	BAD_1336	possible penicillin-binding protein	FU
42.50	L	BL1307	GTP-binding protein	FU
42.40	L	BL1204	DNA-directed RNA polymerase β-subunit	GI
41.00	P	BL1664	widely conserved protein in universal stress protein family	FU
40.00	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
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
32.30	L	BL1251	probable glutamyl-tRNA synthetase	A

31.60	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
31.10	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
28.80	L	BL1518	α-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
24.40	L	BL0544	α-L-arabinosidase	C
24.40	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 α-1,4-glucosidase; maltase-like enzyme	C
22.25	L	BL0871-72	possible ABC transporter component	MT
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
12.77	L	BL1104	possible glycosyltransferase	C
12.74	M	BL1251-52	probable glutamyl-tRNA synthetase; possible phosphodiesterase	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 (*Rhodococcussp.* RHA1)	FU
12.00	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 tuberculosis polyphosphate glucokinase	A, C
10.71	P	BL1695-96	ATP binding protein of ABC transporter for pentoses; probable 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) methylideneamino] imidazole-4-carboxamide isomerase	A
8.58	L	BAD_0608/ BL0338	pyrroline-5-carboxylate reductase; aspartate ammonia-lyase	A
8.51	L	BL1772	sugar kinase in PfkB family	C
8.50	M	BH1867	HP (Bacillus haloduransC-125)	FU
8.40	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 str. 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
5.10	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
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
4.70	L	BL0675	possible cell surface protein similar toFimAfimbrial 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. xylistr.* 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
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
1.70	P	BL0141	possible solute binding protein of ABC transporter	FU
1.56	L	BL0529	probable α-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
1.40	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
1.10	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
1.00	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
0.80	L	BL0613	probable integral membrane transporter	FU
0.74	L	BAD_1065	putative ABC transport system integral membrane protein	FU
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
0.20	L	BL1518	α-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
0.10	P	BL0536	sucrose phosphorylase	C
0.10	L	BL1795	hypothetical proteasome-associated protein;	GI
0.10	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
0.00	L	BL0457-58	narrowly conserved HP; narrowly conserved HP with duf24	FU
0.00	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.

5

The Fecal Bifidobacterial Transcriptome of Adults Taking Dietary Prebiotics Assessed Using Microarrays

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Bifidobacteria are a predominant group present among the adult human intestinal microbiota and are considered to be beneficial to the host health. Both the dynamics and functional activity of bifidobacteria from the intestinal tract of four adults, following ingestion of a specific prebiotic mixture, was investigated. Over the course of a 3 week intervention, PCR-DGGE patterns for the total bacterial community showed increasing change compared to the baseline samples but the bifidobacterial population remained relatively stable. The percentage of total bifidobacteria, as monitored by quantitative real time PCR, altered to a low extent during intake of the specific prebiotic mixture, but marked species specific changes occurred in all individuals over time, indicating a dynamic bifidobacterial community. Insight in the functional activity of the bifidobacteria was acquired using a clone library-based microarray comprising the genomes of various bifidobacteria to reveal the fecal bifidobacterial transcriptome. Total RNA from the fecal microbial community of the adults was hybridized to the microarray of which the vast majority of clone inserts represented protein encoding genes matching closely to bifidobacterial DNA in the database. The obtained sequences belonged to a wide range of functional groups demonstrating substantial metabolic activity. However, most predicted genes were involved in metabolism of carbohydrates of plant origin, house keeping functions such as DNA replication and transcription, and membrane transport of a wide variety of substrates including sugars and metals.

Introduction

The human gut contains an immense number of microorganisms, collectively known as the microbiota which is dominated by anaerobic bacteria and is the most dense in the colon with estimates of at least 10^{12} cells (240). Molecular analyses have substantially unraveled the microbial diversity within the human gastrointestinal tract. The most numerous of the species are the obligate anaerobes of the *Bacteroidetes* and *Firmicutes* phyla (low-G+C Gram-positives) followed by the *Actinobacteria* (high-G+C Gram-positives), which includes bifidobacteria that represent several percent of the total microbiota in adult feces (5, 112). These gut microbes and their genomes, termed the microbiome, endows the host with extra physiologic capacities such as positive nutritional and immuno-modulatory effects (156, 209). There is considerable interest particular in the development of functional foods with specific bifidobacterial strains incorporated as prebiotics. Similarly the intestinal bifidobacteria are targeted by prebiotics, selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confer benefits upon host well-being and health (66). It has been suggested that an increase in bifidobacteria enhances the defense against pathogens, by mechanisms such as lowering the intestinal pH with production of lactic acid and short chain fatty acids, through natural antimicrobial excretion, competition for nutrients, and immune system modulation. Various prebiotics have been shown to selectively increase bifidobacterial species especially when this genus is rare (27, 45, 110). The use of the prebiotics inulin (fructooligosaccharides), galactooligosaccharides, and lactulose clearly stimulated bifidobacteria and lactobacilli, which ordinarily, are not the most numerous organisms in the gut except in breast-fed babies (66, 178). The impact of a dietary supplement of long chain fructooligosaccharides and galactooligosacchardes, demonstrated an increased number and changed metabolic activity of bifidobacteria accompanied by a reduction of pathogens, in infants (102, 104).

Although the studies on the effect of prebiotics on the diversity and quantity of bifidobacteria in the human intestine are increasing, our insight into the molecular activity and function of bifidobacteria in the intestine due to prebiotic treatment remains very sparse. Recently, bifidobacterial-specific microarrays, comprising clone-library containing inserts of seven bifidobacterial species were constructed (25). These were used to determine the gene expression of bifidobacteria within infant feces that were fed with formula or human-milk (Chapter 4, this thesis). In a preliminary intervention a “proprietary prebiotic mix” was demonstrated to have a bifidogenic effect on the indigenous bifidobacteria of adults (234). In the present study the effect of the same “proprietary prebiotic mix” on the indigenous bifidobacteria was followed in four adults both at the level of diversity and functional activity. The total bacterial community as well as the bifidobacterial population was monitored by the qualitative fingerprinting PCR-denaturing gradient gel electrophoresis (DGGE) of the 16S rRNA gene. Subsequently, the gene expression of the fecal

bifidobacterial population was studied by hybridizing total RNA isolated from the fecal samples to the bifidobacterial-specific microarrays. This study demonstrates that adult intestinal bifidobacteria may also be monitored by this microarray technology and the outcome may be used to gain insight in their activity.

Materials and methods

Subjects and sample collection

Four healthy individuals between the ages of 28 and 37 years were selected on the basis of presence of bifidobacteria among their commensal fecal microbiota confirmed by fluorescent *in situ* hybridization (FISH) with flow cytometry (data not shown). Written consent was obtained from each individual. The volunteers received a specific prebiotic mixture in a sachet and dissolved this in water prior to ingestion. The specific prebiotic mixture was ingested for 30 days and fecal samples were collected at baseline (3 samples within week 0), week 1, week 2 week 3 and week 4 following intake. Fecal samples were collected and processed immediately for the RNA isolation and within 1 day for other analyses. The processing of samples for the different analyses is described below.

Analysis of the bacterial and bifidobacterial diversity

DNA was isolated using the QIAamp DNA stool mini kit (Qiagen Sciences, Maryland, USA) from samples stored at -20°C. PCR and denaturing gradient gel electrophoresis (DGGE) of the V6-V8 regions of the 16S ribosomal DNA (rDNA) of the total microbial community was performed as described previously using 16S rDNA-targeted primers 968-f/1401-r. The V6-V8 region of the 16S rDNA of the bifidobacterial population was targeted using Im26-f/Im3-r followed by Bif164-f/Bif662-rGC (189, 250). DGGE profiles were normalized and subjected to computer assisted DNA fingerprint analysis using the GelCompar®II software (BioNumerics, Applied Maths, Kortrijk, Belgium), which measured band position differences against DNA size standards. Band-based dendrograms were produced by using Pearson UPGMA (Unweighted Pair Group Method with Arithmetic mean) similarity coefficients.

Quantitative real-time PCR (qPCR) was performed as described previously (76) to obtain percentages of 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 and hybridization to the bifidobacterial-specific microarrays

Fecal samples were immediately stored in RNAlater (Ambion Inc., Austin, TX, USA) in a ratio of 1: 2, mixed, incubated overnight at 4°C and stored at -20°C until further processing as described in Nature protocols (254). RNA concentrations were measured using the Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE, USA). Furthermore, quality and concentration of the RNA was checked using the Experion Automated Electrophoresis System (Bio-Rad, USA) with RNA StdSens microfluidic chips. Total RNA with a ratio 16S/23S rRNA of at least 1.8 was used for microarray experiments.

The microarray described previously (26) was used for hybridization of Cy5 labeled cDNA and Cy3 labeled DNA samples, and processed as in chapter 2 of this thesis. In short, three genomics libraries were constructed comprising *B. pseudolongum ssp. pseudolongum* LMG 11571, *B. longum* LMG 13197 and a third library of a mix of *B. adolescentis* LMG 10502*, B. animalis ssp. animalis* LMG 10508, *B. bifidum* LMG 11041, *B. catenulatum* LMG 11043, *B. longum* LMG 13197, and *B. pseudolongum ssp. pseudolongum* LMG 11571. Of each library 2000 clones were spotted on the array. Microarrays were handled as described in chapter 4 of this thesis.

Data preprocessing and normalization

The fluorescent signals from the two different labels on the hybridized arrays were quantified with a ScanArray Express scanner (Packard Bioscience) and Imagene 4.2 software (BioDiscovery). Spots of which the quality was approved by the Imagene software package were selected. For each spot, the ratio of the mean signal intensity was divided by the local background intensity and considered as positive hybridization when it was at least 1.25 times greater than the background. The total hybridization intensity values were normalized to the total intensity of the signal on the slide to make comparison of different microarrays possible. Spots that did not show positive hybridization to any of the samples were removed from further analysis. A selection of inserts was sequenced by GATC Biotech (Germany). The Smith & Waterman algorithm (203) was applied to search the sequences against different datasets. All sequences were searched against the complete protein and nucleotide sequences of all completely sequenced genomes present in the NCBI repository at March 18th 2007. In addition, extra searches were performed using only the complete genomes of *B. longum* NCC2705 and *B. adolescentis* ATCC 15703 (Gifu University, Life Science Research Center, Japan). For the nucleotide searches, all matches with the genome sequences were analyzed for annotated features using the protein and RNA annotation files of the genomes.

Principal Component Analysis

Principal Component Analysis (PCA) was performed using Canoco software package 4.5 (Biometris, Wageningen, The Netherlands) to assess the impact of the specific prebiotic mixture on the hybridization of the total RNA to the spotted amplicons of the different bifidobacterial clone libraries on the microarray which indicates the activity of the bifidobacterial species among the fecal microbiota. Redundancy analysis (RDA) was chosen as it explains the structure of the "species" data table (in this case the ratio hybridization intensity divided by the local background intensity for each clone) by the effect of the specific prebiotic mixture (before intake or after intake of the specific prebiotic mixture). 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 to the environmental variable (specific prebiotic mixture). To test significance of the relationship of the hybridizations with the dietary group, unrestricted Monte Carlo permutation tests were performed with 499 random permutations and significance level (p) of 0.05.

Quantitative real time PCR for gene expression

Primers were designed using geneID software which also takes the presence of secondary structures, including possible primer-dimers, 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.nml.nih.gov) (132). *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 species including *L. plantarum* and *E. coli* (data not shown). Target genes were lacto-N-biose phosphorylase (BL1641) with primer set AAC CGT ACA AGG ACG GAT TCG/ CGG AAT ATC GGC GAT CAT GC, α-L-arabinosidase (BL1665) with primer set TAC ACG CAA CGG CCA AGG/ CCA GCA GGA CCA TCT GAC C, and thymidylate synthase (BL0544) with primer set CAC GTG CAT ATT TGG GAT GAG TG/ CCA GGA ACG CCA CTG CAC. iQ SYBR Green Supermix (Bio-Rad) was used in all reactions. iQ5 real-time PCR detection system (Bio-Rad) was used for all real-time Q-PCR. 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 s 95°C denaturation step, a 30 s annealing step (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°C 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

Dynamics of the total bacterial community and bifidobacterial population by PCR-DGGE

Initially, qualitative insight in the effect of intake of the specific prebiotic mixture on the fecal microbiota of the 4 adults was obtained by analyzing both the total community and bifidobacterial population using the PCR-DGGE fingerprinting technique. A comprehensive analysis of the diversity of fecal bacterial communities and bifidobacterial populations were followed in time. Results showed the same trend as observed in the predominant bacterial PCR-DGGE profiles.

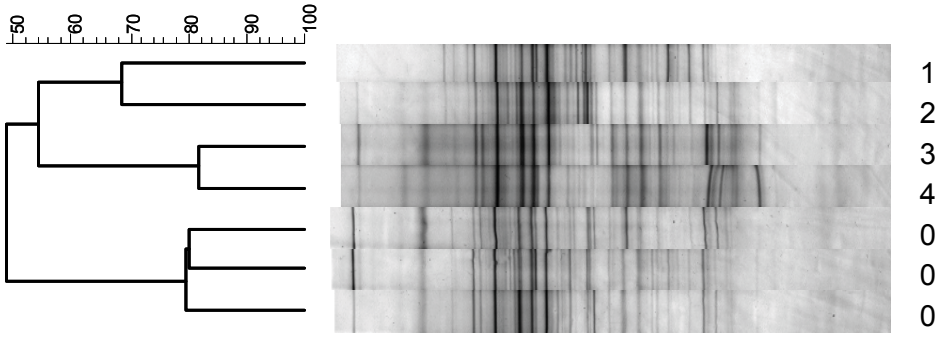

Figure 1 Pearson UPGMA cluster analysis of the universal PCR-DGGE profiles of adult 1 at baseline (week 0) and week 1 to 4. End of baseline was the first day of intake of the specific prebiotic mixture.

The DGGE profiles representing the diversity of the total bacterial community demonstrated an increasing shift in the microbiota during the intake of the specific prebiotic mixture for all individuals while the baseline samples remained very stable. Statistical analysis of the profiles using the Bionumerics software allowed the calculation of a distance matrix presenting the similarity indices of all possible gel tracks within the DGGE patterns by using the Pearson UPGMA correlation coefficient. Based on the values of the resulting matrix, a cluster analysis was performed and samples were visualized in dendrograms. This is illustrated for adult 1 in the similarity dendrograms of DGGE banding patterns in Figure 1. The samples of adult 1 taken before intake of the specific prebiotic mixture (at day 1, 3 and 5) were 80% identical to each other and provide a good baseline to analyze the influence of the intake of the specific prebiotic mixture (53). All further samples shared lower similarity

and in particular differences in bands could be visualized in the lower part of the DGGE gel. The bifidobacterial-specific PCR demonstrated the presence of bifidobacteria in all adults.

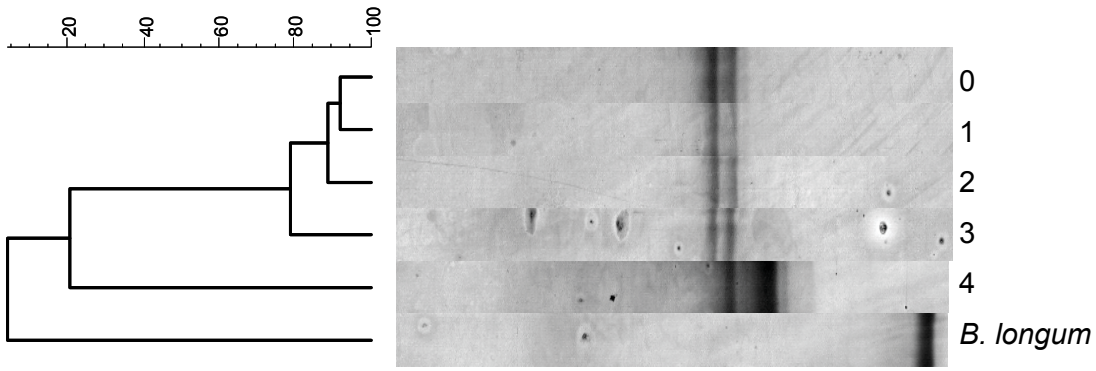

Figure 2 Pearson UPGMA cluster analysis of the bifidobacterial PCR-DGGE profiles of adult 1 at baseline (0) and week 1 to 4. End of baseline was the first day of intake of the specific prebiotic mixture. *B. longum* represents the amplicon of *B. longum*.

In contrast to the total community the bifidobacterial patterns were simpler and showed a rather stable diversity of the dominant bifidobacterial species for all adults (see example for adult 1 in Fig. 2).

Quantitative analysis of the bifidobacteria

The percentage of fecal bifidobacterial population before (baseline) and during the intake of the specific prebiotic mixture was determined by qPCR. The proportion of the total bifidobacteria did not reveal a statistically significant change during the trial in the whole population (Table 1). To get insight into the distribution of the bifidobacterial population at the species level, qPCR was performed targeting 9 bifidobacterial species. In all individuals no *B. catenulatum*, *B. dentium* and very low numbers of *B. angulatum* and *B. animalis* were detected (data not shown). *B. adolescentis*, *B. bifidum*, *B. breve*, *B. infantis*, and *B. longum* were detected in all individuals (Table 1) with *B. adolescentis* being the most dominant species present, an observation that was previously reported for adults (11). During the intervention, the level of *B. adolescentis* decreased to a near level of significance ($p=0.069$). In all four individuals the number of *B. infantis* increased significantly ($p=0.008$) over time from 1.6±0.6% at baseline to 4.3±0.5% at the end of the test period. The levels of *B. longum* increased significantly over time ($p=0.04$) and accounted for 4.7±1.5% to 10.1±2.1% from baseline to the end of the test period, respectively ($p=0.04$). These species are reported to be commonly detected in infants (83). Interestingly, adults 2 and 3 showed a marked increase in *B. bifidum,* and simultaneously showed a decrease in *B. adolescentis* and *B. breve*, respectively. It is noteworthy that only 30-50% of the total bifidobacteria was covered by the selected species. This can partly be explained by the fact that some of the bifidobacterial

species are not detected by qPCR assays ysed for this study and possibly other bifidobacterial species are present. In conclusion, the results of the qualitative and quantitative molecular methods demonstrated the presence of fecal bifidobacteria within the adults. The shift in abundance of the different bifidobacterial species detected by qPCR during the course of the intervention strongly suggests an effect of the specific prebiotic mixture on the activity of specific bifidobacterial species in the feces.

Transcriptomics of the intestinal bifidobacterial community

To gain insight in the functional activity of the bifidobacterial population within the fecal microbiota, RNA transcripts were profiled using the bifidobacterial clone library-based microarrays. Fecal samples of the 4 individuals at baseline (week 0) and week 4, after 24 days of daily intake of the specific prebiotic mixture, were investigated. Total RNA was isolated from the fecal microbiota and hybridized to bifidobacterial-specific microarrays. Scanning of the arrays revealed positive hybridization for 4496 of the 6000 spotted clones with one or more of the labeled samples. The inserts of 310 clones were sequenced and are discussed below. Each insert contained on average 1 to 4 genes, and this translated into a total of 438 genes with a significant match to sequences in the database. Database searches with the sequences resulted in a match in 90% of the cases with one of the annotated bifidobacterial genomes (*B. longum* NCC2705 and *B. adolescentis* ATCC 15703). The predicted genes were grouped according to their KEGG Orthologies (Fig. 3). The mapping of these genes and their predicted products to clusters of orthologous groups or on known metabolic pathways using the KEGG database allows predictions to be made regarding the functional capabilities of a microbial community (69). The groups comprising genetic information processing and carbohydrate metabolism were the most represented, followed by membrane transport, amino acid metabolism and the group of hypothetical genes indicated as function unknown (Fig. 3). Matches with ribosomal genes confirmed the presence of bifidobacterial rRNA in the total RNA extracted from the fecal microbiota. However, these were only found in 28 of the sequenced clones indicating a preference for messenger RNA which comprises only 1 to 10% of the total RNA.

Table 1 Percentage of specific bifidobacterial species relative to total bifidobacteria as determined by qPCR in fecal samples of the 4 individuals for baseline and each week. Baseline (week 0) and week 4, after 24 days of daily intake of the specific prebiotic mixture, were used for microarray analysis. [#]comparing average of each species baseline values to end values for the trial.

	adult	Week 0	Week 1	Week 2	Week 3	Week 4	P-value[#]
B. adolescentis	1	11.0	1.3	9.0	15.2	11.2	
	2	12.5	11.2	7.2	4.2	4.0	
	3	16.4	13.1	12.2	13.2	12.1	
	4	11.4	5.2	7.2	9.2	9.2	
	average	12.9	7.7	8.9	10.4	9.1	0.069
	SD	2.46	5.44	2.36	4.88	3.61	
B. breve	1	3.4	2.5	2.5	2.5	2.4	
	2	12.3	17.2	15.2	13.5	12.2	
	3	16.5	12.5	5.4	3.1	5.2	
	4	14.3	4.8	4.3	18.2	14.8	
	average	11.6	9.3	6.9	9.3	8.7	0.142
	SD	5.75	6.81	5.69	7.78	5.81	
B. bifidum	1	14.2	15.1	17.3	15.2	16.4	
	2	0.2	1.5	7.9	8.2	6.2	
	3	4.2	6.2	10.4	14.4	18.2	
	4	3.4	14.2	7.9	0.1	1.3	
	average	5.5	9.3	10.9	9.5	10.5	0.179
	SD	6.05	6.53	4.44	6.99	8.11	
B. infantis	1	2.0	3.0	5.0	4.5	5.0	
	2	0.9	2.2	3.2	4	4.2	
	3	3.1	2.2	4.5	5	5.1	
	4	0.3	2.1	2	2	2.8	
	average	1.6	2.4	3.7	3.9	4.3	0.008
	SD	1.24	0.42	1.35	1.31	1.06	
B. longum	1	4.6	5.1	7.2	9.5	9	
	2	3.3	4.2	6.4	12.4	14.2	
	3	9	7	6.8	7.4	12.3	
	4	1.9	2.8	5.9	5	4.8	
	average	4.7	4.8	6.6	8.6	10.1	0.041
	SD	3.07	1.76	0.56	3.14	4.12	
total	1	40.9	39.0	44.2	45.0	45.0	
	2	29.3	36.3	39.8	42.3	40.8	
	3	44.2	33.0	34.3	39.1	49.9	
	4	31.0	25.3	29.1	40.5	34.8	

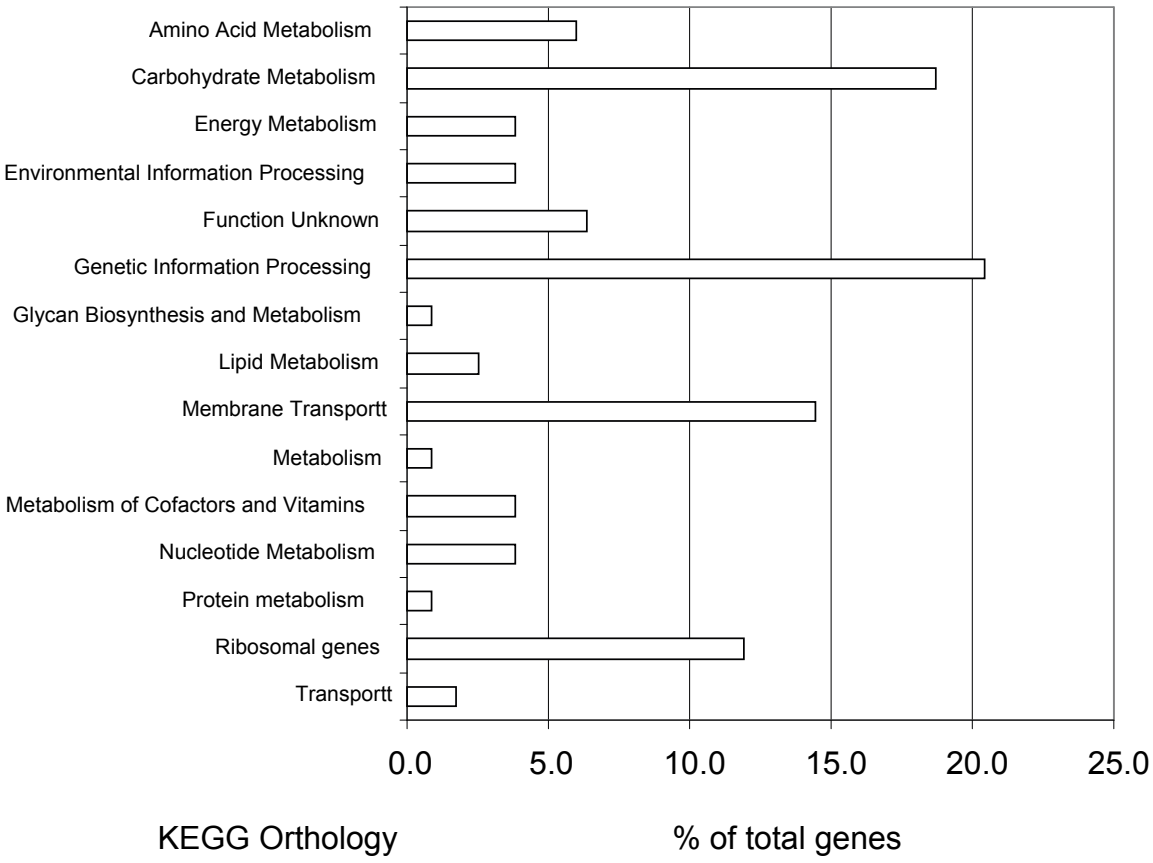

Figure 3 Division of the predicted genes from the sequenced inserts of the different bifidobacterial clone libraries on the microarray and which showed positive hybridization to the labeled total RNA of the adult fecal intestinal bifidobacterial community.

RDA of all signals on the microarray from the 4 individuals bifidobacteria using Canoco software resulted in clustering of samples taken before and after intake of the specific prebiotic mixture although this was not significant ($p = 0.5$) (data not shown). However, t-tests within individuals 1, 2, and 4 showed significant differences between signals before and after intake of the specific prebiotic mixture (p values of 4.9×10^{-12}, 1.5×10^{-12}, 0.6, 1.1×10^{-7} for adults 1, 2 and 4, respectively). This demonstrates individual change, whether or not influenced by the specific prebiotic mixture intake, possibly due to the inter-individual difference in microbiota as well as the host itself. Thus overall there was no clear trend between the patterns of gene expression and the levels of bifidobacterial species. This can be due to the absence of sufficient bifidobacterial species genomic DNA on the microarray and the fact that not all bifidobacterial species were targeted by the qPCR. It is possible that at least a part of these unidentified species hybridize to clones on the microarray because of similarities of the genomes of bifidobacterial species.

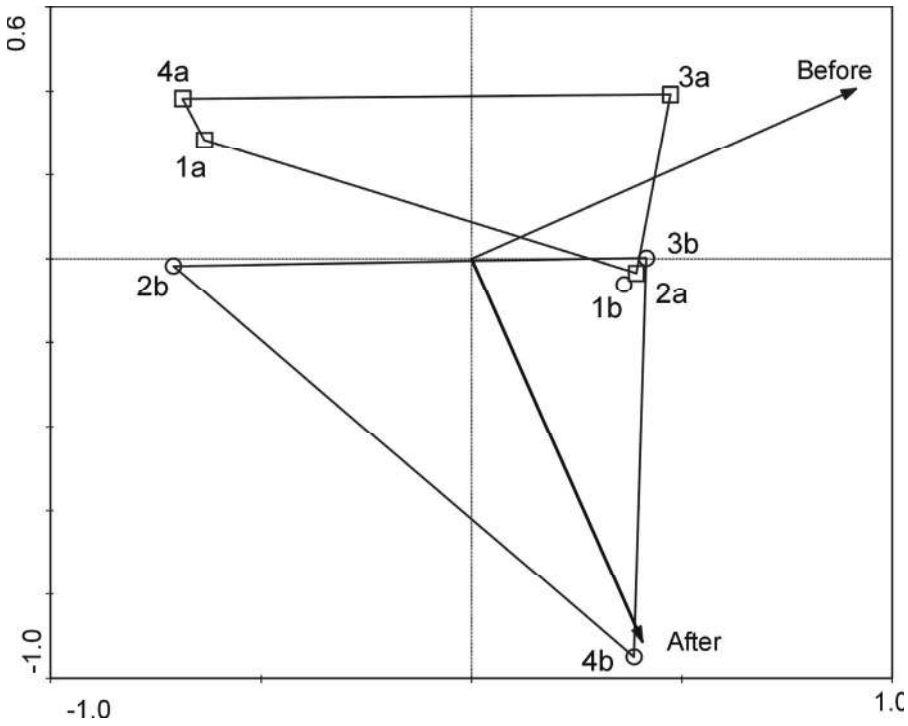

Figure 4 RDA of microarray hybridization values (signal/ background) for each individual (1- 4); ○ before (b) and □ after (a) intake of the specific prebiotic mixture. The vectors represent the time variable of sampling.

Activity of bifidobacteria within the human intestine

The predicted bifidobacterial genes found to be expressed in the fecal samples are described below to give an indication of the activities of the *in vivo* bifidobacteria. Substantial metabolic activity of the fecal bifidobacteria was indicated by hybridization to clones that contain genes annotated for essential housekeeping functions involved in genetic information processing such as DNA replication (in clones coding for BL0742, BL1152-530), and gene transcription (BL1204). Furthermore protein synthesis was inferred from several tRNA synthetases (BAD_0544, BL0116, BAD_0914, BL0017-18), ribosomal proteins (BL1291, BL1549, BL1589) and a translation initiation factor (BL1616) involved in translation of mRNA. Genes involved in nucleotide metabolism (BL0630, BL1752, and BL1148) were also detected which indicates synthesis of amino acids which are necessary in the human intestinal environment. This all indicates an active turnover of DNA, RNA and proteins in the fecal bifidobacterial population.

Several putative response regulators (BL0005-07, BL1402-03 and BAD_0055) and a histidine kinase sensor protein (BL1646-47) of two-component regulatory systems were detected. These systems are likely to be important in detection and adaptation to the conditions within the colonic environment and deserve further investigation. A large number of putative genes were detected for membrane transporters which target a wide variety of

substrates including sugars and metals, such as BL1126-29 that is possibly involved in iron uptake, as well as transporters involved in cell division. Noteworthy is a putative copper-transporting ATPase gene detected for all individuals. This enzyme was also found in the proteome of *B. infantis* (231). In the transcriptome analysis of *L. plantarum* WCFS1 a similar copper transporting protein showed high expression and induction in intestinal samples of human (232) and mice (29), respectively. This gene might transport other metals and be involved in detoxification in the intestinal tract.

Several genes annotated from the folate and folate one carbon pool pathways were detected, namely dihydrofolate reductase (BL1666) and thymidylate synthase (BL1665) involved in the production of folate, as well as intermediary enzymes, bifunctional methylenetetrahydrofolate dehydrogenase and methenyltetrahydrofolate cyclohydrolase (BL0993). Folate is involved in many metabolic pathways, such as methyl group biogenesis and synthesis of nucleotides, vitamins, and various amino acids. Several bifidobacterial species have been demonstrated to produce folate *in vitro* (168). Folate synthesized by bacteria in the human intestine may be absorbed and used by the host, and based on these gene expression studies its likely that that the intestinal bifidobacteria partake in this (185).

Substantial carbohydrate uptake and metabolic genes expressed *in vivo*

The genome of *Bifidobacterium longum* NCC2705 encodes numerous genes for carbohydrate utilization (197) and shows a preference for metabolism of di-, tri and oligosaccharides pointing towards a biased utilization for complex oligosaccharides complemented with transporters for a variety of disaccharides and oligosaccharides (161). The importance of carbohydrate metabolism is in this study reflected by the large number of transcripts detected for sugar metabolism which could be used to infer the substrate utilization in the human intestine by the bifidobacterial population.

The genes from the positively hybridized clones on the microarrays that were functionally annotated to be involved in carbohydrate metabolism and uptake are presented in Appendix 1. A bifidobacterial α-L-arabinosidase was detected in the *B. longum* library (clone BL0544) as well as for other species on the microarray, and a gene highly related to an α-arabinofuranosidase II (SAV1043) of *Streptomyces avermitilis* (a GC-rich actinobacteria). These are hemicellulose-degrading enzymes that hydrolyze terminal α-L-arabinofuranosyl groups from arabinose-containing oligo- and polysaccharides such as arabinans, arabinoxylans, and xylans, which are major components of plant cell walls (184). A predicted pullulanase (BAD0708) detected for all individuals might be involved in hydrolysis of α-1, 6 bonds in amylopectin and pullulan. A putative β-fructofuranosidase (BL0105) gene and associated sugar transport protein detected could be involved in the breakdown of inulin, which is a naturally occurring storage polysaccharide found in many plants, and fibers of plant origin by hydrolysis of terminal non-reducing β-D-fructofuranoside residues in β-D-fructofuranosides as well as acting on sucrose. Arabinose-containing polymers and inulin are

abundant in the diet and reach the colon. The α-arabinofuranosidases are required together with hydrolases with endo- and exo-activities for the complete degradation of polymeric arabinose-containing polymers. It is noteworthy that the *B. longum* genome (197) harbors at least 14 different enzymes predicted to be involved in the catabolism of arabinose-containing polymers, which along with the present gene expression study indicates the importance of these type of enzymes and polysaccharides in the metabolism of intestinal bifidobacteria. Activity of these enzymes was also demonstrated *in vitro* in *B. longum* NIZO B667 and the genes were detected in a wide range of common intestinal bifidobacterial species (74). The expression of the genes for these enzymes by the intestinal bifidobacteria presumably facilitates survival and colonization in the human colon, while concomitantly providing the host with otherwise inaccessible nutrients from the diet.

Many genes encoding enzymes that are predicted to target more simple sugars were also detected. A putative α-galactosidase (BL1518) gene, that can hydrolyze terminal α-D-galactose residues from α-D-galactosides for example from melibiose and raffinose, was identified in inserts of more than one library. Melibiose, raffinose, and sucrose could also be degraded by a putative gene for α-1, 4-glucosidase (BL0529) which hydrolyses terminal non-reducing 1, 4-linked α-D-glucose. In 3 different clones a putative β-galactosidase gene (BL0259) was detected for metabolism of lactose to glucose and galactose. A possible lactaldehyde reductase (BL1673) was detected in all individuals that can anaerobically reduce L-lactaldehyde, a product of both the L-fucose and L-rhamnose catabolic pathways, to the end product L-1,2-propanediol (141). A galactose-1-phosphate uridylyltransferase gene (BL1211) involved in galactose metabolism was also detected.

To complement the carbohydrate metabolic genes above, putative transport genes were also detected, notably *lacS* for lactose transport. LacS (BL0976) is a highly efficient transport protein first described for *S. thermophilus* that transports lactose and can excrete galactose, and a homologue is present in the *B. longum* NCC2705 genome as well as in other bifidobacteria (161, 162). The presence of lactose would account for the expression of the lactose and galactose genes of the fecal bifidobacteria. However, the expression of *lacS* in *B. longum* NCC2705 could be induced by several carbohydrates besides lactose including raffinose, maltose and FOS. Clones BL0033 and BL0034, which encoded genes highly similar to the *E. coli* ribose ABC transporter, were also upregulated by growth on raftilose P95 (FOS, Orafti) *in vitro* (161). The ATP binding protein of ABC transporter BL1692 was predicted to transport multiple sugars with fructose, glucose and mannose moieties. Genes (in clones BL1163-BL1164 and BL1169-BL1170) encoded sugar transporters predicted to be involved in lactose transport and were shown previously to be induced by FOS and lactose (161). The products of clone BL1523 was previously shown to be involved in raffinose uptake (161) (see Appendix 2).

Of special interest were the genes with high identity to the putative lacto-N-biose (LNB) operon, a novel putative operon for release and metabolism of galactose from mucin sugars and human milk oligosaccharides, detected by hybridization with clones from the *B. longum* library (Appendix 1) (98). BL164 (LNBP) was annotated as lacto-*N*-biose phosphorylase and activity was shown in several bifidobacterial species (98). The cluster of genes BL1641-44, which were annotated as lacto-*N*-biose phosphorylase, mucin desulfatase, galactose-1-phosphate uridylyltransferase, and UDP-glucose 4-epimerase, respectively, likely code for a metabolic pathway for mucin sugars, because galacto-*N*-biose (GNB) is the core structure of mucin type sugars (44). In this pathway, galactose 1-P formed by the phosphorolysis of LNB/GNB is converted to UDP-glucose. Genes in clones BL1638-1640 were annotated as component proteins of an ABC-type sugar transporter and the former gene was shown to be induced by FOS (161). The intestinal mucus layer can serve as a nutrient source for the intestinal microbes as well as an initial binding site (97).

Other key energy genes such as a transaldolase (BL0715) were detected, that was also identified in the metaproteome of infant feces (99) and as one of the most abundant proteins of *B. infantis* grown *in vitro* (176, 231). Transaldolase is active in the non-oxidative stage of the pentose phosphate pathway and besides energy metabolism is used for reducing agents, nucleotide formation, and reducing equivalents for synthetic purposes (15). A gene for lactate dehydrogenase (BL1308), involved in the final step in anaerobic glycolysis and formation of lactate, was detected in just one of the samples.

Quantitative real time PCR and sequencing analysis

Three target genes, namely thymidylate synthase (in clone BL1665), lacto-N-biose phosphorylase (BL1641), and α-L-arabinosidase (BL0544), present in the clone inserts with positive hybridization with the labeled total RNA from the fecal microbiota were a target for qPCR to confirm the expression of the genes in the samples. The relative copy numbers obtained with qPCR showed a similar trend as relative signal over background values obtained with the microarray hybridizations (see Fig. 6 for BL0544), confirming the level of messenger RNA of these genes. Sequence analysis of the amplicons of qPCR confirmed amplification of the target genes.

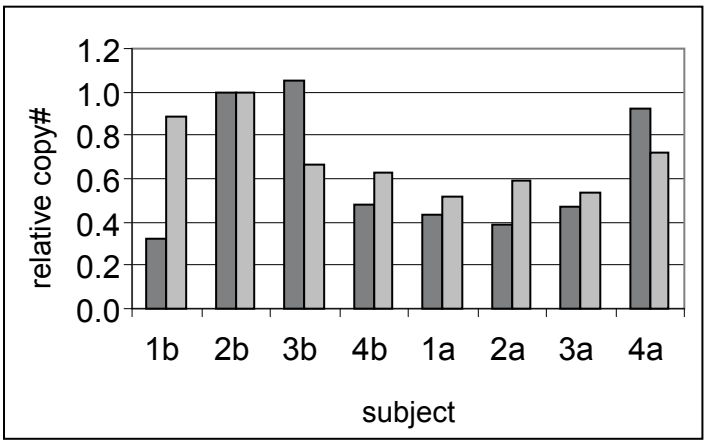

Figure 6: Relative copy numbers of putative α-L-arabinosidase in clone BL0544 upon comparison of microarray (■) and qPCR (■) values for each adult (1-4) before and after intake of the prebiotic.

Conclusions

As metagenomic studies revealed the presence of multiple bifidobacterial strains within the fecal microbiota (69), the present use of a mixed species microarray has enabled us to target a great part of the fecal bifidobacterial population for gene expression studies. In this study, initially molecular tools, PCR-DGGE and qPCR, identified a unique diversity of bifidobacterial species in each individual. Though the total amount of bifidobacterial species did not change over time during the intervention, species specific percentages indicated changing dynamics of the bifidobacterial community. To study the changes on a functional level, mRNA was isolated and hybridized to microarrays consisting of amplicons of mixed bifidobacterial species clone libraries. The application of the mixed microarray to assess the functionality of bifidobacteria in their intestinal habitat revealed a wide range of genes involved in different functional metabolic pathways. These results give a first insight in the activity of the bifidobacterial population in the human colon. Of particular interest are the genes involved in the specific survival and colonization of the human gastrointestinal tract. In this study numerous genes specialized in breakdown of plant fibers were detected which give us insight in the nutritional requirements of the intestinal bifidobacteria in order to compete in this environment. Furthermore, the production of folate by the intestinal bifidobacteria offers us some insight in the molecular basis for host benefits of this group.

The genome-wide transcriptome profiling provides us clues for specific target genes that need to be studied in more detail. Site directed gene knock-out strategies should complement this. Also, newly arising bifidobacterial genomes should be compared to gain insight in the differences between species of the *Bifidobacterium* phylum. Ultimately, the combination of these strategies will provide knowledge required for the construction of molecular models that explain the selectivity of microbe-host interactions, the impact of the microbes on the host well-being and targets for modulation.

Acknowledgements

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Supplementary Materials

Appendix 1 Clones from the three libraries on the microarray, *B. longum* library (L), *B. pseudolongum* (P) library, and mixed bifidobacterial species library (M) containing genes involved in carbohydrate metabolism and membrane transport with signal over background values of the hybridized samples of adult 1- 4 before (b) and after (a) intake of the prebiotic.

	Locus tag	Function	KO	1		2		3		4	
				b	a	b	a	b	a	b	a
P	BAD_0255	propionyl-CoA carboxylase beta chain; JadJ	C, LM	14.2							
M	BAD_0366-67	transcription antiterminator similar to LicT; glucose-specific II BC component	GI, MT		18.2		9	12.4		10.1	
P	BAD_0470	probable solute binding protein of ABC transporter system	MT								
M	BAD_0544	methionyl-tRNA formyltransferase	MT	7	7	16.5	17	8.8	9.3	7.8	7.5
M	BAD_0708	pullulanase	M	12.1	18.5	17.4	6.9	8.1	6.4	9.2	14.4
M	BAD_0956-57	probable aminotransferase; similar to Mycobacterium tuberculosis polyphosphate glucokinase	A, C	8.6	14.9	23.8	9.1	8.3	8.2	9.3	17
L	BAD_1188	galactoside symporter (*lacS*) (similar to BL0976)	T	6.5	6.5	19.4	20.8	8.6	9	7.1	6.5
L	BAD_1188	galactoside symporter (*lacS*) (similar to BL0976)	T	8.21	8.2	10.6	17.3	8.6	7.5	6.4	7.1
M	BAD_1191-92	ABC transporter; ATP-binding protein of ABC transporter	MT	8.7	17.4		7	7.6	4.8	64.1	18.1
P	BAD_1575-76	possible NagC/XylR-type transcriptional regulator; α-galactosidase	C, GB		28						73.8
M	BAD_1578	probable NhaP-type Na(+)/H(+) exchanger	MT	9. 8	9.8	10.9	14.6	9.5	9	17.4	8.1
L	BAD_1605	β -galactosidase	C	15.7				12.2	13.8	14.8	
M	BB3022	probable glycoprotease	C	8.4	13.9	18.7	9.3	7.1	8	9.7	12.3
L	BL0033-34	ATP binding protein of ABC transporter; probable solute binding protein of ABC transporter system possibly for sugars	MT	8.1	11.3	17.1	10	8.4	9.1	10	10.3
L	BL0073-74	possible phospholipase/carboxylesterase; permease protein of ABC transporter system	MT	7.6	12.4	19.3	8.5	8.5	6.6	7	13.5
L	BL0092	probable DNA helicase II	C, CV					8			
L	BL0092-93	probable DNA helicase II; narrowly conserved HP	C, CV	7.2	17.3	17.2	8.4	8.9		10.1	14.6
L	BL0092-93	probable DNA helicase II; narrowly conserved HP	C, CV	6.8	15	18.8		10.2	9.2	7.4	12.6
L	BL0099-100	HP with possible acylase domain; DNA repair protein RecO	MT, GI	7.7		23	6.9	6.7	5.1	6.2	13.5
L	BL0104-05	possible permease protein of ABC transporter system; β -fructofuranosidase (sucrase/invertase); possible inulinase	MT, C	8	13.1	17.8	8.4	7.6	7.3	7.3	10.5
L	BL0105	β-fructofuranosidase (sucrase/invertase); possible inulinase	C		15.7			7			

L	BL0105-06	β -fructofuranosidase (sucrase/invertase); possible inulinase; sucrose transport protein	C	8.6	17.8	17.5		10.6	10.4		
L	BL0116	glycyl-tRNA synthetase	C						7.9		31.5
L	BL0163-64	HP related to thiamine biosynthesis lipoprotein ApbE; probable permease protein of ABC transporter system	CV, MT	5.9	12.5	20.3	8.5	10.6	9	11.3	11.9
L	BL0228	possible fused dTDP-4-keto-L-rhamnose reductase and dTDP-4-keto-6-deoxyglucose-3,5-epimerase enzyme involved in rhamnose biosynthesis	C	5.9	11.6	22.8	7.5	6.6	9.6	9.6	10.6
L	BL0259	β -galactosidase I	C	5.6	17.2	17.7	7.2	6.8	5.3	7.7	15.1
L	BL0268	ATP binding protein of ABC transporter similar to Vex1 (VexP1) of *S. pneumoniae*	MT	6.3	6.3	17.1	21.1	9.7	6.9	6.9	
L	BL0342-43	possible permease of ABC transporter; possible permease protein of ABC transporter system	MT	7	17.3	16.2	9.4	5.8	7.7	7	13.3
L	BL0433-34	protein-pII; uridylyltransferase; nitrogen regulatory protein N-II; phosphoenolpyruvate carboxylase	GI, C	6.9	15.9	22.1	6.4	7.4	6		
L	BL0450-51	ATP binding protein of ABC transporter; possible permease protein of ABC transporter system	MT		14.1	20.6	9.1	7.3			13.8
L	BL0529	probable α-1,4-glucosidase; maltase-like enzyme	C	6.4	18.6	14.1	7.8	8.4	7.5	8.2	13.3
L	BL0529	probable α-1,4-glucosidase; maltase-like enzyme	C	6.8		20.5	7.6	8.1	4.9		17
L	BL0544	α-L-arabinosidase	C					6.7			
M	BL0544	α-L-arabinosidase	C	5.2	16.1	17	7.7	7	6.3	7.6	14.8
M	BL0597	glycogen phosphorylase	C	7.4	13.9	20.4	11.3	13	12.9	10.4	14.4
L	BL0604	phosphoenolpyruvate carboxylase	C	7.7	14.5	22.9					14.1
L	BL0671-72	ribonucleotide-diphosphate reductase beta subunit; probable glycosyltransferase	N, C		18.3		8.6			6.6	
L	BL0694-95	possible ABC transporter permease for cobalt; narrowly conserved protein with unknown function	T, FU	6.9	16.9	19.8	7.2	7.4	7.7	7.4	15
L	BL0715	Transaldolase	C	7.7	18.5	20.3	6.2	6.2	7	98.2	12.3
L	BL0837-38	HP; LacI-type transcriptional regulator	FU, C	5.7	18.4	18.6	6.2	8.2	8.5	7.6	9.6
L	BL0901-02	probable ATP binding protein of ABC transporter; histidine kinase sensor of two-component system	MT, EP	8.1	12.7	24.6	8.5	8	8.3		14.9
L	BL0950-51	pyruvate formate-lyase 1 activating enzyme; formate acetyltransferase	GI, C	9.5	14.1	18.2		11.1	7	9.6	14.3
L	BL0951-52	formate acetyltransferase; HP	C, HP	4.6	13.4	15.3	6.8	6.5			
L	BL1026	peptidyl-tRNA hydrolase	MT	8	8	16.8	15.8	8.2	7.7	5.4	10.2
L	BL1068-69	possible rRNA methylase; possible permease protein of ABC transporter for cobalt	GI, MT						7.8	11.3	71.4
L	BL1069-70	possible permease protein of ABC transporter for cobalt; ATP binding protein of ABC transporter	MT	8.1	18.8	23.1	9	10.3	8.5	8.3	12.7

L	BL1126-29	probable solute binding protein of ABC transporter system; probable metal uptake regulator similar to ferric uptake regulator protein; phosphoribosylaminoimidazole carboxylase ATPase subunit	MT, GI, CV	13.2	19.1	8.6	8.3	10.7	10.5	11.9	11.9
L	BL1128-29	probable metal uptake regulator similar to ferric uptake regulator protein; phosphoribosylaminoimidazole carboxylase ATPase subunit	MT, CV	7.8	18.2	14.6	9.1	7.6	7.7		13.5
M	BL1148-49	deoxyguanosinetriphosphate triphosphohydrolase-like protein; alanine racemase; probable amino acid transporter	N, MT	14.1	18	6.6	8	6.4	9.5	10.8	10.75
M	BL1163	probable solute binding protein of ABC transporter system for sugars	MT	6.8	6.8	17.5	19.3		7.3	8.1	7.2
L	BL1164	probable solute binding protein of ABC transporter system for sugars	MT	6.9	6.9	15.1	18.9	8.8	6.4	7.3	7.7
L	BL1164-65	probable solute binding protein of ABC transporter system for sugars; probable solute binding protein of ABC transporter system for sugars	MT	8.8	19	23.7	7.8	11.3	9.3	9.3	10.3
L	BL1169-70	probable permease of ABC transporter system for sugars; probable permease of ABC transporter system for sugars	MT	6.1	14.8	19.6	8.3	10.2	8.4	6.3	11.9
L	BL1169-70	probable permease of ABC transporter system for sugars; probable permease of ABC transporter system for sugars	MT	7.8	13.2	20.3	8.7	7.4	8.7	6.2	12.4
M	BL1170	probable permease of ABC transporter system for sugars	MT	8.3	8.3	15.2	19.5	10.4	7.2	7.1	8.1
L	BL1181-82	HP with weak C-terminal similarity to TraG; FtsX-like protein involved in cell division	FU, MT	5.9	17.3	17.6	9.6	7.6	7	5.9	11.5
L	BL1211	galactose-1-phosphate uridylyltransferase	C								24
L	BL1276-77	HP with MutT domain; ATP-binding protein of ABC transporter system	FU, MT	7.1	14.1	16	8.4	7.1		9.7	13.9
P	BL1308	lactate dehydrogenase	C			18.2					
L	BL1396-97	probable cation-transporting ATPase; aconitate hydratase	C	5.5	19.4	19	7.8	7.1	7.1	9.4	13.4
L	BL1411	cell division protein FtsK	MT	6.1	6.1	14.1	15.1	8.7		6.9	7.6
L	BL1450/ BL0527	leucyl-tRNA synthetase; 4-α-glucanotransferase	A, C	6.8	15.1	20.7	7.7	7.1	5.3	8.9	10.7
L	BL1518	α-galactosidase	C	5.3	12.4	22.5	6.9	4.9	7.6	6.4	16.9
L	BL1523	sugar permease of ABC transporter system	MT	56	5.6	14.7	16.3	7.8	6.8	5.5	7.3
L	BL1535-36	JadJ; propionyl-CoA carboxylase beta chain	LM, C		14.1	17.6					
L	BL1571-73	30S R protein S9; probable glycogen operon protein GlgX; 50S R protein L13	GI, C	6.5	19	18.6	6.6	7.9	8.8	9.4	15.1
L	BL1630	phosphoglucomutase	C	6.7	15.4	27.7	6.5	7.9	6.6	6.8	12.7
L	BL1638	solute binding protein of ABC transporter for sugars	MT	7.5	7.5	13.6	17.5	8.6	5.9	6.1	8.5
L	BL1639	permease of ABC transporter for sugars	MT				21.8				
L	BL1641	lacto-N-biose phosphorylase	FU	6.8	14.4	17.2	7.9	9			13.1
L	BL1642	putative desulfatase possibly for mucin	C			19.3		5.5	7.1		
L	BL1643-44	galactose-1-phosphate uridylyltransferase; UDP-glucose 4-epimerase	C	8.6	17.9	17.9		9.3			
L	BL1673	possible lactaldehyde reductase	C	7.3	14.9	15.9	7.7	8.2	7.7	7.8	14.5
L	BL1673-74	possible lactaldehyde reductase; probable glycosyltransferase	C	11.3	17.5	18.4	10.2	11.9	7.9	8.2	14.2

L	BL1692-93	ATP binding protein of ABC transporter for pentoses; probable repressor protein in (NagC/XylR) family	MT	7.2		17.8					
L	BL1771-72	C4-dicarboxylate transporter; sugar kinase in PfkB family	MT, C				10.6	9			
L	BL1775	fragment of β-galactosidase	C	10.8	11.6	19.7	11.2	13.2	10.9	11.9	16.7
L	Lxx04970	rhamnosyltransferase	C	7.7	12.7	14.3	6.8	5.8	9	7.6	12.8
L	Meso_2877	glycosyl transferase, group 1	C	10.9	14.8	19.7		11.2			12.5
L	Reut_B4371	AMP-dependent synthetase and ligase (acetyl-CoA synthetase)	EM, C	7.6	15.5	17.3	7	5.7		8.9	14.9
L	SAV1043	α-arabinofuranosidase II	C							6.7	

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.

Appendix 2 Other clones with signal over background values of the hybridized samples of adult 1- 4 before and after intake of the specific prebiotic mixture.

L	Locus tag	Function	KO	1		2		3		4	
				b	a	b	a	b	a	b	a
M	AAur_3512	bacterioferritin comigratory protein (thioredoxin reductase)	GI	11.5	16.9	20.9	11.3	14.8	12.6	10.2	11
M	Arth_2252	2,5-didehydrogluconate reductase	M	7.5	15.8	18.9	8.1	8	6.8	7.6	12.4
M	BAD_0055	response regulator of two-component system; queuine tRNA-ribosyltransferase; putative exodeoxyribonuclease V	EP	8.2	15	17.4	10.6	10.6	7.6	8.2	12.9
M	BAD_0243	glutamate 5-kinase	A	6.4	16.7	16.7	10.2	7.9	7.5	11.4	11
M	BAD_0322-23	50S R protein L4; 50S R protein L23	GI	17.2	20.2	18.6	16.8	18.5	12.1	15.6	13.9
M	BAD_0356-57	riboflavin kinase; tRNA pseudouridine synthase B	GI, CV	8.6	17.7	21	6.9	9.1	6.7	8.4	17.5
M	BAD_0408	hypoxanthine-guanine phosphoribosyltransferase	N	10	20.4	18.6	7.9	8.4	8.9	15.8	9.3
M	BAD_0467	SsrA-binding protein	GI	7.2	13	16.7	7.6	10.6	6.6	8.3	14.5
M	BAD_0490-92	cystathionine gamma-synthase; ATP-dependent DNA helicase RecQ; cystathionine β-synthase	EM, A, GI	6.6	14.2		6.7	6.7	5.4		15.2
L	BAD_0608/ BL0338	pyrroline-5-carboxylate reductase; aspartate ammonia-lyase	EM, A		12.9	18.8	8.1	7.3	8.7	9.2	11.9
M	BAD_0764/ BL0356	dihydroorotate dehydrogenase; ATP synthase subunit A	EM, N	8.3	20.3	16.4	8.3	11.3	12.1	10.3	15.9
M	BAD_0914	tyrosyl-tRNA synthetase	A, GI	25.6	12.7	20.4	13	14.7	10.7	14.1	18.1
M	BAD_1028	DP-diacylglycerol--glycerol-3-phosphate 3- phosphatidyltransferase	LM	9.3	15	19.2	9	7.1	7.9	8.2	12.3
M	BAD_1105-07	peptidoglycan synthetase; penicillin-binding protein 3; UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D- alanine ligase	GB	8.7	16.1	19.1	10	7.5	8.8	10.8	13.4
M	BAD_1120	ATP-dependent helicase	GI	8.8	15.9	17.4	11	9.4	8.5	7.6	15.3
M	BAD_1124	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino) methylideneamino] imidazole-4-carboxamide isomerase	A	9.4	16.8	23.3	10.9	9.1	8.1	9.7	6.8
P	BAD_1130	DNA polymerase III α subunit	GI		30.8						65.8
M	BAD_1132	possible lipoprotein signal peptidase	GI	6.9	14.1	15.4	8	7	8.3	7.2	13
M	BAD_1319-20	DNA-directed RNA polymerase β chain; HP	GI, FU	7.2	14.3	19.2	8.8	8.1	7.5	10	11.6
M	BAD_1614-15	dimethyladenosine transferase; HP	GI, FU	7.2	18.1	19.5	7.9	9.3	5.7	7.6	13
L	BL0005-06	response regulator of two-component system; histidine kinase sensor of two-component system	EP	5.6	10.6	19.8	9	9.3	7.2	7.8	15.2
M	BL0006-07	cold shock protein; histidine kinase sensor of two-component system	GI, EP	8.2	17	17.3	9.8	7.3	9.2	8.5	13.8
L	BL0012-13	HP weakly similar to putative transcriptional regulator from Streptomyces; proline/betaine transporter	FU, T	8	15.7	25.7	6.4	8.5	5.8	7.1	14.5

M	BL0017-18	histidyl-tRNA synthetase; aspartyl-tRNA synthetase	GI, A	7.9	13.2	18.8	8.3	9.9	5.8	6	13.4
L	BL0307-08	possible Rim-like protein involved in efficient processing of 16S rRNA; inosine-uridine preferring nucleoside hydrolase	GI, FU		17.2	14.1	8.6	7	7.4	8.9	11.4
L	BL0356-57	ATP synthase subunit B; ATP synthase epsilon chain	EM	6.9	14.9	20	10.2	7.3	8	8.2	19.1
M	BL0395	valyl-tRNA synthetase	GI	7.4	17	21.9	10.5	10.5	8.5	8.6	10.2
M	BL0409	copper-transporting ATPase	EM	11.8	18.9	16.8	11.1	10.8	13.1	15.7	13.3
L	BL0485	DNA topoisomerase I	GI		50.2						66.6
M	BL0485	DNA topoisomerase I	GI	5.8	14.5	21	8.2	6.8	8.3	23.1	10.1
L	BL0552	probable ferredoxin/ferredoxin-NADP reductase	EM	7	16.1	18.3	8	8.3	8.3		13.7
M	BL0552-53	probable ferredoxin/ferredoxin-NADP reductase; HP	EM	7.1	15.4	20.1	6.9	7.8	6.7	7.8	14
M	BL0630	glutamate dehydrogenase	A	6	15	18.1	8.1	8.9	5.9	9	17.5
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		17.8	16	6.2	6.6	7	8.3	
L	BL0670	ribonucleotide-diphosphate reductase a subunit	N	6.2	16.5	20	8.9	8.1	7.6	10.7	13.1
L	BL0670	ribonucleotide-diphosphate reductase a subunit	N					12.1			
L	BL0742	probable helicase	GI	6.5	12.7	15.9	7.8	6.3	7.4	6.4	12.7
L	BL0795-96	glutamate-ammonia-ligase adenylyltransferase; choloylglycine hydrolase	LM, A		14.1						
L	BL0820-21	HP; possible thioredoxin-dependent thiol peroxidase	FU, GI	7.5	16.8	23	6.8	6.4	6.3	7	11.7
M	BL0934	possible pyridoxine kinase	CV	8.8	13.8	20	11.2	10	6	8.9	14.3
L	BL0949-50	narrowly conserved HP; pyruvate formate-lyase 1 activating enzyme	GI	6.2	15	18.8	8.1	6.2	5.9	7.5	11.7
L	BL1148	deoxyguanosinetriphosphate triphosphohydrolase-like protein	N					7.9			
L	BL1152-53	S-ribosylhomocysteinase; ATP-dependent DNA helicase RecQ	A, GI	6.8	14.7	20.6	8.2	8	7.7	7.5	16
L	BL1204	DNA-directed RNA polymerase β subunit	GI	6.2							
L	BL1207-08	probable RNA methyltransferase;probable A/G-specific adenine glycosylase	GI	7	12.1	18.7	7.2	7.4	7.9	8.6	14.5
L	BL1275-76	possible homoserine kinase; HP with MutT domain	A, FU						6.7	10.4	
L	BL1280-81	succinyl-diaminopimelate desuccinylase; ribonuclease G	A, GI	8.4	17.6	21.6	9.8	9.3	9.8	7.9	13.4
L	BL1287-89	probable transcription antitermination protein; HP;50S R protein L11; preprotein translocase SecE subunit	GI	8.4	16.4	19.5	8.8	6.7	8.6	8.6	13.6
L	BL1291	50S R protein L1	GI								41.4
L	BL1402-03	response regulator of two-component system; atypical histidine kinase sensor of two-component system	EP	6.7	18.3	18.2	8.9	5.9	6.3	7.8	11.8
M	BL1422	anthranilate phosphoribosyltransferase 1	A		28.2		19.6				73
M	BL1429	DNA gyrase subunit B	GI	11.5	16.2	18.1	9	13.6	12.4	13	11.1

L	BL1537	Fas (fatty acid synthase)	LM	7.6	14.3	22.6	10.2	9.8	11.8	10	11.5
L	BL1549-50	50S R protein L10; 50S R protein L7/L12	GI, PM	8.7	13.9	15.3	10.1	9.8	7.4	7.6	9.4
M	BL1564-65	HP; DNA-damage-inducible protein P	FU, GI	9.5	15.1	17.4	9.1	9.9	7.8	7.4	14.1
L	BL1589	30S R protein S17	GI	6.7	15.5		7.9	8.1			10.4
L	BL1616	translation initiation factor IF-2	GI	4.8	14.7	18.5	7.6	7.3	6.4	5.7	11.7
L	BL1646-47	possible histidine kinase sensor of two component system; HP	EP, FU	6.8	12	22.3	9.3	8.1	8.2	9.3	15.4
L	BL1665-66	thymidylate synthase; dihydrofolate reductase	CV	5.4	16.1	22.9	8.4	6.8	7.2	7.7	12.3
L	BL1732	methionine aminopeptidase	GI	5.2	15.6	20.4	7.7	7.5	6.4	7.8	12.6
L	BL1751-52	exodeoxyribonuclease VII large subunit; anaerobic ribonucleoside triphosphate reductase	GI, N	6.7	17	18.3	9.3	10.3	8.4	9.8	11.5
L	BL1752	anaerobic ribonucleoside triphosphate reductase	N	9.8		18.2	8.6	9.7	10.2	9.3	13.6
L	BL1811-13	uracil-DNA glycosylase; HP; widely conserved MoxR-like protein in magnesium chelatase family	GI	10	17.8	19.8	7.2		5.6	8.8	16.2
M	BLt02	Trp tRNA	PM								11.8
M	Francci3_2963	putative transposase, IS891/IS1136/IS1341	GI	9.3	14.3	17.1	13.1	10.8	8.2	11.4	13.6
L	Francci3_4210	Integrase	GI	7.2	13.5	17.6	7.4	8.2	5.8	6.9	13.6
L	MPN198	adenine-specific methyltransferase EcoR	GI					10.7		10.3	
L	NTHI1839	putative type I restriction enzyme HindVIIPspecificity protein	EM	6.9	13.5	19.7	8	9.2	7.7	10.3	11.6
M	str0679	MutT/nudix family protein	GI	8.3	20.1	22.1	9	7	7	8.2	13.5

Abbreviations as for Appendix 1

6

Human Milk, Formula Milk, and Prebiotic Galactooligosaccharide-Induced Gene Expression in the Intestinal Bacterium *Bifidobacterium longum* Analyzed with Clone-Based Microarrays

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In order to gain insight in the effects of human breast milk on the development of the intestinal bifidobacteria and associated health effects, the transcriptome of *Bifidobacterium longum* LMG 13197 grown in breast milk, and formula milk containing galactooligosaccharides (GOS) and long chain fructooligosaccharides (lcFOS) was compared to that obtained in a semi-synthetic medium with glucose. Total RNA was isolated from exponentially-growing cells from the various media and hybridized to a clone library-based microarray. Inserts of significantly hybridizing clones were sequenced and tentatively identified. Although the *B. longum* transcriptomes obtained following growth on human and formula milk were more similar than that on glucose medium, there were remarkably few genes amongst those sequenced for sugar utilization shared between the two milks, in spite of the high concentration of oligosaccharides present in the formula milk. Notable common highly upregulated genes included putative genes for cell surface type 2 glycoprotein-binding fimbriae that are implicated in attachment and colonization in the intestine. Genes involved in carbohydrate metabolism formed the dominant upregulated group specifically for breast milk. These included putative genes for N-acetylglucosamine degradation, and metabolism of mucin and human milk oligosaccharides via the novel galactose/ lacto-N-biose gene cluster that were specific to human milk. This supports the notion that the bifidogenic effect of human milk is to a great extent based on its oligosaccharides. The transcriptional effect of specifically added GOS, which is formed of transglactosylated lactose and galactose, on the *B. longum* transcriptome, was also studied and revealed quite some overlap in up-regulation of carbohydrate utilization genes between the GOS medium and human milk. This knowledge provides leads to optimize formula milk to better simulate the observed bifidogenic effects of human breast milk.

Introduction

Intestinal colonization by microorganisms has a pronounced impact on the maturation of the infants' intestinal immune system and the gut microbiota is profoundly influenced by the feeding regime of the human infant. Consequently within a few days, human milk stimulates bifidobacteria to be amongst the dominant microorganisms, and may comprise up to 90% of the infant gut microbiota in breast-fed infants. In contrast, formula-fed infants have a more complex microbiota (58). The postnatal maturation of a balanced immune system requires constant microbial stimulation from the developing gut microbiota (30, 85). Many beneficial effects have been claimed specifically for the bifidobacteria, including protection against pathogens (68), normal development and maintenance of a balanced immune system (30, 85, 198), and positive nutritional effects for the intestinal cells and the host (154).

Complex neutral oligosaccharides have been identified as the most likely prebiotic factor in human milk that stimulates the growth of bifidobacteria in the infant gut (22, 35, 55). The oligosaccharides are quantitatively one of the main components of human milk and are only partially digested in the small intestine, so they reach the colon, where they stimulate selectively the development of microbiota dominated by bifidobacteria (35). Furthermore, proteolytic fragments of major human milk proteins are also effective as growth factor for bifidobacteria (117). Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve host health (65). Like human milk oligosaccharides, the supplementation of infant formula with certain prebiotics stimulates the number of fecal bifidobacteria (76, 178). Several investigators have reported the bifidogenic effect of human milk oligosaccharides (6, 24, 35). Feeding infants formula supplemented with galactooligosaccharides and long chain fructooligosaccharides (GOS/lcFOS) (1:9) significantly increased the number of bifidobacteria accompanied by a reduction of pathogens in both preterm infants and term infants (23, 102, 104). Moreover, the GOS/lcFOS supplement caused the microbial diversity to closely resemble the microbiota of breast-fed infants, also at the level of the different *Bifidobacterium* species (6, 76).

The molecular mechanisms behind the influence of breast feeding on the development of the intestinal bifidobacterial community is not yet completely understood. The publication of the genome sequence from *Bifidobacterium longum* NCC2705 revealed that the chromosome encodes numerous genes for carbohydrate utilization (197). There was a preference for di, -tri and oligosaccharides pointing towards a biased utilization for complex oligosaccharides (161, 162) complemented with transporters for a variety of disaccharides and oligosaccharides (161). This suggests adaptation to a special colonic niche and utilization of specific dietary components (197). Recently, bifidobacterial-specific microarrays, comprising clone-library containing inserts of seven bifidobacterial species were constructed (26). These were used to determine the gene expression of bifidobacteria within infant feces

that were fed with formula or human-milk, which suggested the importance of several genes for intestinal colonization (Chapter 4 this thesis).

In the present study the effects of human milk and formula milk on the global gene expression of bifidobacteria was investigated to gain some insight on the impact of the milks on the development and colonization of intestinal bifidobacteria within the infant gut. Transcriptomics was performed on *B. longum* LMG 13197 following growth in human and formula milk and also in comparison to semi-synthetic medium. This was complemented with the metabolic effect of galactooligosaccharides, a component of human milk oligosaccharides, on the transcriptome of *B. longum* LMG 13197.

Materials and Methods

Bacterial strains and cultivation conditions

Bifidobacterium longum LMG 13197 was obtained from the LMG Collection (Ghent Belgium) and has been isolated from human infant feces. *B. longum* LMG 13197 was maintained in MRS medium and glycerol (1:1 v/v) at -80ºC. An overnight culture (no more than 12 h old) grown in TPYG, pH 7 (17), was used as inoculum (5% v/v).

Growth in milk and semi-synthetic medium with prebiotics

Human milk was aseptically collected from 2 volunteers with a sterile pump and immediately frozen at -20°C. Commercial formula milk (Nutrilon 1 with galacto oligosaccharides and polyfructose; Nutricia, Zoetermeer, Netherlands) was dissolved to 10% solution demi-water. Both milks were centrifuged for 5 minutes' at 5000 rpm (4ºC) and filtered to remove fat. Filtered sterilized cysteine was added (0.5 g/L) and the pH was adjusted to 6.5. Fifty ml of milk was added to sterile flasks with stoppers, sealed with aluminum rings and placed in a water bath for 20 minutes at 80 °C. After cooling to room temperature, flasks were flushed with CO_2/N_2 using sterile filters. They were placed a second time in a water bath for 20 minutes at 80°C and cooled to room temperature overnight.

A semi-synthetic medium was prepared with a minimal carbon source (6.7 g/L yeast nitrogen base (Difco), 0.5 g/L cysteine, 1 ml Tween 80 and 40% v/v salt solution: 0.2 g/L $CaCl_2$, 1 g/L K_2HPO_4, 1 g/L KH_2PO_4, 10 g/L $NaHCO_3$, 2 g/L NaCl, 0.5 g/L casein enzymatic hydrolysate, 0.05 g/L sodium thioglycolate) such that it could be supplemented with different carbon sources to be monitored (91). Transcriptomics of *B. longum* in HM and FM was compared to the semi-synthetic medium containing 5 g/l glucose as sole carbon source. Colony forming units (CFUs) were determined by plating on MRS agar medium (Difco, France) with cysteine (0.5g/L) followed by incubation at 37°C anaerobically.

The semi-synthetic medium, supplemented with GOS (composed of 33% disaccharides, 39% trisaccharides, 18% tetrasaccharides, 7% pentasaccharides, 3% hexa-, hepta-, and octasaccharides; Friesland Foods, The Netherlands) as sole carbon source was used to study transcriptomics of *B. longum* in prebiotics. For comparative purposes *B. longum* was grown in semi-synthetic medium with glucose or no carbon source. Growth was followed by measuring optical density A_{600}. Carbon solutions were sterilized separately and added prior to fermentation at a final concentration of 5 g/L. All media and carbon solutions were flushed with CO_2/N_2, closed with rubber stoppers, sealed with aluminium rings and sterilized at 120°C for 15 minutes. All tests were performed in duplicate at 37°C with agitation (200 rpm) during 10 hours and samples were taken every 2 hours for pH measurements and determination of CFUs by plating on MRS with cysteine (0.5g/L) as described above.

RNA isolation and purification

Cells for RNA isolation (50 ml) were harvested at mid exponential phase and concentrated immediately by centrifugation at 12,000 rpm for 10 min at 4ºC. After centrifugation, the supernatant was discarded and the cell pellet was snap frozen in liquid nitrogen and stored at -80°C. Samples for RNA isolation were taken from milk and glucose cultures after 4 h, and from GOS cultures after 6 h (see Figure 2, 3). RNA isolation was performed using the Macaloid method (166). Briefly, cell pellets were resuspended in 0.5 ml of TE buffer (10 mM; pH 7.5) added to tubes containing 0.8 g of 0.1 mm Zirkonia beads (Biospec Products, Bartlesville), 0.18 g Macaloid in TE (Kronos Titan GmbH Leverkusen); 50 µl of 10% SDS and 0.5 ml acidic phenol (pH 3.75). Cells were disrupted using 3 treatments of 45 sec in a Fastprep (Qbiogene, USA) interspersed by 90 sec incubation on ice. After disruption the samples were centrifuged at 4ºC for 10 min at 12 000 rpm,. The aqueous phase was used for RNA isolation by extraction with chloroform: phenol: isoamylalcohol (1:1:1) twice and chlorophorm: isoamylalcohol (1: 24) once. This was followed by a cleaning step using RNeasy columns (Quiagen Sciences, Maryland, USA) according to the RNeasy cleaning protocol and treatment with DNAse (Roche Diagnostics, Mannheim, Germany) on the column. After elution from the column, RNA concentrations were measured using the Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE, USA). Additionally, quality and concentration of the RNA was measured using the Experion Automated Electrophoresis System (Bio-Rad, USA) with RNA StdSens microfluidic chips. Total RNA samples with a 16S/ 23S ribosomal RNA ratio of at least 2.0 was used for microarray experiments.

Reverse transcription of RNA

Five µg of RNA was mixed with 2.5 µl random p6 primer (2 µg/µl, Roche Diagnostics, Mannheim, Germany) and 2 U RNasin (Promega, Madison, WI, USA). This was incubated on ice for 10 min following an incubation of 5 min at 70°C and cooled to room

temperature. On ice, 5 U RNAsin, 0.1 μM DTT, aa-dNTP, and 200 U Superscript II RT enzyme (Life Technologies S.A., Merelbeke, Belgium) was added and incubated for 3 h at 42°C. RNA was hydrolyzed by adding 6.25 μmol NaOH and incubated at 37°C for 30 min. To neutralize pH 6.25 μmol HAc was added. Unincorporated aa-dUTP and free amines were removed using QIAquick columns (Qiagen Sciences) according to the supplier's protocol.

Fluorescent labeling and hybridization to bifidobacteria microarrays

The microarray slides were provided by TNO Nutrition and Food Research, The Netherlands (26). The array contained 2000 spots of a *B. longum* LMG 13197 clone library containing an average insert size of approximately 1.5 kb covering 85% of the genome. Slides were incubated in preheated prehybridization solution (1% BSA, 5 x SSC, 0.1% SDS; 0.45 μm filtered) at 42 ºC for 45 min under rotation, washed and dried under N_2-flow. cDNA was dissolved in 4.5 μl 0.1 M sodium carbonate buffer, pH 9.0, for 10 min at room temperature. An aliquot of 4.5 μl of the appropriate NHS ester Cy-dye was added, and incubated in dark at room temperature for 1 h. After incubation, unincorporated dyes were removed using autoseq G50 columns (AmershamBiosciences, Freiburg, Germany). The Cy5- and Cy3-labeled probes were combined and dried using vacuum centrifugation. The samples were dissolved in 45 μl of Easyhyb buffer at 42 ºC for 15 min. The preparations were mixed well and spun down briefly. The subsequent steps were performed at 42 ºC. The prehybridized microarray slides were placed on hybridization chambers (Corning) moistened with milliQ water. Hybridization mixtures were pipetted onto the slides, and covered with glass lids. The chambers were immediately sealed and the hybridization was then allowed to take place overnight at 42 ºC. Afterwards, the slides were washed by placing them in 50 ml tubes containing the following wash solutions at 37 ºC and agitated for 10 sec: 1x SSC / 0.2% SDS, 0.5x SSC, 0.2x SSC, and finally washed twice in 0.2x SSC with agitation on a rotation plateau for 10 min. The slides were dried under N_2-flow and scanned using ScanArray (Perkin Elmer), with two wavelengths corresponding to the excitation wavelengths of Cy3 and Cy5 dyes. Scanned images of the slides were further processed with ImaGene 5.6 software.

Analysis of microarray data

The hybridization scheme for the various samples is presented in Figure 1. Human milk samples were compared to formula milk and semi-synthetic medium with glucose samples; glucose samples were compared to GOS samples.

For data analysis, flagged spots were eliminated followed by background subtraction, log transformation and lowess fit normalization, using the Arraynorm program (http://genome.tugraz.at). Log 2 data of the microarray fraction corresponding to *B. longum*

LMG 13197 and presented signal in biological replicates, were analyzed for significant differential expression, through Significance Analysis of Microarray (SAM) with the Tiger Multiarray viewer program (183).

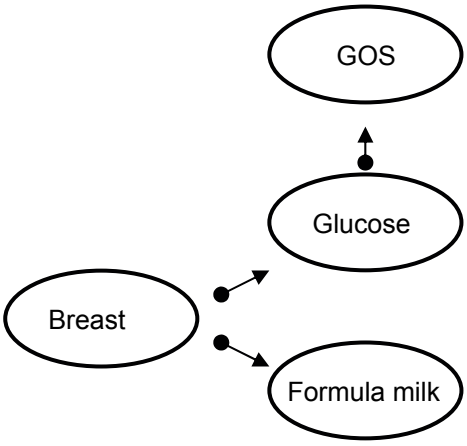

Figure 1 Hybridization scheme for the different growth media tested. The arrowheads correspond to the Cy5 labeled samples, and the dot on the arrow ends corresponds to the Cy3 labeled samples.

Nucleotide sequencing and analysis

A selection of inserts was sequenced by GATC Biotech (Germany). The Smith & Waterman algorithm (203) was applied to search the sequences against different data sets. All sequences were searched against the complete protein and nucleotide sequences of all completely sequenced genomes present in the NCBI repository at March 18th 2007. In addition, extra searches were performed using only the complete genomes of *B. longum* NCC2705 and *B. adolescentis* ATCC 15703 (Gifu University, Life Science Research Center, Japan). For the nucleotide searches, all matches with the genome sequences were analyzed for annotated features using the protein and RNA annotation files of the genomes.

Results and discussion

Growth and gene expression of *B. longum* in human and formula milks and glucose medium

To identify the different effects of human milk and formula milk on the metabolism of *B. longum*, the transcriptomes obtained after growth in the milks were compared to that obtained following growth in semi-synthetic medium with glucose. Insight in the activity of genes could give insight on the impact of the diet on the development of the bifidobacterial population within the infant intestine. Remarkably, *B. longum* displayed a slower growth rate in the human milk compared to the formula milk and semi-synthetic medium (Figure 2). Growth appeared most rapid in the formula milk and there was a corresponding rapid drop in

the pH. The pH profiles for human milk and semi-synthetic medium were comparable. Mid-exponential phase for all media was reached after approximately 4 h and at this time samples were taken for RNA isolation.

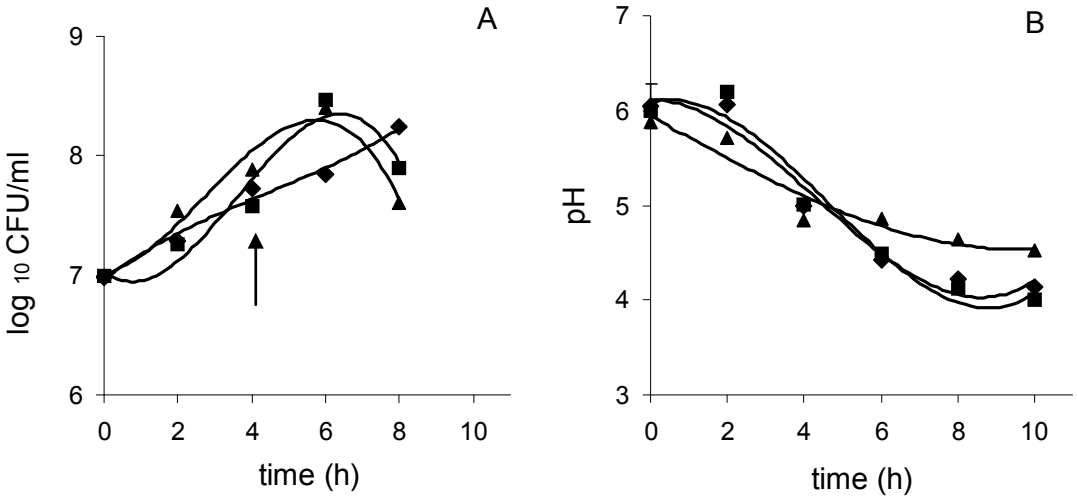

Figure 2 Growth (A) and pH curves (B) for *B. longum* in (♦) semi-synthetic medium with glucose, (■) human milk and (▲) formula milk. An arrow indicates the harvest time for RNA isolation.

Analysis of the microarrays identified 92 clones that showed significant hybridization with cDNA from RNA samples isolated from human milk compared to formula and glucose medium, of which 35 were sequenced and the genes tentatively identified (Table 1). Of these clones, 21 contained genes involved in carbohydrate metabolism and sugar membrane transport, eight were categorized as function unknown and six coded for general cellular processes.

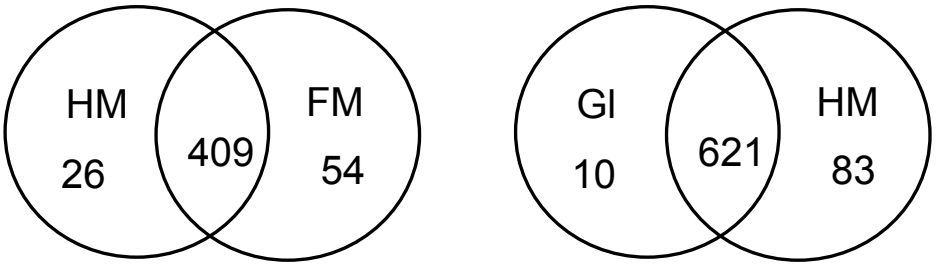

Figure 3 Venn diagram showing the number of differential and commonly hybridized clones in human milk (HM) compared to formula milk (FM), and human milk compared to glucose (Gl).

In order to identify genes induced specific to a condition, the number of clones that hybridized significantly under the different conditions were grouped and are presented in the Venn diagrams (Figure 3). Growth in human or formula milks resulted in the expression of *B.*

longum RNA that shared significant hybridization with 409 clones. In the sections below the genes expression for the various conditions are described and compared.

Genes expressed in both human and formula milks versus semi-synthetic medium with glucose

Carbohydrate metabolism. Despite the addition of oligosaccharides in the formula milk there were only a few genes implicated in carbohydrate metabolism that were similarly upregulated following growth in the two milks. These included two putative glycosyl hydrolases (BL0529 and BL0544; see Table 1 and 2), an ABC-type transporter (BL1164-65), which were reported to be induced by FOS and lactose in semisynthetic medium (161), and a solute binding protein of ABC transporter for sugars (BL1638) (Table 1).

Protein synthesis. Genes predicted to encode for a protein-PII-uridyltransferase (*glnD*) and a nitrogen regulatory protein N-II (*glnB*) (BL0433-34) were upregulated following growth of *B. longum* in human milk and even more in formula milk compared to glucose medium. These genes are involved in regulation of glutamine synthetase activity that plays an essential role in the metabolism of nitrogen by catalyzing the condensation of glutamate and ammonia to form glutamine. The *B. longum* genome contains all homologs needed for biosynthesis of pyrimidine and purine nucleotides from glutamine (197). Glutamate synthetase is regulated through a complex series of events in *E. coli* and many bacteria, in response to a low ammonia concentration in the environment (147). This could be related with the ammonia liberated through the glucosamine-6-P isomerase (BL1343) activity, mentioned below, which was significantly upregulated in human milk. A clone coding for a hypothetical protein and a putative methionine aminopeptidase (BL1731-32) could indicate removal of N-terminal methionine from nascent proteins and was upregulated in both milks. Two of the other putative specific transport systems for peptides in the *B. longum* DJ010A (118) were not detected, either due to low signals or possibly due to absence of these genes in the clone library. The nitrogen content in formula milk originates mainly from proteins and oligopeptides. The nitrogen regulatory proteins as well as genes involved in amino acid metabolism indicate assimilation of nitrogen by *B. longum* via peptide hydrolysis. Another upregulated gene shared by growth both milks was a putative ABC transporter for zinc and manganese (BL0993-94).

Colonization factors. The genes for rather unusual cell surface proteins with high similarity to the type 2 glycoprotein-binding FimA fimbrial subunit and sortase-like fimbria-associated protein of the oral inhabitant *Actinomyces naeslundii* (BL0675-76 Table 1, 2) were highly upregulated following growth of *B. longum* in human milk and even more in formula milk (Table 2). These proteins are involved in the adhesion of *A. naeslundii* to microbial oral biofilms and interaction with epithelial surfaces (135, 160). The genes contained several motifs: a putative Ig domain found in other cell-surface proteins; a Cna protein B-type

domain found in *Staphylococcus aureus* collagen-binding surface protein which forms a repetitive β-sandwich structure that presents the ligand-binding domain away from the bacterial cell surface; and a surface protein with the sortase LPxTG motif, which can function as an anchor on the cell wall. There is some tentative evidence that bifidobacteria have fimbria-like structures (197), however, further characterization is required to determine if they contribute to attachment or retention in the human intestine.

Genes upregulated specifically in human breast milk

Complex neutral oligosaccharides have been identified as the most likely prebiotic factor in human milk that stimulates the growth of bifidobacteria in the infant gut. This was supported by the high percentage of genes upregulated with predicted functions in transport and metabolism of oligosaccharides in human compared to formula milk (Table 1). Significantly upregulated genes included those for putative N-acetylglucosamine-6-P deacetylase (BL1344) that catalyses the deacetylation of N-acetyl-D-glucosamine 6-P, and glucosamine-6-phosphate isomerase 1 (BL1343) which converts D-glucosamine-6-P to D-fructose-6-P and ammonia. N-acetylglucosamine-containing oligosaccharides are regarded as the most bifidogenic oligosaccharides in human milk (106). Consequently *B. longum* would be able to use the *N*-acetyl-glucosamine-6-P in the human milk as a source of carbon and nitrogen.

Other significant genes involved in carbohydrate metabolism upregulated specifically in human milk included a putative desulfatase (BL1642) and sugar permease subunit (BL1639, Table 1). The latter form part of a novel putative operon for metabolism of mucin sugars and human milk oligosaccharides in *B. longum* JCM1217. The complete operon encodes an ATP-binding cassette (ABC)-type sugar transporters, lactose-N-biose phosphorylase, mucin desulfatase, galactose-1-phosphate uridylyltransferase, and UDP-glucose 4-epimerase. It is speculated that this operon plays a critical role in the bifidogenic effect of human milk as the lacto-N-biose structure appears to be unique to human milk oligosaccharides (98). Moreover, intestinal colonization by bifidobacteria would be enhanced by metabolism of mucin sugars. The putative (galacto) oligosaccharide transporter genes (BL1638 and BL1639) were previously found to be induced *in vitro* by FOS, and expressed but not induced by lactose, raffinose and maltose (161). Another gene involved in galactose metabolism, a galactose-1-phosphate uridylyltransferase gene (BL1211) was also specifically upregulated in breast milk.

Table 1 *B. longum* genes upregulated in human milk compared to formula milk and semi-synthetic medium with glucose.

Locus tag	Function	Kegg	Fold change (log2 ratio)	
			Glucose	Formula
BL1638	solute binding protein of ABC transporter for sugars	MT	5.54	[b] 0
[a] **DVU_1515**	type II DNA modification methyltransferase, putative (*Desulfovibrio vulgaris* subsp. *vulgaris* str. *Hildenborough*)	FU	5.07	6.46
BL1639	permease of ABC transporter for sugars	MT	4.52	4.39
BL1642	putative desulfatase possibly for mucin	FU	4.47	4.56
BL1673-74	possible lactaldehyde reductase, probable glycosyltransferase	C, FU	4.41	4.09
BL0978	β –galactosidase (lacZ)	C	4.29	2.40
EF2229	HP (*Enterococcus faecalis* V583)	FU	3.85	[c] -
BL1344	*N*-acetylglucosamine-6-phosphate deacetylase	C	3.26	3.62
BL0919-20	possible efflux transporter protein	FU	3.22	3.53
BL1343	glucosamine-6-phosphate deaminase	C	3.13	4.0
BL1673	possible lactaldehyde reductase	C	2.98	-
BL0454-55	narrowly conserved HP, widely conserved hypothetical membrane protein	FU	2.93	5.06
BL1674	probable glycosyltransferase	C	2.61	-
BL1211	galactose-1-phosphate uridylyltransferase	C	2.11	-
BL0992	30S ribosomal protein S1	TR	2.09	-
BL1411	cell division protein FtsK	PS	1.99	2.04
BL0951-53	formate acetyltransferase, HP, glutamine-dependent NAD(+) synthetase	C, FU, NM	1.90	2.27
BL0675	possible cell surface protein similar to FimA fimbrial subunit of *Actinomyces naeslundii*	FU	1.73	-1.78
BL0676	sortase-like protein similar to fimbria-associated protein of *Actinomyces naeslundii*	FU	1.69	-1.71
BL1164	probable solute binding protein of ABC transporter system for sugars	MT	1.44	0
BL0949-50	narrowly conserved HP, pyruvate formate-lyase 1 activating enzyme	FU, EM	1.40	-
BL1402-03	response regulator of two-component system, atypical histidine kinase sensor of two-component system	EP	1.24	-
BL1589	30S ribosomal protein S17	TR	1.22	-
BL0197-198	possible ATP binding protein of ABC transporter, hypothetical membrane protein with unknown function	FU	1.17	-
BL1164-65	probable solute binding protein of ABC transporter system for sugars, probable solute binding protein of ABC transporter system for sugars	MT	1.11	0
BL0716	transketolase	C	0.99	-
BL0715	transaldolase	C	0.81	-
BL0157	narrowly conserved HP	FU	0.56	-
BL0671-72	ribonucleotide-diphosphate reductase beta subunit, probable glycosyltransferase	N, FU	0.47	-1.73
BL0433-34	protein-pII; uridylyltransferase, nitrogen regulatory protein N-II	EP, NM, EM	0.36	-2.34
BL1545-46	30S ribosomal protein S15, A polyribonucleotide nucleotidyl-transferase	TR, N	0.22	-
BL0976	galactoside symporter (*lacS*)	MT	0.20	-
BL0742	probable helicase	RR	0.16	-

BL0284	Fem AB-like protein for formation of peptidoglycan	CP	-	0
BL0529	probable α-1,4-glucosidase	C	-	0
BL0544	α-L arabinosidase	C	-	0
BL0872	possible ABC transporter component	MT	-	0
BL0966	phosphoglycerate mutase	C	-	0
BL0993-94	methylenetetrahydrofolate dehydrogenase (NADP+), solute binding protein (zinc/manganese) of ABC transporter system	CV, MT	-	0
BL1169-70	probable permease of ABC transporter system for sugars	MT	-	0
BL1731-32	HP, methionine aminopeptidase	NM	-	0

[a] Clones that were significantly upregulated in human milk compared to both glucose medium and formula milk are in bold, [b] 0 implies expressed but not upregulated, [c] no significant signal detected.
A: Amino Acid Metabolism, C: Carbohydrate Metabolism, CV: Metabolism of Cofactors and Vitamins, EM: Energy Metabolism, EP: Environmental Information Processing, FU: Function Unknown, GI: Genetic Information Processing, HP: hypothetical protein. LM: Lipid Metabolism, MT: Membrane Transport, N: Nucleotide Metabolism, NM: Nitrogen metabolism, PM: protein metabolism, PS: Cellular Processes and Signaling, PyM: Pyruvate Metabolism, RR: Replication and Repair, TR: translation.

As expected, since lactose is the predominant sugar in the milk, a putative gene for lactose transport, galactoside transporter *lacS* (in clone BL0976), was upregulated in human milk, although rather surprisingly it was not detected in formula milk. Note the expression of *lacS* in *B. longum* NCC2705 was induced by several carbohydrates besides lactose including raffinose, maltose and FOS (161, 162) so perhaps alternative sugar sources in human milk promoted the strong up-regulation. On the other hand the amount of lactose reaching the newborn colon also from formula milks is low (36). Significant up-regulation of a putative β-galactosidase gene (*LacZ*, BL0978) was also detected in human milk; the latter may be involved in hydrolysis of lactose to glucose and galactose and/ or alternatively in hydrolysis of terminal non-reducing β-D-galactose residues in milk oligosaccharides (24) since up-regulation of the gene was not detected in formula milk. Only in human milk, a tentative lactaldehyde reductase (BL1673) gene, that anaerobically reduces L-lactaldehyde (a product of both the L-fucose and L-rhamnose catabolic pathways to L-1,2-propanediol was highly upregulated (141). It is noteworthy that L-fucose can be derived from human milk oligosaccharides (106) or mucin sugars. Putative genes for transketolase and transaldolase, involved in the pentose phosphate pathway (BL0715-16 and BL0966) were upregulated to a great extent in the human milk compared to glucose medium. The pentose phosphate pathway, besides energy metabolism, is used for reducing agents, nucleotide formation, and reducing equivalents for synthetic purposes (15), suggesting an enhanced requirement for the latter in human milk. A bifidobacterial transaldolase protein was previously identified *in vivo* in infants feces via a proteomics approach (99). This founds the assumption that *in vitro* measured activities can also be expected *in vivo*.

Table 2 *B. longum* genes highly upregulated in formula compared to human milk.

Locus tag	Function	Kegg	Fold change (*log 2* ratio)
BL0012-13	HP weakly similar to putative transcriptional regulator from *Streptomyces,* proline/betaine transporter	FU	4.30
BL1647	HP	FU	4.23
BL1534	putative biotin biosynthesis protein BioY	FU	3.45
BL0841-42	HP, widely conserved hypothetical transmembrane protein with duf013	FU	3.01
BL1537	fatty acid synthetase (*Fas*)	LM	2.86
BL1775	fragment of β- galactosidase	C	2.83
BL1148	deoxyguanosinetriphosphate triphosphohydrolase-like protein	N	2.63
BL1114-15	HP in ImpB/MucB/SamB family of UV repair proteins, S-adenosyl-L-homocysteine hydrolase	RR, A	2.45
BL0841-42	HP, widely conserved hypothetical transmembrane protein with *duf*013	FU	2.45
BL0433-35	protein-pII; uridylyltransferase, nitrogen regulatory protein N-II, possible ammonium ion transporter	NM, MT	2.34
BL0613	probable integral membrane transporter	MT	1.96
[a]BL0675	possible cell surface protein similar to FimA fimbrial subunit of *Actinomyces naeslundii*	FU	1.78
BL0671-72	ribonucleotide-diphosphate reductase β subunit, probable glycosyltransferase	N, FU	1.73
BL0676	sortase-like protein similar to fimbria-associated protein of *Actinomyces naeslundii*	FU	1.71
BL0433-434	protein-pII; uridylyltransferase, nitrogen regulatory protein N-II	NM	1.63
BL1647-48	HP	FU	1.60
BL0604	phosphoenolpyruvate carboxylase	C, EM	0.69

[a]In bold, clones also induced in human milk compared to glucose. Abbreviations for KEGG, as in Table 1.

Highly upregulated genes in formula milk versus human milk

There were 54 genes significantly upregulated in formula milk compared to human milk (and glucose medium) and the sequence analysis of 17 clones are presented in Table 2. Amongst the genes tentatively identified were those annotated for carbohydrate utilization including a fragment of LacZ and a glucosyltransferase (BL1775, BL0672). Overall the number of differentially expressed genes involved in carbohydrate metabolism in formula milk was much less than those upregulated in human milk. One could speculate that this is due to a lower carbohydrate metabolism in formula compared to human milk by *B. longum*, however, only a minor amount of the induced clones were sequenced and identified.

A few of the genes were also upregulated in human milk but more strongly in formula milk (Table 2). Namely, the putative genes for regulation of glutamine synthase activity (BL0433-34) were highly upregulated, more than in human milk. The same was observed for the potential fimbriae genes discussed above. Other formula-specific upregulated genes were involved in membrane transporter such as a possible oxalate/ formate transporter (BL0613), lipid metabolism (BL1537) and several genes with unknown function. A nucleotide hydrolyzing enzyme (BL1148) was found to be upregulated, as well as a ribonucleotide-2P reductase (BL0671), possibly to the influence of the high level of nucleotides in formula compared to human milk. A putative *bioY* gene (BL1534), encoding part of a group of proteins BioMNY which are considered to constitute tripartite biotin ABC-transporters in

prokaryotes, was significantly upregulated in formula milk (84). This might be due to the presence of a high concentration of biotin in the formula milk of (17 µg/ L) compared to the human milk used in this study, as the level can vary between 5 to 11 µg/ L (9). Enzymes needed for synthesis of biotin appeared to be missing from the *B. longum* genome sequence (197).

Transcriptomics of *B. longum* on galactooligosaccharides

It was interesting to gain deeper insight in the effect of specific β-galactooligosaccharides (GOS) on the transcriptome of intestinal bifidobacteria, since like human oligosaccharides, they harbor a large amount of lactose and galactose molecules. β-galactooligosaccharides are manufactured from lactose by glycosyl transfer catalyzed β-galactosidase and occur as complex mixtures with various glycosidic linkages (41). Note the core molecule in human milk is characterized by repetitive attachment of galactose and N-acetyl glucosamine in β-glycosidic linkage to lactose (24). Galactooligosaccharides have previously been demonstrated to be metabolized by bifidobacteria *in vitro* (229) besides increase fecal bifidobacteria numbers *in vivo* (121). In this study, the transcriptome of *B. longum* growing on purified GOS compared with glucose was studied at mid-exponential growth (Figure 4). *B. longum* grew more slowly on GOS than on glucose and with a concomitant slower production of acids. No growth was observed when growing on semi-synthetic medium without a carbon source (Figure 4).

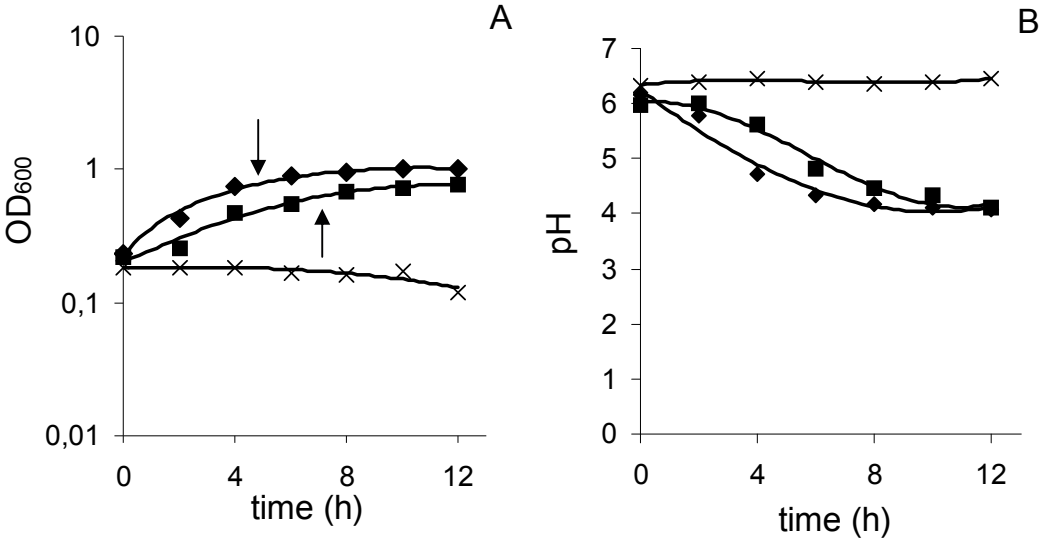

Figure 4: Growth (A) and pH (B) of *B. longum* on (♦) glucose, (■) galactooligosaccharides (GOS) and (x) no carbon source with standard deviation. An arrow indicates the harvest time for RNA isolation.

Comparison of the transcriptome of *B. longum* grown in medium with GOS to glucose showed 91 significantly induced clones (Table 3). Sequencing of 36 clones revealed 25 clones involved in carbohydrate metabolism and transport, one for amino acid biosynthesis, one for ribosomal genes, one for genetic information, one for cellular processes and 17 with unknown functions.

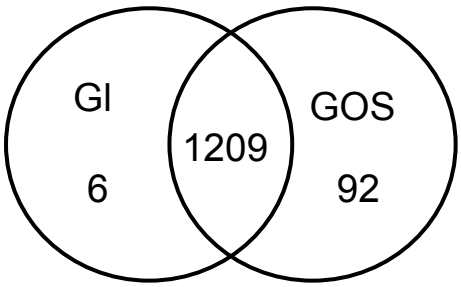

Figure 5 Venn diagrams showing the number of differential and commonly hybridized clones of GOS compared to glucose.

The Venn diagram (Figure 5) indicates that under GOS conditions many more genes are upregulated compared to the glucose condition probably due to the complex nature of GOS. The degradation of GOS requires a wide range of enzymes and transporters for uptake into the cell which are discussed below.

Genes specifically up-regulated in GOS. In comparison to glucose medium and the human and formula milks, putative genes unique to GOS included β-galactosidase I (BL0259) and genes coding for an ABC sugar permease transporter (BL0260-61) within the same gene cluster. These proteins were reported to be similar to the predicted lactose ABC permease of Streptomyces coelicolor and were shown to be expressed by growth on lactose, raffinose, maltose and FOS by B. longum NCC2705 (161). Another sugar permease transport system (BL1523) was also specifically induced by GOS; the latter was previously shown to be induced by raffinose in vitro (161) and also in this study (data not shown).

Shared upregulated genes in GOS with human or formula milks. There was quite some overlap in up-regulation of carbohydrate utilization genes between the GOS medium and human milk. Other putative genes for β-galactosidase (BL0978), galactoside transport (BL0976), galactose-1-phosphate uridylyltransferase (BL1211), glycosyltransferase (BL1674), and possible lactaldehyde reductase (BL1673) were detected that were also upregulated human milk (Table 1 and 4) which indicate specifically induced carbohydrate metabolism. Genes BL1638 and BL1639 that form part of a predicted transporter in a novel gene cluster for metabolism of mucin sugars and human milk oligosaccharides as described above (Table 3) (98) and other genes for transport of sugars (BL1164-BL1165) were significantly upregulated in GOS compared to glucose and induced by growth in human and formula milks as well.

Table 3 *B. longum* induced genes by growth on GOS compared to glucose in semi synthetic medium.

Locus tag	function	Kegg	Fold change (log 2 ratio)
BL0978	β –galactosidase (*LacZ*)	C	3.9
BL1673-74	possible lactaldehyde reductase, probable glycosyltransferase	C, FU	3.83
BL1164*	probable solute binding protein of ABC transporter system for sugars	MT	3.75
BL1164-65*	probable solute binding protein of ABC transporter system for sugars, probable solute binding protein of ABC transporter system for sugars	MT	3.43
BL1673	possible lactaldehyde reductase	C	3.38
BL1402-03	response regulator of two-component system, atypical histidine kinase sensor of two-component system	FU	3.12
BL0157	narrowly conserved HP	FU	3.09
BL0157-58	narrowly conserved HP, very narrowly conserved HP	FU	2.96
BL0919	possible efflux transporter protein	FU	2.78
BL0976	galactoside symporter (*LacS)*	C	2.66
BL0949-50	narrowly conserved HP, *pflA* pyruvate formate-lyase 1 activating enzyme	FU, PyM	2.46
BL0260-61	sugar transport system permease protein; sugar transport system permease protein	MT	2.42
BL0950-51	pyruvate formate-lyase 1 activating enzyme, formate acetyltransferase	PyM	2.24
BL0259	β -galactosidase I	C	2.20
BL0951-53	formate acetyltransferase (*Pfl*), HP, glutamine-dependent NAD(+) synthetase (*NadE*)	PyM, FU, CV	2.17
BL1638	solute binding protein of ABC transporter for sugars	MT	2.13
BL1523	sugar permease of ABC transporter system	MT	2.01
DVU_1515	putative type II DNA modification methyltransferase (*Desulfovibrio vulgaris* subsp. *vulgaris str. Hildenborough*)	FU	1.77
BL0157	narrowly conserved HP	FU	1.58
BL1639	permease of ABC transporter for sugars	MT	1.57
BL1674	probable glycosyltransferase	FU	1.49
BL0978 b	β –galactosidase (*LacZ*)	C	1.45
BL0699	hypothetical myosin-like protein with unknown function	FU	1.39
BL0454-55	narrowly conserved HP; widely conserved hypothetical membrane protein	FU	1.30
BL1211	galactose-1-phosphate uridylyltransferase	C	1.24
BL1411	cell division protein FtsK	PS	1.17
BL1571-73	50S ribosomal protein L13, *rpsI* 30S ribosomal protein S9, probable glycogen operon protein *GlgX*	R, C	1.05
BL1098	elongation factor G	GI	1.04
BL1646-47	possible histidine kinase sensor of two component system, HP	FU	0.92
BL1647-48*	HP	FU	0.89*
BL1396-97	probable cation-transporting ATPase, aconitate hydratase	FU, C	0.69
BL1731-32 #	HP, methionine aminopeptidase (map)	PM	0.57

In bold, clones also induced in human milk compared to glucose. Underlined, clones induced in human compared to formula milk. *Induced in formula compared to human milk. #Expressed but not induced under all other conditions. Abbreviations as in table 1.

Conclusions

The gene expression of *B. longum*, a common intestinal bacterium, was studied to gain insight in the effects of human breast milk versus formula milk on its functionality and associated health effects within the human intestine. Transcriptomes of *B. longum* grown in breast milk, formula milk containing GOS/lcFOS, and laboratory medium with GOS or glucose as the control were compared. The human milk and GOS medium showed quite some overlap in up-regulation of carbohydrate utilization genes presumably due to the large amount of lactose- and galactose units shared between the milk and GOS oligosaccharides. In contrast amongst the clones sequenced, there were fewer genes for sugar utilization shared between the human and formula milks despite the oligosaccharides present in the formula milk. Moreover, genes involved in carbohydrate metabolism formed the most dominant upregulated group specifically for breast milk. These unique genes upregulated for breast milk in this study included putative gene BL1642, for N-acetylglucosamine degradation, and metabolism of mucin and human milk oligosaccharides via the novel galactose / lacto-N-biose gene cluster with corresponding sugar transporters (BL1638-39) of which the transporters are also induced by GOS and formula milk. This supports the reported findings that the bifidogenic effect of human milk is to a great extent based on its unique and complex oligosaccharides. It was interesting to note that the putative genes for cell surface type 2 glycoprotein-binding fimbriae that are implicated in attachment and colonization in the intestine appeared to be only upregulated in both milks. Importantly, the present study generates leads for further investigation of genes for metabolism and colonization of *B. longum* in the human intestine.

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7

Summary, Concluding Remarks and Future Perspectives

Introduction

From the first day of life, the colonization of the human body by myriads of microbes commences. This especially holds for the gastrointestinal tract, of which the colon represents the most densely colonized interface reported (5). In particular, bifidobacteria are an important group within our intestinal microbiota and can comprise up to 90% of the breast-fed infants' intestinal microbiota. Using traditional techniques, bifidobacteria were first isolated and described from human infant feces over a century ago and quickly associated with a healthy intestinal tract due to their numerical dominance in breast-fed infants and relation with reduced diarrhea symptoms compared to bottle-fed infants (218, 219). The underlying assumption is that at least part of the protective effect of breast-feeding is linked to the high level of bifidobacteria. As we grow up and mature the intestinal microbiota becomes more diverse and hundreds of different species emerge, but the phyla of bifidobacteria still accounts for several percentage in the adult intestine which is significant. Whereas the function of bifidobacteria still remains somewhat enigmatic, clinical trials have led to their wide application aiming at improving the hosts' health, especially in fermented dairy products in the form of yoghurts and daily shots. They are also the target of prebiotic-containing products, typically oligosaccharides selectively fermented by bifidobacterial species, aiming at improving the hosts' health. Although foods containing probiotic bifidobacteria and bifidogenic prebiotics are widely consumed, there is only fragmentary information about the physiology, ecology, and genetics of *Bifidobacterium* group members within the human host as well as on many isolated *Bifidobacterium* strains marketed as probiotics.

Bifidobacterial diversity

Only the last 15 years, molecular approaches have heightened our awareness of the diversity and metabolic activity of the intestinal microbiota (51). This may be a consequence of the renewed interest in microbes, which represent the most abundant and diverse life from on our planet (240). The most recent estimates suggest that only 30% of the gastrointestinal tract microbes have been enumerated and isolated in pure cultures due to insufficient suitable conditions and challenges in cultivating anaerobic microbes (81). Typically lactic acid bacteria and bifidobacteria were considered to be culturable. However, following the recent discovery of bifidobacterial 16S ribosomal RNA gene sequences coding for new unique OTUs (operational taxonomic units), that share less than 97% similarity to the identified 16S rRNA genes of other bifidobacterial species in the public databases, it is recognized that not all bifidobacteria have been cultured and described (11, 188, 222). Consequently, molecular approaches are being applied to reveal the complete architecture of the intestinal microbial ecosystem based on targeting the 16S rRNA genes (12). Nowadays, a wealth of technologies to track and study the bifidobacteria are available ranging from basic diversity techniques,

such as 16S rRNA PCR-DGGE fingerprinting and quantitative real time PCR, up to developing genome-wide methodologies such as DNA microarrays. The principles and applications of these techniques to reveal the identity and diversity of bifidobacterial species in the human intestine are discussed in **Chapter 1**, as well as specifying future post-genomics studies to determine their functionality.

In **Chapters 2, 4** and **5,** bifidobacteria targeted PCR-DGGE profiles and 16S rRNA clone libraries were used to determine the dynamics of the intestinal bifidobacteria and identify the bifidobacterial species involved, respectively. Most of the obtained 16S rRNA sequences closely matched with known bifidobacterial species. However, several shared less than 97% similarity to known sequences in the database indicating the presence of novel bifidobacterial species within these intestinal samples. This was further supported by the qPCR results targeting the most common intestinal species in the infant and adult human intestine in **Chapters 4** and **5**. From the total bifidobacteria population in these samples only 40% was found to be detected by the used bifidobacteria qPCR primer sets that covered the known intestinal bifidobacteria. Besides the less common species not targeted here, it is likely that these other, not yet described bifidobacteria, are forming a significant part of the total bifidobacterial population. Overall, these studies provide a dynamic view of bifidobacterial species consisting of stable species as well as erratically changing species. The observed shift as well as persistent colonizing species reveal active turnover and suggest functional activity of bifidobacteria.

A major obstacle in relating microbial diversity with function is the fact that each of us is inhabited by an individual-specific microbial community composed of hundreds of distinct species, shaped namely by host factors (maternal imprinting host genotype, secretory products such as gastric acid and bile, peristalsis and food transit time) and environmental factors (dietary components, ingestion of microorganisms, food structure and viscosity, lifestyle and drugs) (51, 116, 142). This challenges the feasibility of aiming for a detailed description of all the individual species when studying the function of the intestinal microbiota. Recent studies indicate that a stable core of intestinal microbes is present throughout life that impacts functionality and is shaped by genetic, environmental and stochastic factors (48, 170). Hence, an alternative approach would be to address the microbiota as a whole, specifically focusing on the stable core. This has been become feasible with the development of post- and metagenomics tools, such as metagenomics and metaproteomics.

Genomics and Metagenomics

To avoid the incomplete description of the intestinal fecal microbial diversity, the microbial community as a whole may be targeted. The collective microbial genome, termed the microbiome, has a coding capacity that vastly exceeds that of the human genome and encodes biochemical pathways that humans have not evolved (21). Therefore, the human intestinal microbiota can be approached as an organ located in the intestinal tract (151) that is capable of more biochemical conversions than the liver, which is perceived as the most versatile human organ (49).

Metagenomics refers to the study of the collective genomes in an environmental community and typically involves cloning fragments of DNA isolated directly from microbes in natural environments, followed by sequencing and functional analysis of the cloned fragments. This application has provided insight into the genetic potential of complex microbial communities, including previously uncultured species (77). Assuming that there are approximately 1000 bacterial species with an average genome size of 3 Mb in each person, the human intestinal microbiome probably comprises several thousand Mb (5, 175). This equals the size of the human genome but vastly exceeds its coding capacity because of a much higher gene density and is estimated to code for over three million genes (175, 240). Different metagenomic studies in humans were used to investigate the microbial diversity among healthy and diseased individuals and detected numerous viral genotypes in feces (28, 247) and a reduced complexity of the bacterial phylum of the *Firmicutes* in Crohns patients (124). Metagenomics can provide an inventory of all potential functions whereby analysis of expression products can link the functional gene traits to the encoded metagenomic sequences (222). Therefore, several screening approaches of intestinal metagenomic libraries have been established (69, 71, 124). This is demonstrated by the discovery of novel hydrolase genes in uncultured rumen bacteria (59), β-glucanase genes affiliated with uncultured bacteria that colonize the large bowel of mice (235) and several genes involved in modulation of eukaryotic cell growth (71).

Above all, genomics and metagenomics sequence information form the essential basis for successful functional studies that exploit post-genomics strategies such as proteomics and transcriptomics as applied in this thesis.

Post-genomic strategies

Because protein expression is a reflection of specific microbial activities in a given ecosystem, metaproteomics has great potential for the functional analysis of microbial communities, such as the fecal microbiota, and benefits from the relative stability of protein extracts. To study rapid changes within the fecal bifidobacterial community a transcriptome analysis using a mixed species clone-library based micro array, was performed as well.

Fecal metaproteomics

Metaproteomics of fecal samples has not been described previously, possibly because of the complexity of the microbial communities and their encoded proteins. Based on the notion that neonates are colonized after birth by an in complexity increasing microbial community and the infant gut is not so well developed yet, the proteome of fecal samples from young infants was studied. **Chapter 2** describes the metaproteomics approach, comprising two-dimensional (2D) gel electrophoresis and Maldi-Tof/Tof Mass Spectrometry (MS) that was applied to the infant fecal microbiota that contains many uncultured microbial species. Unique profiles of each infants' microbiota were observed in line with the unique PCR-DGGE profiles. A comparison of the metaproteome of two 3 month-old infants over a period of 33 days revealed changes in number and intensities of protein spots although the overall patterns remained similar and individual-specific. Sequencing of peptides of selected protein spots revealed the presence of a bifidobacterial transaldolase protein being produced within the host. This enzyme, also detected at high levels *in vitro*, was already produced in the first weeks of life confirming the active bifidobacterial community. Transcription of the transaldolase messenger RNA of this gene was also detected in other infants (**Chapter 4**) as well as in adults (**Chapter 5**) using a microarray derived from a bifidobacterial mixed species clone-library based microarray. Several other peptide sequences were obtained as well but these did not match significantly with any sequence present in the public databases. Currently insufficient microbiome sequence information confounds identification of the proteins.

It is estimated that only 50,000 of the genes out of an expected pool of over 3 million genes expected in the microbial intestinal metagenome have been deposited into the public databases (69). Fortunately, ongoing metagenomic library construction and analysis will enable meaningful identification in the coming years (71, 115, 124). Peptide mass fingerprints will lead to significant database matches to identify proteins when more complete metagenome data is present (173). Hence, metaproteomics approaches have the potential to become a useful tool to monitor the functional products of the microbiota in feces over time as affected by dietary intervention, length life, health, and disease. Various improvements of proteomics techniques are being implemented. This includes the application of larger SDS-PAGE gels that will greatly improve the resolution. In addition, 2D Fluorescence Difference Gel Electrophoresis (2D DIGE), a method that labels protein samples with fluorescent dyes before electrophoresis, enables accurate analysis of differences and similarities in protein abundance between samples of individuals (48). However, it remains to be established whether co-migrating proteins from different samples indeed contain identical proteins, notably when the protein complexity is high. Finally, there is a growing number of so-called non-gel based proteomics approaches that are exploiting the development of high throughput mass spectrometry equipment with increased sensitivity (179). By targeting whole cell

extracts it is possible to dig deeper into complex proteomes and gain better insights into the composition, quantitative response, covalent modifications and macromolecular interactions of proteins that collectively drive cellular function.

Fecal bifidobacterial community transcriptomics

Besides the metaproteomics approach, a mixed bifidobacterial species microarray was applied to gain insight in the gene activity of the bifidobacterial community. As metagenomic studies revealed presence of multiple bifidobacterial strains within the fecal microbiota (69), the application of a mixed species microarray allowed to target a great part of the fecal bifidobacterial community and compare fecal bifidobacterial gene expression in individuals who all carry unique fecal microbial composition.

Chapter 3 describes the method that was developed to extract high quality total RNA from intestinal samples. As snap-frozen samples showed extensive degradation of ribosomal RNA, sampling in RNA protective buffer is very import for successful transcriptomics studies, as applied in **Chapters 4** and **5**.

The potential of micro array analysis of intestinal samples was exemplified by studies reporting full-genome transcriptome profiles of *Bacteroides thetaiotaomicron* and *B. longum* residing in the caeca of germfree mice (205, 206), as well as for *L. plantarum* WCFS1 in regular mice (125). Moreover in human individuals, the transcriptome of *L. plantarum* revealed differential gene expression at different sites of the intestine (232). In **Chapter 4,** the hybridization profiles, of total RNA isolated from infants' fecal microbiota, were analyzed by a redundancy analysis (RDA, Canoco software package) to identify whether the host, environment or stochastic effects could explain the grouping of the samples observed. The RDA showed that 44% of the difference could be explained significantly (p-value of 0.004) by the difference in feeding, either breast or formula milk. Approximately 250 clones that showed the most prominent hybridization with the samples were selected for sequencing to reveal the genes encoded on the cloned fragment. The vast majority of the inserts (90%) matched to protein-encoding bifidobacterial genes in the database. A wide range of functional groups was covered by the obtained sequences although most were found to be involved in carbohydrate metabolism or processing of information. Several of these were selected for a qPCR and sequence analysis that confirmed expression of the corresponding transcript with the expected nucleotide sequence. On the whole the detected transcripts indicate functional activity and give insight in the life style of bifidobacteria that colonize the infant intestine. The expressed genes in infant feces starts to explain the growth promoting effect of oligosaccharides which are described as prebiotics or are present in human breast milk.

In **Chapter 5** the mixed species microarrays were used to analyze the bifidobacterial transcriptome in adult feces who consumed a prebiotic mixture of oligosaccharides. Total RNA from the fecal microbial community of the adults was hybridized to the microarray, and

as observed for the infants above, the vast majority of clone inserts represented protein encoding genes matching closely to bifidobacterial DNA in the database. The obtained sequences belonged to a wide range of functional groups demonstrating substantial metabolic activity. However, most predicted genes were involved in metabolism of carbohydrates of plant origin, housekeeping functions such as DNA replication and transcription, and membrane transport of a wide variety of substrates including sugars and metals. Of special interest are the set of genes that together target plant derived fibers and might explain a way for bifidobacteria to out compete other microbes in the gastrointestinal tract. These genes were complemented with genes involved in metabolism of simple sugars and transporters. The ability to process a wide variety of indigestible components may contribute to the competitiveness of a given strain in the gastrointestinal tract (2). Overall the percentage of genes involved in the glycobiome was greater in adults compared to infant, 19 and 14% respectively, possibly due to the more complex and diverse oligosaccharides in the diet of adults compared to the milk based diet in infants. However, among the clones significantly hybridized with infants' cDNA, a larger percentages of genes could not yet be linked with a function. In both studies genes involved in the production of folate were detected that likely have a beneficial impact on the host. In addition, the expression was detected of genes 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. These were only detected in infants as well as in milk fermentations discussed below, possibly due to components in the milk based diets of these subjects, either formula or human milk.

Remarkably, the fraction of detected clones containing ribosomal genes was higher in the adult samples compared to the infants, 12 and 9% respectively. This could be explained by the fact that bifidobacterial species make up a much lower fraction of the total microbiota in adults compared to infants, an average 3-5% versus up to 60%, respectively. Possibly the RNA isolated from infants feces contains more messenger RNA as the transit time through the infants intestine is faster and the fecal bacteria closer resemble the more viable *in vivo* situation and will therefore contain more messenger RNA. Nevertheless, the quality of the total RNA fractions used for the microarray experiments was equally good for both adult and infant samples.

Dietary influences on the *B. longum* transcriptome

To get a complete insight in the impact of the bifidobacterial community on the host and total gut microbiota, an effort should be made to identify and preferable also isolate such species for detailed metabolic and functional studies (50). The *in silico* analysis of the *B. longum* NCC2705 genome (197) and gene expression studies (161, 162) provided a road map to

describe activity of distinct metabolic routes. Nowadays, there is considerable interest in modulating the new-born infants' microbiota towards a healthier composition. The diet, either breast milk or formula milk, of new born babies has a distinct influence on the number and diversity of the intestinal microbiota and specifically on the bifidobacterial population. In **Chapter 6** the transcriptome of *B. longum* LMG 13197 growing in breast milk, formula milk (supplemented with GOS and long chain FOS (in a ratio of 1 to 9) and chemically defined medium containing GOS was studied. When focusing on differential induced genes among the tested condition, expression of specific genes was detected. Overall, the transcription of a set of genes involved in carbohydrate metabolism and uptake is specifically induced by breast milk in *B. longum*. Genes uniquely upregulated for breast milk included putative genes for N-acetylglucosamine degradation, and metabolism of mucin and human milk oligosaccharides via the novel galactose/ lacto-N-biose gene cluster. It was interesting to note that the putative genes for cell surface type 2 glycoprotein-binding fimbriae that are implicated in attachment and colonization in the intestine appeared to be upregulated in both milks. This supports the hypothesis that the bifidogenic effect of breast milk is to a great extend based on its complex mixture of oligosaccharides. Human milk contains much higher concentrations of oligosaccharides (12-14 g/L) compared to cows milk and (1 g/L) and has a unique complex biochemical composition (36). Galactose is one of the core sugars of human milk oligosaccharides (150) and therefore relevant to study as well. There was quite some overlap between the human milk and GOS-induced genes which indicate specifically upregulated carbohydrate metabolism. The studies described above generate important leads for further investigation of genes for metabolism and colonization of *B. longum* in the human intestine and to enhance our understanding of the complex intestinal ecosystem.

Perspectives and recommendations

The post-genomic era has arrived. Within this thesis, the focus of studies on the bifidobacterial population has shifted from composition and activity to include the study of the functional products of gene expression. This thesis has used post-genomic technologies, transcriptomics and proteomics to get insight in the molecular activity of bifidobacteria on different levels. The metaproteome and transcriptome reveal *in situ* activity of the fecal bifidobacterial population. Diversity studies in healthy adults, over 4 to 9 years, show that bifidobacteria are part of the stable core of the fecal microbiota and are highly similar between twins. The functional activity together with the host specificity and stability of this species (31) suggests active crosstalk with the host. There is considerable more research yet needed in order to support the role of bifidobacteria in functional foods as well as substantiate the potential health benefits of bifidogenic prebiotics.

Based on this study and previous ones, there is a large group of bifidobacteria colonizing the intestine that have not yet been cultured or described. Similarly, a part of the

bifidobacterial transcriptome is not detected by the mixed species bifidobacterial microarray, as only the more common human intestinal species have been arrayed and the unique genomes of these not-yet-cultured species are also missing. Further approaches are necessary to encompass the whole population including the not-yet–cultured members. Application of sorting strategies such as FISH probes and flow cytometry might be helpful to separate all the fecal bifidobacterial cells from the complex background, which will consist partly of undescribed and yet not-cultured species. Combination of FISH techniques and sorting to target specific microbial populations for proteomics analysis will increase the resolution of the proteomics technique such as the analysis of the alive, injured and dead subsets of intestinal bacteria (11), and the development of functional gene targeted CPRINS (cycling primed *in situ* amplification)-FISH (95) or RING (recognition of individual genes)-FISH (256). Recently specific sorting of *L. plantarum* from ileum samples using specific antibody coated magnetic beads and 2D gel electrophoresis of the proteins demonstrated the application for gastrointestinal samples (33). To address the gene expression of the bifidobacteria population from infant and adult intestinal samples in the absence of genomic information, a functional genomics approach using arrays comprising genomic libraries of the most common intestinal bifidobacterial species, was applied (**Chapters 4 and 5).** Specific sorted population may also be used for subsequently constructing specific bifidobacterial genomic libraries for production of microarrays. At the same time, the gap of the unique unknown bifidobacterial genes could be closed by expansion of sequence data from metagenomic libraries of intestinal microbiota will very likely generate data on not yet cultured or detected bifidobacterial species.

There has been a wealth of functional studies to get more insight in the metabolism, colonization and interaction of bifidobacteria with the human host. The increasing number of bifidobacterial genomes published as well as sequencing projects (100) are expected to open up a new era of comparative genomics in bifidobacterial biology. The microarray technology can be used for the global comparative analysis of gene content between different bifidobacterial isolates without the necessity of sequencing many strains, and can provide information about the degree of relatedness and variability. Variable regions may harbor important traits that contribute to the individual lifestyle of a strain (113) and give leads in knowledge regarding specific adaptations and evolution. Uncovering the diverse capabilities of *Bifidobacterium* through its genome will greatly enhance our knowledge of the role bifidobacteria play and should place us one step closer to a more comprehensive understanding of the biology of these bacteria. However, genome sequencing is merely the starting point on the road to fully understanding the biology of a bacterium. Functional studies and genome trait matching complement each other (169) and give leads for setting up predictive models. These models could be used to predict functions of the microbiota, as well as deviations from the balanced microbiota in terms of their functionality besides diversity.

The knowledge will advance the further development of new and specific prebiotics or probiotics that impact host health. Such approaches may prove to be a significant step in reducing the risk of intestinal diseases and reconstructing the specific gut microbiota balance of the host.

Comparison of targeted mutants and wild type species would give a deeper understanding of the many complex issues surrounding the relationship between humans and their resident microbial populations. Today, genetic tools to test hypotheses of bifidobacterial function are scarce especially in comparison to many other microbes of food industry interest. Efficient gene transfer systems stifle further advances in the genetics of bifidobacteria and this is a serious limitation for genetic approaches aiming at establishing cause-effect relations between genes and function. preferably with improved ability to create mutants to investigate the functionality of particular genes but the application is successful in other organism (31, 210).

Finally, the impact of bifidobacteria on the host and its neighboring microbes is an essential area for future studies. There is an increasing prevalence of a number of disorders, both within and outside of the gastrointestinal tract that are believed to be influenced by the gut microbiota, including inflammatory bowel disease and atopic states such as asthma and food allergies. Especially the presumed interactions with the immune and neuroimmune systems (210) are highly relevant as are other epithelial interactions with the host. Besides being studied in neonates, there role should also be studied in children and adults, and this research requires novel or non-invasive methods as well as useful animal and other alternative models. Further multi-disciplinary research involving combinations of technologies such as microbiomics and metabolomics will be necessary to fully decipher bifidobacteria impact on the human health (128). In conclusion, since the discovery of bifidobacteria by Henry Tissier in 1899, within the last decade a firm framework for understanding the role of bifidobacteria within the human microbial intestinal ecosystem has been realized and facilitated by this present study. However, these post-genomics approaches mark just the beginning of a new era that is required to further develop bifidobacteria beyond this level and harvest the fruits of these bacteria that are close to our heart since the beginning of our life!

8

Nederlandse Samenvatting

Ons gehele maag-darm kanaal is bevolkt met micro-organismen en de aantallen variëren enorm (zie figuur 1). Wetenschappelijk omschrijven we het geheel van deze darm-micro-organismen met de term darmmicrobiota in plaats van darmflora (het zijn immers microben en geen plantjes). Het overgrote deel van deze microbiota bestaat uit bacteriën. Vooral in de dikke darm zijn de aantallen bacteriën heel hoog. Hier bevinden zich in totaal 10^{14} (100 000 000 000 000) bacteriën, samen zo'n 1,5 kg. Het komt er op neer dat we in ons lichaam tien keer meer bacteriecellen hebben dan onze eigen lichaamscellen. In dit proefschrift onderzoeken we de soort "*Bifidobacterium"*. Bifidobacteriën leven bijna bij ieder mens in de dikke darm en vooral bij baby's kan het percentage bifidobacteriën oplopen tot wel 90 % van het totaal. Maar ook bij volwassenen zijn dit nog enkele procenten wat aangeeft dat de bifidobacterie een belangrijke inwoner van onze dikke darm is.

	Bacteriën per ml
Maag	$10^1 – 10^2$
Dunne darm	$10^4 – 10^7$
Dikke darm	$10^{10} – 10^{12}$

Afbeelding 1. Aantallen bacteriën in de verschillende compartimenten van het maag-darm kanaal.

Bifidobacteriën zijn al ontdekt in 1899 door Henry Tissier in babypoep. Al snel werden deze bacteriën als gezonde bacteriën gezien omdat ze voornamelijk in baby's die borstvoeding krijgen worden gevonden. Baby's die borstvoeding krijgen hebben over het algemeen minder last van infecties en andere darmproblemen. Het immuunsysteem van baby's is dan nog volop in ontwikkeling. Het natuurlijke immuun systeem bevindt zich voor 70 % rond de darmen en de ontwikkeling hiervan wordt voor een groot deel beïnvloed door de darmmicrobiota. De precieze rol en levenswijze van bifidobacteriën is nog niet helemaal duidelijk, desondanks zijn er veel producten op de markt die deze bacteriën bevatten en het bevorderen van een gezonde "darmflora", spijsvertering en stoelgang claimen.

Pas sinds de afgelopen 15 jaar is de interesse in het onderzoek naar de darmmicrobiota flink gestegen. Dit heeft natuurlijk te maken met het feit dat het onderzoek van poepbacteriën niet altijd zo'n fris werkje is. Bovendien werd er tot voor kort gedacht dat de dikke darm slechts een opslagplaats voor onverteerbare voedingsbestanddelen was. Het

onderzoek naar de soorten bacteriën in onze poep heeft aangetoond dat dit de meest diverse samenstelling van bacteriën is op onze planeet en dat er wel honderden soorten te vinden zijn. Deze samenstelling verschilt van mens tot mens. Dit heeft te maken met de genetische opbouw van de mens zelf, zijn dieet, medicijngebruik, leefgewoontes en omgeving waarin hij leeft. De verschillende enzymatische (biochemische) reacties van al die verschillende micro-organismen worden minstens even groot geschat als die van de lever. De darmmicrobiota spelen een belangrijke rol bij het omzetten van voedsel dat terechtkomt in de dikke darm, zoals vezels die we zelf niet kunnen afbreken. Daarnaast zijn deze bacteriën erg belangrijk voor de ontwikkeling van ons immuunsysteem.

Zoals ik al eerder zei, bifidobacteriën maken bijna altijd een significant onderdeel uit van deze diverse groep bacteriën. Nu we weten dat deze bifidobacteriën in groten getale aanwezig zijn, willen we ook weten wat ze doen en hoe ze invloed uitoefenen op ons lichaam en de rest van de bacteriën in de darm. Zijn ze levend, dood, actief, passief, wat voor producten maken ze, waar leven ze van, etc.? **Hoofdstuk 1** van dit proefschrift geeft een overzicht van wat er nu bekend is over bifidobacteriën en verschillende technieken waarmee je onderzoek kunt doen naar deze bacteriën.

Technieken gebaseerd op DNA

In dit proefschrift wordt gebruik gemaakt van moleculaire technieken. Omdat veel van de bacteriën die onze darm leven nog niet gekweekt kunnen worden in het laboratorium is het erg lastig om iets over de aantallen en leefgewoontes van deze microben te weten te komen. Daarom gebruiken we andere technieken die zich richten op het DNA, erfelijk materiaal, van de darmbacteriën. We halen geen levende bacteriën uit de darm maar alleen het DNA van deze bacteriën. We kunnen bacterie soorten detecteren met behulp van het DNA. Deze techniek is in dit proefschrift gebruikt om te identificeren of de onderzochte poepmonsters bifidobacteriën bevatten.

Activiteit van bifidobacteriën in de darm van de mens

Omdat we niet alleen willen weten of er bifidobacteriën aanwezig zijn maar ook wat ze doen hebben we naast DNA ook naar andere moleculen gekeken. Hiertoe eerst een kleine algemene introductie over de werking van DNA in een bacteriecel. De streng met DNA in de bifidobacterie noemen we de *genoomsequentie* en bevat ongeveer 1800 genen. Elk gen draagt de informatie voor een specifieke eigenschap. Niet elk gen is ook actief, dit ligt aan de

omstandigheden waarin deze bacterie zich bevindt. Elk gen dat nodig is voor de bacterie wordt geactiveerd en omgezet in een RNA molecuul. In een volgende stap wordt een RNA molecuul omgezet in een eiwit (zie figuur 2). Dit eiwit is een stof die actief is in de cel zoals bijvoorbeeld een enzym dat voedsel voor de bacterie kan afbreken. Om te onderzoeken welke genen actief zijn in de bifidobacteriën in de darm kunnen we dus RNA moleculen en eiwitten onderzoeken.

DNA → RNA → eiwit

Figuur 2 Omzetting van DNA (erfelijk materiaal) in RNA en vervolgens in eiwit (actieve stof).

Door poepbacteriën open te breken en in een speciale vloeistof te mixen kun je eiwit of RNA uit deze bacteriën halen. Eiwitten zijn erg stabiel en daardoor is het makkelijk om eiwit te isoleren uit bacterie cellen. Deze techniek heet *proteomics*. RNA moleculen breken heel snel af maar geven daardoor een meer dynamisch beeld van de activiteit van de bifidobacteriën. Deze techniek heet *transcriptomics*. Om de functie van de gevonden RNA moleculen en eiwitten te achterhalen is het erg belangrijk dat er al genoomsequenties van bifidobacteriën bekend zijn. Op dit moment zijn er genoomsequenties van twee soorten bifidobacteriën openbaar toegankelijk in de database. Van zes andere soorten zijn de genoomsequenties wel bekend maar niet gepubliceerd dus nog niet beschikbaar voor ons.

Proteomics (eiwit onderzoek)

In **Hoofstuk 2** worden de eiwitten van poepbacteriën onderzocht in poep van 2 baby's die veel bifidobacteriën in hun poep hebben. Omdat we de bifidobacteriën niet van de andere bacteriën hebben gescheiden noemen we het metaproteomics. Met "meta" bedoelen we proteomics (eiwit onderzoek) van de totale darmmicrobiota. We zagen dat de eiwitten gemaakt door de bacteriën over een tijd van 33 dagen veranderingen vertoonden maar dat de algemene eiwit samenstelling stabiel was en ook dat de eiwitsamenstelling van baby tot baby verschillend was. Enkele eiwitten konden deels geïdentificeerd worden. Veel eiwitten kunnen nog niet geïdentificeerd worden omdat de bijbehorende DNA sequentie ontbreekt in de databases omdat deze eenvoudigweg nog niet eerder gevonden is. Eén eiwit was een bifidobacterie eiwit, een transaldolase. Activiteit van het hiermee corresponderende gen werd ook gevonden met transcriptomics (RNA) onderzoek in andere baby's en volwassenen. Dit

betekent dat de bifidobacteriën aanwezig zijn in de darm en ook actief stoffen produceren in de darm.

Transcriptomics (RNA onderzoek)

Omdat RNA erg snel afbreekt is het erg belangrijk dat er schoon en met een goed protocol gewerkt wordt. **Hoofdstuk 3** beschrijft het protocol dat ontwikkeld is om RNA uit poep en stoma monsters te halen.

Met behulp van deze methode hebben we RNA uit meerdere poepmonsters van verschillende baby's gehaald (**Hoofdstuk 4**). Een deel van deze baby's kreeg een speciale flesvoeding en een deel alleen borstvoeding. Het was duidelijk dat de aantallen bifidobacteriën hoger waren in baby's die borstvoeding kregen, en na verloop van tijd steeg ook het aantal bifidobacteriën in de baby's die flesvoeding kregen. De soorten bifidobacteriën verschilden ook tussen de beide groepen. Dit resultaat was vergelijkbaar met eerder onderzoek. Ook de RNA moleculen waren significant verschillend als we alle baby's vergeleken en verdeelden in 2 groepen, fles- en borstvoeding. De functies van de genen die actief waren in de baby's hadden vooral te maken met de afbraak van vezels en suikers die aanwezig zijn in de darm. Ook een aantal genen die betrokken zijn bij de afbraak van suikers die voorkomen in moedermelk en ook in de slijmlaag van onze darm waren actief. Dit verklaart mogelijk het hoge aantal bifidobacteriën in de darmen van baby's die moedermelk ontvangen.

Naast baby's hebben we ook volwassen onderzocht (**Hoofdstuk 5**). Vier volwassenen aten 3 weken lang een mix met vezels die de groei of activiteit van bifidobacteriën zou kunnen bevorderen in de darm van de mens. RNA is onderzocht in poepmonsters voor en na 3 weken inname van de mix. In ieder persoon werden er wel verschillen gevonden tussen beide monsters. Maar als we de monsters voor inname en na inname groepeerden was er geen algemeen verschil. Dit komt waarschijnlijk door grote persoonlijke verschillen en de rest van het dieet. Er zouden meer personen gevolgd moeten worden om invloed van het supplement te meten op de activiteit van bifidobacteriën. Over het algemeen waren er veel genen actief die betrokken zijn bij de afbraak van vezels. Dit was een hoger percentage dan in de baby's, waarschijnlijk veroorzaakt door de grotere variëteit in vezels in het dieet vergeleken met het melkdieet van de baby's. Deze kennis geeft inzicht in de levenswijze van de bifidobacteriën in de darm. Doordat ze zich kunnen voeden met de vezels uit onze voeding hebben ze een groot voordeel ten opzichte van bacteriën die deze vezels niet kunnen afbreken.

Om in meer detail de reactie van bifidobacteriën te onderzoeken hebben we deze bacteriën ook in het laboratorium gekweekt (**Hoofdstuk 6**). Dus in een pure kweek zonder andere bacteriën erbij. Omdat we verschillen konden waarnemen tussen baby's die borstvoeding en flesvoeding kregen hebben we bifidobacteriën gekweekt in moedermelk en flesvoeding. Als vergelijkingsmateriaal hebben we bifidobacteriën in een laboratoriumvoeding met glucose gekweekt. Daarnaast hebben we de bacteriën ook in een laboratoriumvoeding met galactooligosaccharides (GOS) gekweekt. Dit zijn vezels die ons lichaam zelf niet kan verteren en dus in de dikke darm terechtkomen, waar ze kunnen dienen als voedsel van de darmmicrobiota. Het is in eerder onderzoek aangetoond dat na het eten van GOS de hoeveelheid bifidobacteriën toeneemt. Ook hierbij hebben we de RNA moleculen onderzocht om de activiteit van de bifidobacteriën te bepalen veroorzaakt door verschillende voedselcomponenten. In moedermelk werden een aantal overeenkomstige genen gevonden als in de baby's, zoals de genen die te maken hebben met de afbraak van suikers uit moedermelk en suikers uit de slijmlaag van de darm. Ook was er overlap tussen de activiteit in moedermelk en GOS, waarschijnlijk omdat suikers uit moedermelk ook GOS bevatten en door dezelfde enzymen af gebroken kan worden. Beide soorten melk toonden ook activiteit van een gen dat te maken heeft met het aanhechten van bifidobacteriën. Dit verklaart deels de hoge aantallen van bifidobacteriën in de darm van baby's. Deze resultaten geven meer inzicht in de levensstijl van bifidobacteriën in het laboratorium en hierdoor ook inzicht in hun activiteit in de darm van de mens.

Conclusies

In eerdere studies is aangetoond dat bifidobacteriën deel uit maken van de vaste stabiele kern van de darmmicrobiota. Dit duidt op een interactie met de gastheer (de mens) en actieve levensstijl van de bifidobacteriën in de darm. In dit proefschrift hebben we het gangbare onderzoek naar welke soorten bacteriën aanwezig zijn in de darm verrijkt met onderzoek naar de functionele activiteit van de darmbacteriën. Met behulp van de nieuwe moleculaire technieken, die gebruik maken van de DNA sequenties die nu voor handen zijn, kunnen we de activiteit op verschillende niveaus meten. Het is duidelijk, uit dit en andere onderzoeken, dat veel bifidobacteriën nog niet in het laboratorium gekweekt kunnen worden omdat we niet de juiste leefomstandigheden kennen en kunnen nabootsen. Dit is een extra reden om met moleculaire technieken te werken waarbij bacteriën niet eerst opgekweekt hoeven te worden in het laboratorium.

Toekomstperspectieven

Hoewel er een aantal genoomsequenties beschikbaar is van bifidobacteriën kunnen we nog lang niet altijd de DNA- en RNA moleculen en eiwitten van darmmicrobiota terugvinden in de database. Hierdoor kunnen deze moleculen niet altijd gerelateerd worden aan een functie. Op dit moment lopen er grote projecten waarbij DNA sequenties van darmmicrobiota worden onthuld, dus in de nabije toekomst worden de databases aangevuld met nieuwe gegevens. Dit zal onderzoek zoals beschreven in dit proefschrift sterk versnellen.

Daarnaast zijn er mogelijkheden om specifiek alle bifidobacteriën uit de complexe mix van darmmicrobiota te vissen voordat RNA en eiwit eruit worden gehaald. Hierdoor kunnen we meer te weten komen over de tot nu toe onbekende bifidobacteriën en worden het iets makkelijker om de actieve producten (RNA en eiwit) te bestuderen.

Om de functie van een gen specifiek te onderzoeken wordt zo'n gen vaak kapot gemaakt (mutant) en vergeleken met de bacterie waarin dit gen nog functioneel is. Deze methodes werken helaas nog niet goed in bifidobacteriën. Het is dan ook van groot belang om deze methodes verder te ontwikkelen.

Het is essentieel om meer over de invloed van bifidobacteriën op de mens en andere darmbacteriën te weten te komen omdat het aantal mensen dat lijdt aan klachten aan het maag- darmkanaal nog steeds stijgt. Met het gebruik van DNA sequenties en moleculaire technieken is een nieuw tijdperk aangebroken. Deze technieken zijn nog steeds sterk in ontwikkeling en noodzakelijk om meer te weten te komen over bifidobacteriën om uiteindelijk gebruik te kunnen maken van deze bacteriën die vanaf onze geboorte nauw met ons verbonden zijn.

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About the author

Eline Suzanne Klaassens was born on the second of February, 1976 in Delft. In 1994 she graduated at high-school (VWO) at the Bonaventura College in Leiden, The Netherlands. In 1995 she obtained a Chemistry Testimonium (VWO) at the Boerhaave College, Leiden. In the same year she started the study Food Science and Technology at the Wageningen University were she completed two MSc theses. The first thesis entitled "The influence of soy and milk proteins and soy non-nutrients on coloncancer in the rat" was performed at the Group of Toxicology and Product Design and Quality Management. A traineeship studying the rheology of soy protein isolates was performed at the Department of Life Science at the University of Limerick in Ireland. An industrial traineeship was performed at DSM Food Specialties in Delft at the Genetics group. September 2001 she graduated after finishing a second MSc thesis on the expression of Mn(II) induced transport systems in *Lactobacillus plantarum* at the Industrial Microbiology Group at the Wageningen University which was performed at NIZO food research B. V. in Ede. In 2002 she continued practicing science as a PhD student in the Laboratory of Microbiology at Wageningen University and Research Centre (The Netherlands) under the supervision of Prof. Willem M. de Vos and Dr. Elaine E. Vaughan. This research was part of the IOP Genomics Project "A genomics approach towards gut health". The result of the project is presented in this thesis. By August 2006 she has been appointed as a post-doctoral researcher at TI Food & Nutrition, located at NIZO food research B. V. in Ede.

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Activities in the frame of VLAG Graduate School

- Member of VLAG PhD council
- Mass spectrometry in Biology (Nova Knowledge, 2002)
- Symposium LAB-7, LAB-8 (2002; 2005)
- 2-D electrophoresis/ Proteomics course (University of München, 2002)
- Ecophysiology of the gastrointestinal tract (VLAG, 2003)
- Darmendag (2003; 2004; 2005)
- Proteomics Forum (University of München, 2003)
- Scientific English writing (WUR, 2003)
- Microbiology PhD students trip (Japan, 2004)
- Bioinformation Technology 1 (VLAG/ WU, 2004)
- INRA-RRI Symposium on Gut Microbiology (France, 2004; UK, 2006)
- Workshop metabolomics (VLAG/ EPS, 2005)

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