Molecular identification and characterisation of bifidobacteria and lactobacilli in the human gastrointestinal tract

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Propositions

1. Bacterial species can not be reliably identified based on the migration position of their 16S rDNA PCR-fragments in the denaturing gradient gel.
This thesis

2. The *Bifidobacterium* species found in infants are different from those in adults.
This thesis

3. It is not surprising that a novel DNA extraction method is found to be superior when it is only compared to a decade-old method instead of recent methods.
Orsini and Romano-Spica (2001) Lett. Appl. Microbiol. 33:17-20

4. Gut bacteria act in cooperation against environmental threats by distributing accessory genes to each other.
Shoemaker et al. (2001) Appl. Environ. Microbiol. 67:561-568

5. *Bacillus anthracis* is used as a biological weapon, but furthermore is an effective psychological and economic weapon.

6. Dutch is not a difficult language, but learning Dutch is difficult among Dutch people who speak such good English.

7. Good abdominal movement is essential for belly-dancers.

Propositions belonging to the thesis: '*Molecular identification and characterisation of bifidobacteria and lactobacilli in the human gastrointestinal tract*'.

Reetta Satokari
Wageningen, 11 January 2002

Contents

Chapter 1

General introduction

General introduction

The objective of this chapter is to provide the background information and rationale for the studies described in the following chapters that deal mainly with the molecular diversity of bifidobacteria and lactobacilli in the human gastrointestinal tract. First, a general overview of the intestinal microbial community is given, followed by a description of the role of *Bifidobacterium* and *Lactobacillus* spp. in the gut ecosystem and the taxonomy of these bacteria. Furthermore, new molecular methods to study the intestinal bacteria and the achievements obtained with new techniques are reviewed with specific emphasis on bifidobacteria and lactobacilli. Finally, approaches to modify the intestinal microbial communities by diet are summarised, which is followed by the outline of this thesis.

The normal human intestinal microbiota

Acquisition and composition of the intestinal microbiota

Microbes colonise the external surface of the human body, the skin and mucosal epithelium in different locations, including the oral cavity and the gastrointestinal (GI) tract (78, 140). The microbial communities in different body sites are commonly referred to as the normal indigenous microbiota. The composition of this microbiota varies between different body locations and individuals. The most abundant indigenous microbiota can be found in the intestinal tract, particularly in the colon, where the microbial cells reach a density up to 10^{11} to 10^{12} colony forming units (cfu) per gram of faeces (37, 140). Our present knowledge about the GI-tract microbiota is largely based on cultivation studies, but in the past decade molecular approaches based on rRNA are increasingly being applied. These new approaches also allow detection of the members of the uncultured part of the microbiota, which may constitute up to 85 % of the total community (73, 134).

The acquisition of the normal microbiota starts at birth when the germ-free newborn comes into contact with the environment. The colonisation of the

intestine of a newborn proceeds in several stages (29). The first one or two weeks of life are considered as the initial period of microbial colonisation, during which diverse microbes transit through the infant's GI-tract and can be found in its faeces. Facultative anaerobic bacteria, *Escherichia coli*, *Streptococcus* and *Enterococcus* colonise the gut during this period. They create a reduced environment favourable for the subsequent colonisation by anaerobes, such as bifidobacteria, *Bacteroides* and clostridia, which are typically cultivated from faeces. Molecular methods, such as fluorescent *in situ* hybridisation (FISH) of 16S rRNA (see below), have confirmed the presence of these bacterial groups (47). In addition bacteria of the *Coriobacterium*-group (*Coriobacterium* and *Collinsella*) were found to be present in high numbers in faeces of formula-fed infants (49). In nursing infants dietary supplementation, the so-called weaning stage, causes shifts in the intestinal microbiota (133). By about the second year of life the composition of the intestinal microbiota resembles that of an adult in the level of major microbial groups (133), although in the level of species and strains prominent changes are still likely to occur for several years.

The adult intestinal microbiota is an extremely complex ecosystem both in itself and in the network of factors affecting it that include host factors, the environment of the host and microbial interactions (37, 66, 137). Microbial communities vary in different parts of the GI-tract both in composition and numbers, becoming richer and more abundant from the practically germ-free stomach and upper small intestine to the colon (37, 62, 66) (Table 1). The very low pH in the stomach, normally less than pH 3.0 in resting stage, prevents microbial growth and in the upper part of the small intestine (duodenum) digestive secretions such as bile and pancreatic enzymes also have a bactericidal effect. Moreover, the strong flow of intestinal contents prevents extensive colonisation in the small intestine. In the lower parts of the small intestine (jejunum and ileum) the pH becomes more neutral, oxygen tension drops, digestive secretions are diluted and the flow is reduced. The microbial community becomes more diverse and increases in numbers towards the end of the small intestine. In the large intestine the pH is near to neutral, the flow rate is low and the oxygen tension drops, which creates favourable conditions for a rich anaerobic microbial ecosystem. In addition to the above mentioned factors, the gut associated lymphoid tissue (GALT) also plays an important role in

controlling the microbiota by producing secretory immunoglobulin A (sIgA), which is released to the gut lumen and coats bacteria thereby preventing adhesion and facilitating removal of bacteria from the intestine (90).

Initially, most knowledge of bacterial numbers and diversity in the GI-tract was derived from culturing studies. Sophisticated anaerobic culturing techniques and selective media were developed to determine the viable counts of different bacterial groups, and subsequent genus and species identification was done by using phenotypic characterisation. *Bacteroides*, *Bifidobacterium*, *Eubacterium, Ruminococcus, Peptococcus,* and *Peptostreptococcus* are the main cultivable bacterial genera in the large intestine and in many subjects they are present at populations of at least 10^{10} cfu per gram of faeces (37). These and other important groups of bacteria in the large intestine are listed in Table 1. In the large intestine anaerobic bacteria outnumber aerobes and facultative anaerobes more than thousand fold (37, 66). Also yeast, fungi and protozoa were found in the colon by cultivation (37). Presently, molecular methods such as FISH and dot blot hybridisation, which rely on the use of phylogenetic probes targeting 16S rRNA or the corresponding genes (16S rDNA) are increasingly used to enumerate and quantify microbial groups in faeces (40, 85, 126, 135). It is noteworthy that 16S rRNA probes for bacterial groups target phylogenetic groups rather than taxonomically defined families or genera. The probes available today cover up to 70 % of the faecal bacterial community (126) and detect also uncultured bacteria (151). FISH and dot blot hybridisation studies have shown that *Bacteroides*, *Clostridium coccoides - Eubacterium rectale*, and *Clostridium leptum* groups constitute together 44 to 68 % of the total bacterial community in faeces (40, 85, 126). In addition, *Fusobacterium prausnitzii* and related species belong to the dominant microbiota accounting for 17 % of total bacteria in faeces (135).

Cultivation studies indicated that more than 85 % of the bacterial community is composed of 30 to 40 species and it was estimated that at least 400 bacterial species reside in the human large intestine (94). In a recent molecular study the diversity of a single individuals faecal microbiota was investigated by large scale cloning and sequencing of the 16S rDNA sequences (134). It was found that 76 % of the 284 retrieved rDNA sequences derived from hitherto unknown species.

Table 1. Composition of the human GI-tract microbiota.

Location	Total Microbial Count (cfu)	Main Microbial Groups
Stomach	$< 10^3$ / ml	*Lactobacillus, Streptococcus, Candida*
Duodenum and jejunum	$< 10^3$ / ml	*Lactobacillus, Streptococcus, Veillonella, Candida*
Ileum	10^3 - 10^8 / ml	*Bacteroides, Bifidobacterium, Enterobacteriaceae, Veillonella, Enterococcus, Lactobacillus, Streptococcus*
Colon	10^{10} - 10^{12} / g	*Bacteroides, Clostridium, Eubacterium, Ruminococcus, Fusobacterium, Bifidobacterium, Peptostreptococcus, Atopobium*, Enterobacteriaceae, Lactobacillus, Enterococcus, Streptococcus, Veillonella, Peptococcus, Propionibacterium, Actinomyces*

* *Atopobium*-cluster that includes *Atopobium, Coriobacterium, Eggerthella* and *Collinsella* (49).

Activity of the intestinal microbiota

The gut microbiota has a variety of important functions that may be beneficial to the host, including colonisation resistance against pathogenic organisms, positive effects on host's nutrition and stimulation of the immune system (78). Non-digested food components that are not degraded and absorbed during the passage through the stomach and small intestine reach the colon, where they serve as substrates for microbial fermentation. During carbohydrate fermentation gases, ethanol, lactate and short-chain fatty acids (SCFA) such as butyrate, propionate, and acetate are produced. Butyrate is metabolised by the colonic epithelium, whereas acetate and propionate are generally absorbed and used in other host tissues (30). The metabolic end products from the carbohydrate fermentation are considered harmless or even beneficial to the host whereas the

end products from protein fermentation that include phenolic and indolic compounds and ammonia, are potentially harmful (30, 77). Besides non-digested food components, the GI-tract microbiota has another major source of nutrients, which is host-derived. Mucus and dead enterocytes that are released into the gut lumen as a result of epithelial cell renewal become nutrients for the microbiota (106).

Recently, new techniques have been developed that allow the assessment of functionality of microbiota and the host-microbial interactions at molecular level. Hooper *et al.* (56) used DNA microarray technology to monitor the expression of genes in the enterocytes of mice in response to the presence of gut commensal bacteria. It was found that commensal bacteria modulate expression of genes involved in several fundamental intestinal functions such as nutritional absorption, mucosal barrier fortification and postnatal intestinal maturation.

The role of bifidobacteria and lactobacilli in the microbial balance of intestine

It is assumed that both beneficial and potentially harmful microbes constitute a balanced GI-tract community where the overgrowth of opportunistic pathogenic microbes is normally suppressed by other members of the microbiota. This microbial balance may be disturbed by antimicrobial agents, ingested pathogenic microorganisms or changes in the hosts physiological condition such as stress or ageing (66, 118). Bifidobacteria and lactobacilli are considered to be members of the beneficial microbiota that is important in maintaining a healthy, balanced microbial community in the GI-tract and they are also proposed to induce systemic effects that improve the host's health (14, 103, 117). Proposed health effects of bifidobacteria and lactobacilli and possible mechanisms are illustrated in Figure 1.

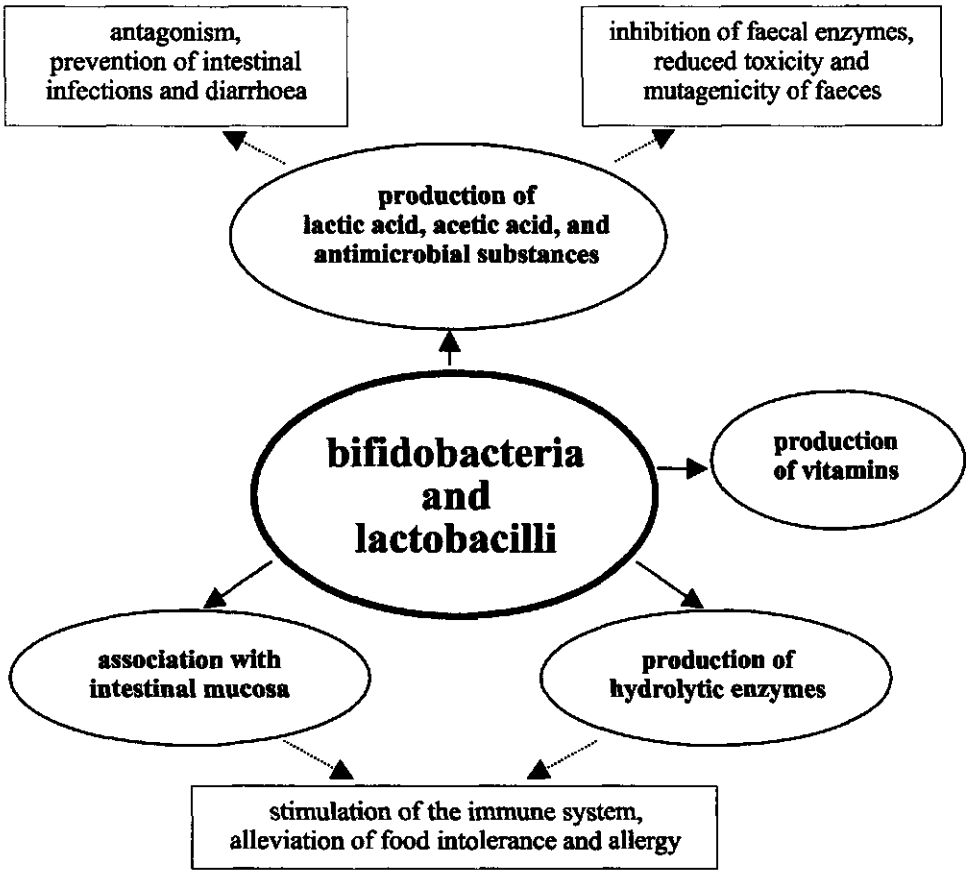

Figure 1. Hypotheses on the health-promoting effects of bifidobacteria and lactobacilli.

Taxonomy of lactobacilli and bifidobacteria

The genus *Lactobacillus* is comprised of Gram-positive, catalase-negative, non-sporulating, microaerophilic rods, which produce lactic acid as the major end product during the fermentation of carbohydrates (9). The genus *Lactobacillus* is one of the largest genera and contains presently around eighty species (5). Traditionally lactobacilli have been divided into three groups according to the type of sugar fermentation. Obligately homofermentative lactobacilli ferment hexose sugars by glycolysis and produce mainly lactic acid, while obligately

heterofermentative lactobacilli use the 6-phospho-gluconate/phosphoketolase (6PG/PK) pathway and produce other end products (CO_2, ethanol) in addition to lactic acid (9). The third group includes the facultative heterofermentative lactobacilli that ferment hexoses via the glycolysis and pentoses via the 6PG/PK pathway. Further classification into species has been largely based on phenotypic and biochemical characteristics, but in many cases it is further supported by genotypic analyses, usually by DNA-DNA homology or 16S rDNA (45, 68).

Modern bacterial phylogeny is based on the idea that the degree of similarity of rRNA sequences between two organisms is comparable to their evolutionary distance (76, 153). Due to the conservation of the process of protein synthesis in living cells and the fact that rRNAs consist of alternating conserved and variable regions, these molecules are extremely suitable as evolutionary chronometers (76, 153). The division of lactobacilli into three main groups according to the type of fermentation is not in accordance with their natural relations as revealed by analysis of their 16S rDNA sequences (9). In the latter phylogenetic analysis, lactobacilli cluster in the subdivision of the low G + C Gram-positive bacteria together with other lactic acid bacteria, anaerobes such as clostridia, peptococci and ruminococci, as well as aerobes and facultative aerobes such as bacilli, staphylococci and listeria. *Lactobacillus* species branch further into several groups and they do not form a coherent phylogenetic unit (Figure 2). Thus, the taxonomy of the genus *Lactobacillus* is unsatisfactory.

The species belonging to the genus *Bifidobacterium*, unlike lactobacilli, form a coherent phylogenetic unit and show generally over 93 % similarity of 16S rRNA sequences with other members of the genus (74, 93) (Figure 2). In the phylogenetic tree of bacteria the *Bifidobacterium* cluster is in the subdivision of high G + C Gram-positive bacteria together with other genera such as *Propionibacterium, Actinomyces* and *Streptomyces.*

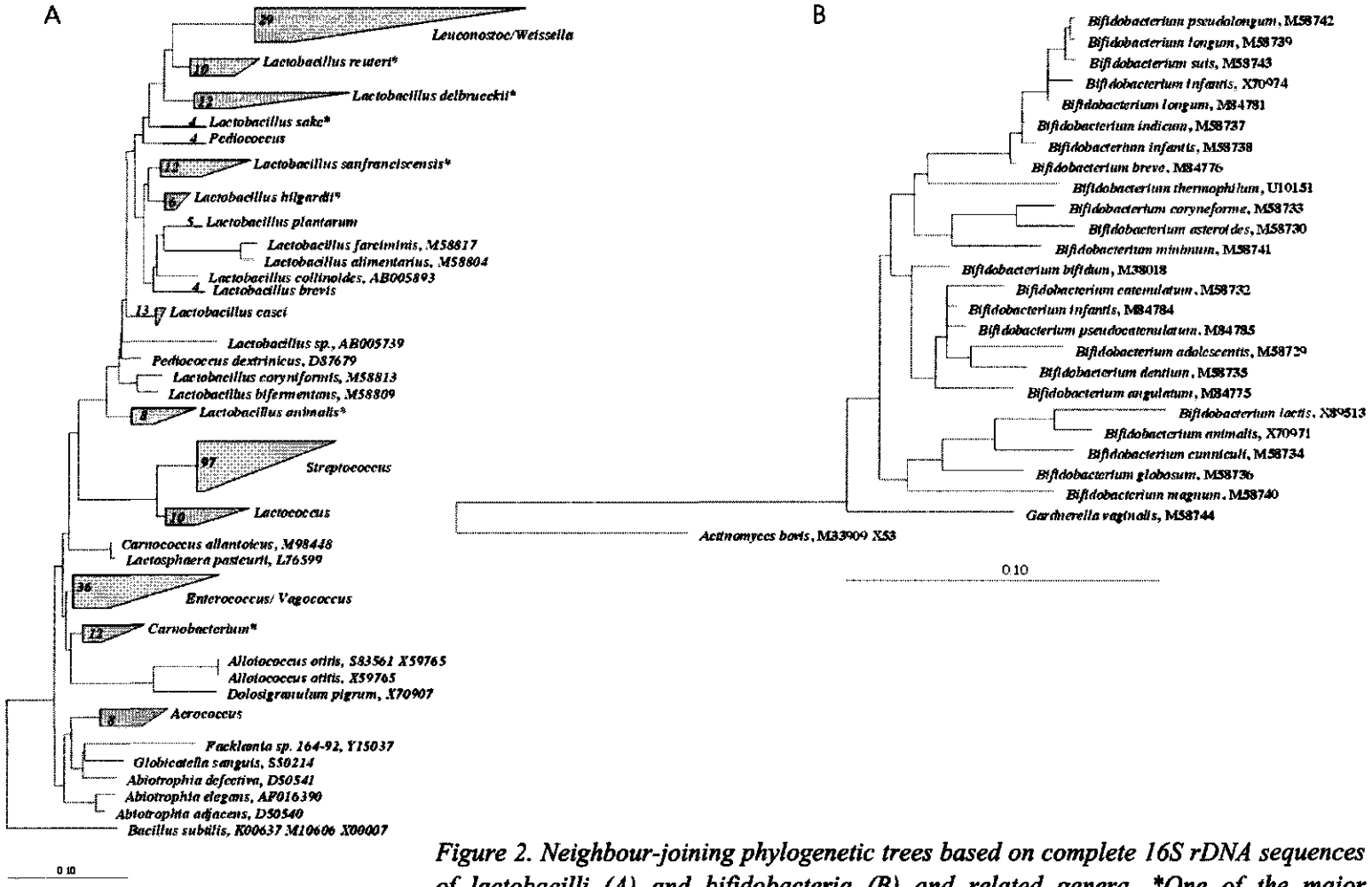

*Figure 2. Neighbour-joining phylogenetic trees based on complete 16S rDNA sequences of lactobacilli (A) and bifidobacteria (B) and related genera. *One of the major representatives of the group. (Trees kindly provided by Dr. E. Zoetendal.)*

Bifidobacteria are gram-positive, strictly anaerobic, non-motile and non spore-forming pleomorphic rods with a particular cell morphology ranging from regular rods to various branched and club-shaped forms. Fructose-6-phosphate phosphoketolase (F6PPK) is the key enzyme of hexose fermentation and F6PPK activity is one of the main phenotypic features used to identify bifidobacteria at the genus level (10). Lactic and acetic acid are produced as metabolic end products from hexose fermentation. Genus-specific probes are also available for the recognition of strains belonging to bifidobacteria (64, 73). The identification of *Bifidobacterium* species by phenotypic features such as sugar fermentation patterns, cell morphology and electrophoretic mobility of enzymes is difficult and therefore DNA-DNA reassociation studies have been widely used in bifidobacterial taxonomy (10, 41, 42). Species identification by 16S rRNA sequence analysis is hampered by the high level of sequence relatedness between closely related bifidobacterial species that show more than 97 % sequence similarity (93). However, in many cases subtle differences in the 16S rRNA sequences have been successfully utilised to design species specific probes and PCR primers that can be applied in species identification (see below). The sequence analysis of conserved genes other than 16S rDNA such as *recA*, coding for the recombinase RecA, and *ldh*, coding for L-lactate dehydrogenase, has been proposed as a method for identification of closely related bifidobacteria (71, 112).

Molecular tools for the detection and identification of bifidobacteria and lactobacilli

This section provides an overview of the different molecular techniques presently used in studying the microbial ecology of the GI-tract and their applications to bifidobacteria and lactobacilli. Specific attention is given to the design, validation and use of 16S rDNA-based oligonucleotides as hybridisation probes or primers in PCR amplification. The main strengths and weak points of the various techniques are summarised in Table 2.

Table 2. Advantages and drawbacks of various techniques for analysis of intestinal microbiota.

Method	Advantages	Drawbacks
Culturing	"Standard" method, recovery of isolates for further analysis	Laborious, requires immediate processing of samples and anaerobic techniques, inadequate selective media, part of the gut microbiota so far unculturable
•Colony hybridisation	Improved selectivity with probes	Requires probe design and validation
•Molecular fingerprinting	Superior strain discrimination	Laborious and slow
Direct molecular approaches	Pre-treated samples can be stored for later analysis, no culturing required, uncultured microbes can be detected	Isolates not recovered
•FISH	Enumeration without cultivation, potential for high throughput	Requires probe design and validation, laborious without automation
•Dot blot hybridisation	Relative quantification without culturing	Requires probe design and validation, relative abundance difficult to relate to cell numbers
•(RT)PCR-T/DGGE	Rapid diversity assessment and comparative analysis	Further identification of fragments by sequencing laborious, PCR-biases
•16S rDNA sequencing	Identification, sequence data of unculturable microbes obtained	Large scale monitoring laborious
•Microarrays	High throughput, automated analysis	No validation yet available

Molecular fingerprinting of bacterial strains

The application of fingerprinting techniques requires prior cultivation in order to obtain separate colonies of bacterial strains or pure cultures. DNA-based typing methods of pure cultures differ in many respects from the conventional phenotypic methods. The nucleotide sequence of the DNA is kept constant during growth and thus DNA-based typing methods are not influenced by the physiological state of the cells. However, extrachromosomal elements, such as plasmids, can be lost or acquired while chromosomal sequences may be subject to rearrangements. DNA-based methods are usually more rapid than the phenotypic methods, since they do not depend on microbial growth. Moreover, they are often also more discriminating and allow identification below the species level. The most commonly used typing methods, which have been adapted to bifidobacteria and lactobacilli are summarised below.

Amplified ribosomal DNA restriction analysis (ARDRA) is essentially restriction fragment length polymorphism analysis of the amplified rRNA genes. The entire rRNA gene or part of it is amplified by PCR and cut with one or more restriction endonucleases. The number and size of resulting DNA fragments varies according to the restriction sites within the specific rDNA sequences and thus, sequence-specific fragment patterns are produced. The discrimination power of the method to distinguish between different bacterial species or strains is dependent on the restriction enzymes used and length of the amplified fragment. A longer fragment contains potentially more restriction sites and produces a more complex pattern upon restriction. Some bacterial species show high rDNA sequence similarity and therefore it may be difficult to select restriction endonucleases that produce distinct restriction patterns for closely related species and strains (112). Typing of *L. acidophilus, L. helveticus, L. delbrueckii* and *L. sake* by ARDRA resulted in good discrimination at the species but not at the strain level (45, 120). ARDRA patterns are highly reproducible and comparable between laboratories.

In the randomly amplified polymorphic DNA (RAPD) technique, also known as arbitrarily primed PCR (AP-PCR), oligonucleotides with a random sequence are used as a primer in low stringency PCR. The primer(s) anneals to the complementary or partially complementary sequences in the target DNA (the

complete genome) and the DNA between the binding sites on opposite strands can be amplified. The RAPD fingerprint pattern consists of an array of anonymous DNA amplicons. Generally, RAPD fingerprinting allows the differentiation between species and, to some extent, also between strains within the same species. It has been widely applied for the typing of lactobacilli and bifidobacteria from various environments, including the intestinal tract (34, 61, 95, 105, 114, 144, 146).

Apart from RAPD, other PCR fingerprinting techniques have been developed such as the so-called rep-PCR techniques which are based on the use of repetitive DNA sequences that are dispersed throughout the bacterial genome (13). PCR-based fingerprinting techniques are simple and rapid, but for the reproducibility of the results careful optimisation and standardisation are needed. Differences in thermal cyclers, DNA polymerase suppliers and concentrations, DNA preparation methods, primer-to-template ratios and magnesium concentrations can cause variations in the RAPD and rep-PCR patterns (143). Consequently, the fingerprint patterns obtained in different laboratories are not always comparable.

Pulsed field gel electrophoresis (PFGE) allows the separation of large DNA fragments in a continuously reorienting electric field. In a uniform electric field, very large DNA molecules tend to migrate independently of their size, but in the changing electric field large molecules need more time to reorientate than smaller molecules and the migration velocity in the net field direction depends primarily on the size of the DNA molecule. PFGE has been widely applied in the analysis of bacterial genomes. The complete genome is digested with rare cutting enzymes such as *Apa*I, *Sma*I, *Bgl*I, *Sac*II, *Not*I, *Sfi*I, and *Xba*I, and the resulting macrofragments are separated by PFGE. PFGE protocols have been well established for both lactobacilli and bifidobacteria and the techniques have shown superior discriminatory power in comparison over other typing methods in strain differentiation (16, 23, 65, 89, 97, 101, 111, 113, 144, 158). Macrorestriction fragment analysis by PFGE is highly discriminating and a very reproducible typing method, but rather laborious, which restricts the number of isolates that can be typed.

Ribotyping is a variation of the restriction fragment length polymorphism (RFLP) analysis of the genomic DNA, where certain fragments are high-lighted

by probing in order to obtain less complex patterns that are easier to interpret. In ribotyping, the fingerprint pattern consists of chromosomal DNA fragments that are derived from the rDNA operon and its adjacent regions that hydridise to the rDNA probe. First, genomic DNA is cleaved by restriction enzymes and the resulting fragments are separated by agarose gel electrophoresis. The fragments are then transferred to a membrane and hydridised with a rDNA fragment. The probes used in ribotyping vary from partial sequences of the rDNA genes or their spacer regions (46, 83, 97) to the whole rDNA operon. The latter is also used as a probe in the automated ribotyping device, marketed as the RiboPrinter^{R} by Qualicon, USA. If the probe contains conserved regions of rDNA, it can be used for the ribotyping of a wide range of bacteria, even those that are phylogenetically distant. Evidently, more fragments hybridise with probes that encompass a larger region of the rDNA operon than with shorter probes (109). Thus, the discriminatory power of the technique is dependent on the size of the probe, but also on the restriction enzyme(s) used. Using this approach it was possible to divide 26 *L. helveticus* strains into five, nine and ten ribotypes using *Eco*RI, *Pvu*II and *Mlu*I, respectively using the intergenic 16S-23S rDNA as a probe (46). Moreover, ribotyping has been shown to be a useful tool in differentiating human intestinal lactobacilli and bifidobacteria at both the species and strain level (65, 83, 89, 144).

Probe and primer design based on ribosomal RNA sequences

The prokaryotic ribosomes consist of proteins and three ribonucleic acids: the 5S rRNA (120 nucleotides), 16S rRNA (1540 nucleotides) and the 23S rRNA (2900 nucleotides). The rRNA sequences consist of alternating conserved and variable regions with nine variable regions being found in the 16S rRNA. The rRNA genes (rDNA) are organised into one or more *rrn* operons, where they are separated by hypervariable spacer regions, and in many cases also one or more tRNA genes are found between the 16S and 23S rRNA genes (25, 100). Most organisms carry several *rrn* operons in their genome and in most cases the intragenomic sequences of the structural rRNA genes are highly similar. So far bifidobacteria and lactobacilli have been found to harbour from two to six *rrn* operons (23, 28, 97, 121, 157). Microheterogeneities have been described for the

16S rDNA of *Bifidobacterium adolescentis* (121). Analysis of rDNA sequence data, especially that of 16S rDNA, has revealed variable regions in the gene sequences that contain information specific for different phylogenetic levels; group, genus, species or subspecies. Thus, sequences unique to certain organisms or a group of organisms can be found. This has been utilised extensively to design species- and group-specific nucleic acid probes for the detection and identification of bacteria (Table 3).

Specific oligonucleotide probes and primers targeting rRNA or rDNA have been designed for different species of *Bifidobacterium* and *Lactobacillus* that occur in the human intestine and these have been used in various applications. Examples of validated probes and primers for bifidobacteria and lactobacilli are presented in Table 3. As mentioned above lactobacilli are phylogenetically heterogeneous, which causes difficulties in the design of a strictly genus-specific probe, but group-specific probes and primers have been designed. Two *Lactobacillus* group-specific primers cover in addition to *Lactobacillus* also *Leuconostoc, Pediococcus,* and *Weissella* (52, 148). In addition, two probes cover also other related genera such as *Enterococcus, Streptococcus, Vagococcus* and *Oenococcus* (Table 3). Presently, probes are available for all *Bifidobacterium* species found in the human intestine. However, the probe panel for lactobacilli is incomplete and lacks specific probes for intestinal species such as *L. vaginalis, L. mucosae, L. catenaformis, L. buchneri* and *L. rogosae.*

Table 3. rRNA/rDNA targeted oligonucleotide probes and PCR primers for Bifidobacterium *and* Lactobacillus *species found in the human intestine.*

Specificity	Sequence (5′to 3′)	Target	Target site[a]	Use	Ref.
Bifidobacterium-genus	CATCCGGCATTACCACCC CCACCGTTACACCGGGAA	16S	181-164 679-662	FISH	73
	CGGGTGCTI*CCCACTTTCATG GATTCTGGCTCAGGATGAACG	16S	1432-1412 15-35	PCR; CH	64
B. adolescentis	GCTCCCAGTCAAAAGCG	16S	451-435	DBH	156
	CTCCAGTTGGATGCATGTC CGAAGGCTTGCTCCCAGT	16S	182-200 474-442	PCR	88
B. angulatum	CAGTCCATCGCATGGTGGT GAAGGCTTGCTCCCCAAC	16S	185-203 473-441	PCR	88
B. bifidum	GCAGGCTCCGATCCGA	16S	1317-1303	DBH	156
	CCACATGATCGCATGTGATTG CCGAAGGCTTGCTCCCAAA	16S	184-204 475-442	PCR	88
B. breve	AAGGTACACTCAACACA	16S	491-475	DBH	156
	CCGGATGCTCCATCACAC ACAAAGTGCCTTGCTCCCT	16S	175-192 475-444	PCR	88
B. catenulatum/ pseudocatenulatum	CGGATGCTCCGACTCCT CGAAGGCTTGCTCCCGAT	16S	176-192 474-442	PCR	88
B. dentium	ATCCCGGGGGTTCGCCT GAAGGGCTTGCTCCCGA	16S	72-89 473-443	PCR	87
B. gallicum	TAATACCGGATGTTCCGCTC ACATCCCCGAAAGGACGC	16S	170-189 479-454	PCR	87
B. infantis	TCACGCTTGCTCCCCGATA	16S	459-441	DBH	156
	TTCCAGTTGATCGCATGGTC GGAAACCCCATCTCTGGGAT	16S	182-201 1027-1007	PCR	87
B. longum	TCTCGCTTGCTCCCCGATA	16S	459-441	DBH	156
	TTCCAGTTGATCGCATGGTC GGGAAGCCGTATCTCTACGA	16S	182-201 1028-1008	PCR	87
B. lactis[c]	GTGGAGACACGGTTTCCC CACACCACACAATCCAATAC	16S 16S-23S IS	991-1009[b] 1671-1651[b]	PCR	145

Table 3. Continued.

Specificity	Sequence (5'to 3')	Target	Target site[a]	Use	Ref.
Lactobacillus-group[d]	CACCGCTACACATGGAG	16S	683-667	PCR	52
	AGCAGTAGGGAATCTTCCA ATTY*CACCGCTACACATG	16S	362-380 705-688	PCR	148
	Y*CACCGCTACACATGR*AGTTCCACT[e]	16S	746-722	DBH	125
	GGTATTAGCAYCTGTTTCCA[f]	16S	177-158	FISH	48
L. acidophilus	GAATCTGTTGGTTCAGCTCGC	16S	86-66	DBH	53
	AGCTGAACCAACAGATTCAC	16S	70-89[b]	PCR	149
	TCTAAGGAAGCGAAGGAT CTCTTCTCGGTCGCTCTA	16S-23S IS		PCR	142
	TGCAAAGTGGTAGCGTAAGC	23S		PCR	130
L. brevis	TGTTGAAATCAGTGCAAG	16S	107-90[b]	DBH	147
L. casei	TGATCTCTCAGGTGATCAAAA	16S	1049-1028[b]	PCR	33
	TGCACTGAGATTCGACTTAA	16S	65-84[b]	PCR	150
	GCGATGCGAATTTCTTTTTC	16S-23S IS		PCR	149
L. casei/paracasei	GCACCGAGATTCAACATGGAA	16S	89-110[b]	PCR	27
L. casei/ rhamnosus	GCAGGCAATACACTGATG	23S		DBH	55
L. casei/paracasei/ rhamnosus	CTGATGTGTACTGGGTTC	23S		DBH	55
L. crispatus	GTAATGACGTTAGGAAAGCG	16S	66-85[b]	PCR	149
	CAATCTCTTGGCTAGCAC	23S		Reverse DBH	35
	AGGATATGGAGAGCAGGAAT CAACTATCTCTTACACTGCC	16S-23S IS 23S		PCR	130
L. delbrueckii	ACCTATCTCTAGGTGTAGCGCA	16S	1046-1024[b]	PCR	33
	ACGGATGGATGGAGAGCAG GCAAGTTTGTTCTTTCGAACTC	16S-23S IS		PCR	142
	AAGGATAGCATGTCTGCA	23S		DBH	55
	ACAGATGGATGGAGAGCAGA	16S-23S IS		PCR	130
L. fermentum	CAATCAATTGGGCCAACGCGT	16S	86-66	DBH	53
	GCGACCAAAATCAATCAGG	16S	110-92[b]	DBH	147
	GTTGTTCGCATGAACAACGCTTAA	16S	160-183[b]	PCR	27
	CTGATCGTAGATCAGTCAAG	16S-23S IS		PCR	149
	ACTAACTTGACTGATCTACGA TTCACTGCTCAAGTAATCATC	16S-23S IS 23S		PCR	130
L. gasseri	GAGTGCGAGAGCACTAAAG CTATTTCAAGTTGAGTTTCTCT	16S-23S IS		PCR	149
	AGCGACCGAGAAGAGAGAGA TGCTATCGCTTCAAGTGCTT	23S		PCR	130

Table 3 continued.

Specificity	Sequence (5'to 3')	Target	Target site[a]	Use	Ref.
L. helveticus	GAAGTGATGGAGAGTAGAGATA CTCTTCTCGGTCGCCTTG	16S-23S IS		PCR	142
L. jensenii	AAGAAGGCACTGAGTACGGA	16S-23S IS		PCR	130
L. johnsonii	GAGCTTGCCTAGATGATTTTA	16S	61-81[b]	PCR	149
L. paracasei	CACCGAGATTCAACATGG	16S	67-84[b]	PCR	150
	CACTGACAAGCAATACAC	23S		DBH	55
	GCGATGCGAATTTCTTTTTC	16S-23S IS		PCR	142
	GGCCAGCTATGTATTCACTGA	23S		PCR	130
L. paracasei/casei	CCGAGATTCAACATGG	16S	69-84[b]	PCR	27
L. paracasei/ rhamnosus	CAGACTGAAAGTCTGACGG GTACTGACTTGCGTCAGCGG	16S-23S IS		PCR	142
L. plantarum	CCAATCAATACCAGAGTTCG	16S	86-67	DBH	53
	ATCATGATTTACATTTGAGTG	16S	96-117[b]	PCR	27
	TTACCTAACGGTAAATGCGA	16S-23S IS		PCR	149
	ATTCATAGTCTAGTTGGAGGT CCTGAACTGAGAGAATTTGA	23S		PCR	130
L. reuteri	GATCCATCGTCAATCAGG	16S	109-92[b]	DBH	147
	TGAATTGACGATGGATCACCAGTG	16S	91-114[b]	PCR	27
	AACACTCAAGGATTGTCTGA	16S-23S IS		PCR	149
	CAGACAATCTTTGATTGTTTAG GCTTGTTGGTTTGGGCTCTTC	16S-23S IS 23S		PCR	130
L. rhamnosus	GCGATGCGAATTTCTATTATT	16S-23S IS		PCR	142
	CTTGCATCTTGATTTAATTTTG	16S	91-112	PCR	2
	TGCATCTTGATTTAATTTTG	16S	65-84[b]	PCR	150
	GCGATGCGAATTTCTATTATT	16S-23S IS		PCR	149
L. ruminis	TTCGGTGAAAGAAAGCTTCG	16S	86-67	DBH	53
L. sakei	TTAATGATAATACTCGATT	23S		DBH	54
L. salivarius	ATTCACTCGTAAGAAGT	16S	95-111[b]	PCR	27
	AATCGCTAAACTCATAACCT CACTCTCTTTGGCTAATCTT	16S-23S IS 23S		PCR	130
L. zeae	TGTTTAGTTTTGAGGGGACG ATGCGATGCGAATTTCTAAATT	16S-23S IS		PCR	149

[a] Corresponds to the *E. coli* numbering or [b] corresponds to the numbering of the respective species sequence
[c] Not a human intestinal species, but used as a probiotic. Reclassified as *B. animalis*.
[d] Includes genera *Lactobacillus, Leuconostoc, Pediococcus,* and *Weissella*
[e] Includes genera *Lactobacillus, Leuconostoc, Pediococcus, Weissella, Enterococcus* and *Streptococcus*
[f] Includes genera *Lactobacillus, Leuconostoc, Pediococcus, Weissella, Enterococcus, Oenococcus* and *Vagococcus*
* I = Inosine, matches all four nucleotides (A, C, G, and T); Y = C or T; R = A or G
IS = intergenic sequence
CH = colony hybridisation; DBH = dot blot hybridisation; FISH = fluorescent *in situ* hybridisation; PCR = polymerase chain reaction

Direct detection of bacteria by using specific primers and probes

Amplification of DNA by the polymerase chain reaction (PCR) is one of the easiest and most rapid ways of detecting specific sequences. rRNA may be used as a measure of cellular activity and reverse transcription-PCR (RT-PCR) of the rRNA is performed prior to the PCR in order to detect this molecule. In (RT-)PCR nucleic acids are amplified rapidly *in vitro* using a thermostable DNA polymerase enzyme (e.g. *Taq* polymerase) and synthetic oligonucleotide primers in a thermo-cyclic process. The specificity of (RT-)PCR is mainly dependent on the primers, but also reaction conditions and thermo-cycling programs have to be adjusted for optimal performance. Due to the exponential amplification of nucleic acids in PCR, the sensitivity of detection is high. Specific PCRs are widely used in detecting the presence of a certain microbe in a sample or to verify the identity of a cultivated isolate. Quantification of PCR is possible by using an internal standard, which is added to the reaction in a known amount. The amount of the amplification products is then measured and the initial amount of the target sequence is calculated using the amplification efficiency of the standard as a reference. In real-time PCR, fluorescent markers are incorporated into the amplicons and the kinetics of amplification of products with different melting temperatures is observed directly, which greatly facilitates the quantification. A set of species-specific 16S rDNA primers and real-time PCR was used to successfully monitor and quantify several bacterial species in the rumen of cows (138). However, due to the many factors affecting the amplification reaction (152) quantification of nucleic acids by PCR is only approximate and the determination of bacterial numbers is hampered by variation of the *rrn* operon copy number and the ribosomal content of cells in different bacteria.

Direct hybridisation of an oligonucleotide probe to the target microbial nucleic acid (dot blot hybridisation) is a straightforward method to detect specific nucleic acid sequences or genes. DNA or RNA is extracted from a sample or cultured microbes, fixed to a positively charged membrane (nylon, nitrocellulose) and hybridised with an oligonucleotide or a fragment of DNA that has a radioactive, chemiluminescent or digoxigenin label. The dot blot technique can be quantified to measure the amount of specific target

rRNA/rDNA in a mixture relative to the total amount of rRNA/rDNA (32, 126). Microbial cells can be also directly transferred to a membrane after which cells are lysed and DNA fixed to the membrane. In colony hybridisation, bacterial colonies are transferred to a membrane, lysed, and hybridised to a probe. This allows one to enumerate the target microbes and to recover the colonies from replica plates for further analysis.

Ehrmann *et al.* (35) presented a reverse dot blot hybridisation technique for simultaneous detection of different lactic acid bacteria. A sample was first subjected to a non-specific PCR amplification of rDNA and the labelled PCR products were then hybridised with *Lactobacillus* species-specific capture probes that were bound to filter membranes. An important further step in hybridisation techniques is the development of DNA-chips, also called DNA microarrays (38). With this new technique up to thousands of probes can be bound on small glass slides and samples can be tested simultaneously against many probes, analogously to the reverse dot blot hybridisation. Hybridisations with fluorescent sequences are detected by using a laser. Recently, DNA microarrays containing 16S rDNA probes have been reported and they may be promising tools for the characterisation of complex microbial communities (4).

In FISH technique, the detection of rRNA sequences within morphologically intact cells is achieved using fluorescently labelled oligonucleotide probes. Special permeabilisation procedures may be required in order to aid passage of the probe into cells of some bacteria (12, 110). The multiple rRNA molecules present in bacterial cells capture the probe, the signal is intensified and the cells can be detected and enumerated by using an epifluorescence microscope or flow cytometry (58, 159). The detection limit of FISH with automated, microscopy-based enumeration is approximately $1x10^7$ cells per gram of faeces (58). FISH and dot blot techniques have proved to be invaluable tools for the quantification of bacteria in faeces (40, 49, 85, 126, 135).

Profiling of bacterial communities

Nucleotide sequence analysis of 16S rRNA or rDNA provides a direct way of assessing the microbial diversity in an ecosystem. In practise, 16S rDNA (or rRNA) sequences present in the sample are PCR-amplified and cloned in order to construct a rDNA sequence library. Subsequently, the clones are sequenced and compared to sequences available in the rRNA databases such as GenBank, EMBL and Ribosomal Database Project (RDP) (79). In general, 16S rRNA sequences having more than 97 % similarity are considered to belong to the same molecular species or operational taxonomic unit (OTU). Molecular species and OTU are terms used to describe sequences with low sequence divergence, that are likely to be derived from the same species. Thus, it is reasonable to assume that a sequence has derived from a novel, hitherto undescribed species if its similarity is less than 97 % with the sequences of known species (132). The number of clones that needs to be sequenced in order to get a reasonable estimate of the diversity depends on the complexity of the ecosystem. An estimated 85 % coverage of one person's faecal 16S rDNA diversity was obtained when 284 clones were sequenced (134). The clones were classified into 82 molecular species, the majority of which were found to be novel. While direct sequencing can indeed provide significant new information about the microbial diversity in the GI-tract, the approach is rather laborious and hence, appropriate only for the screening of a limited number of samples.

The rRNA heterogeneity among bacterial species can also be utilised in fingerprinting of bacterial communities (98, 99). In practise, RNA or DNA is extracted from an environmental sample and a fragment of the rRNA or rDNA is amplified using primers for bacteria or other phylogenetic groups. The PCR amplicons, all of approximately equal size but varying in sequence, are then separated by using specific electrophoretic techniques, temperature gradient gel electrophoresis (TGGE) or denaturing gradient gel electrophoresis (DGGE). During electrophoresis the DNA fragments migrate in an increasing gradient of denaturing agents (temperature or denaturing chemicals) which gradually leads to the separation of the two strands of double-stranded DNA (melting). One end of the fragment is held together with a so-called CG-clamp, which is an approximately 30 to 50 bp long C + G -rich sequence that is attached to the 5′-

end of one of the primers. In theory, a DNA fragment stops migrating in the T/DGGE gel when it reaches its melting point. PCR amplicons with different sequences have different melting behaviour and consequently migrate unequal distances in the T/DGGE gel. After staining, the amplicons with different sequences are seen as separate fragments in the gel forming a "fingerprint" of the microbial community.

The PCR-T/DGGE of 16S rRNA and rDNA is now widely used in molecular ecological studies to assess, in a culture-independent way, the diversity and community dynamics in microbial communities (99). In general, these techniques detect only the predominant community members that make up at least 1 % or more of the total community (99). Application of this method to study human adult faeces revealed unique TGGE patterns of bacterial 16S rRNA for each individual, reflecting a host-specific community of dominant bacteria (161). Furthermore, it was found that the host genotype contributes to determining the bacterial composition in the GI-tract (160). PCR-DGGE was likewise successfully used to monitor day by day the succession of bacteria in the faeces of new-born infants (36). The achievements in profiling predominant bacterial communities lead to the further refinement of the method. In order to obtain a better sensitivity of detection for less abundant bacteria, PCR reactions that target restricted bacterial groups were used. Recently, PCR-DGGE methods for the selective monitoring of bifidobacteria and lactic acid bacteria were developed (52, 121, 148). The T/DGGE method is a convenient tool to monitor the complexity of microbial community and changes occurring in it, while individual members of the community can be identified by subsequent cloning and sequencing of the fragments. Another possibility to obtain additional information about particular species or bacterial groups is to hybridise T/DGGE profiles with phylogenetic probes.

As with every method, there are also problems associated with the use of PCR for the analysis of microbial community structure, primarily due to the formation of artefacts such as chimeric and heteroduplex molecules when a particular gene is amplified from a mixed community of DNA (152). In the subsequent sequence analysis, such chimeras can be readily discovered by performing a chimera-check and subsequently omitted from the diversity estimates. However, artefact fragments can not be directly pointed out from the

T/DGGE profiles, and therefore may lead to an overestimation of the microbial diversity. On the other hand, 16S rDNA fragments with different sequences can have similar melting behaviour and co-migrate to the same position in the gel. Thus, T/DGGE profiles provide a general picture of the diversity rather than the exact number of community members. Other, more recent community fingerprinting methods such as single strand conformation polymorphism (SSCP) of DNA (124), terminal restriction fragment length polymorphism (T-RFLP) (84), and RAPD-based community fingerprinting (39) have not gained great popularity in GI-tract ecology so far. Overall, it is easy to see the benefits of molecular methods, used either directly or in combination with cultivation, in acquisition of new and more detailed information about the GI-tract microbiota.

Molecular studies of bifidobacteria and lactobacilli in the GI-tract

Traditionally bacterial numbers and diversity in the GI-tract were assessed by culturing on selective media and subsequent identification to genus and species level by phenotypic characterisation. The realisation that a considerable part of the intestinal microbiota is not culturable using present techniques and media (73, 94), and advances in molecular biology have lead to the development and application of culture-independent molecular methods in GI-tract ecology studies. Some common molecular approaches and the roles of different techniques in analysing the GI-tract microbiota are schematically illustrated in Figure 3.

The traditional culturing strategy is well applicable to study the ecology of bifidobacteria and lactobacilli, which are culturable and can be adequately recovered from various selective media (11, 50, 51, 108). Recently, a number of molecular fingerprinting methods have been adapted to bifidobacteria and lactobacilli leading to a more detailed description of these populations in the GI-tract and new ecological observations. The composition of the *Bifidobacterium* and *Lactobacillus* populations was found to be host-specific and in general, bifidobacterial populations seem to be rather stable both in numbers and composition over time, whereas more fluctuation is seen in the lactobacilli

populations (65, 82, 89, 141). Interestingly, an ingested *Lactobacillus* strain established itself better in individuals lacking a stable indigenous lactobacilli population than in those stably harbouring lactobacilli (141). The culturing approach is often used in monitoring of dietary intervention studies, because for example an ingested probiotic (see below) strain can be recovered from the plates and identified unequivocally by using highly discriminating typing methods (70, 141). Analysis of the microbial diversity by this approach is, however, laborious and the effective recovery of bifidobacteria requires strict anaerobic practise. A major advantage of culturing is the acquisition of isolates, whose activity and properties can be studied further.

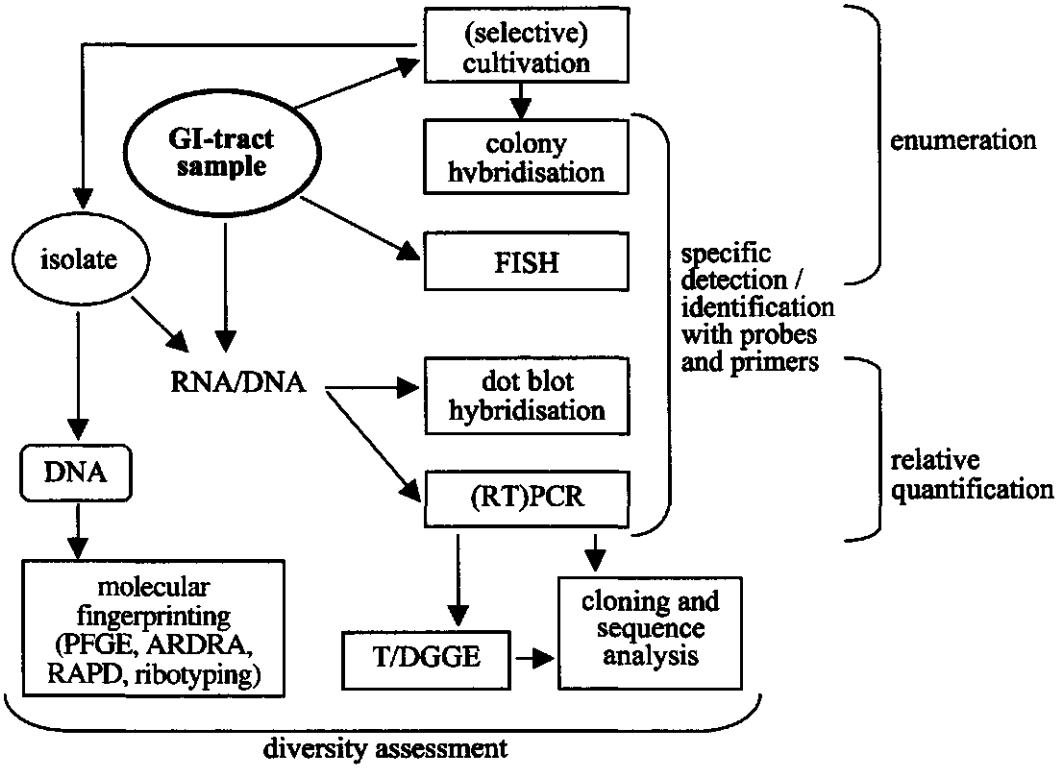

Figure 3. Molecular approaches to study the intestinal microbiota.

Recently developed genus and group-specific PCR-DGGE methods allow a convenient way to monitor the diversity and dynamics of bifidobacterial and lactobacilli populations (52, 121, 148). The PCR-DGGE approach confirmed the

earlier observations that adult faecal bifidobacterial populations are host-specific and stable in time (121). PCR-DGGE monitoring of faecal bifidobacteria in a probiotic and prebiotic feeding trial revealed that while a probiotic strain *B. lactis* Bb12 transiently colonised the gut the indigenous populations of bifidobacteria remained unaffected (122). Likewise galactooligosaccharide administration did not affect the qualitative composition of indigenous *Bifidobacterium* populations (122).

The problems related to the enumeration of bifidobacteria by cultivation can be bypassed by using FISH, which has shown counts that are comparable to those obtained by cultivation (typically 10^9 to 10^{10} cfu/g faeces in adults), yet slightly lower due to better selectivity (40, 73, 141). However, due to the improved enumeration of other bacterial groups by FISH, it was discovered that bifidobacteria usually constitute only 1 to 3 % of the total faecal bacterial community in adults (40, 73) instead of 7 to 10 % estimated by cultivation (32, 73, 94). The smaller proportion of bifidobacteria has also been confirmed by quantitative rRNA dot blot studies (32, 85, 126). Likewise the proportion of lactobacilli was found to represent less than 1 % of the total faecal bacteria by dot blot technique (126) in comparison to the approximately 2 % obtained by cultivation (94).

Species-specific PCR and dot blot hybridisation have mostly been used in combination with cultivation to confirm the identity of (tentatively identified) faecal isolates, either indigenous or probiotic strains (2, 24, 96, 149). However, when applied directly to faecal samples these techniques can also reveal minor species, which would not be detected when a limited number of colonies are analysed, due to the abundance of other dominant species (87). Similarly, *L. sakei* and *Leuconostoc mesenteroides* and other species that are typically detected in food products but not in human faeces, were revealed in faecal samples by using culture-independent community profiling based on *Lactobacillus*-group-specific PCR and DGGE and subsequent identification of PCR amplicons by cloning and sequencing (52, 148). The pitfall of PCR approaches is that they can also detect DNA from dead bacterial cells, if present in sufficient amounts.

Approaches to increase the numbers of bifidobacteria and lactobacilli in the gut

The general recognition of bifidobacteria and lactobacilli as beneficial members of the intestinal microbiota has lead to attempts to increase their numbers in the GI-tract by administration of living bifidobacteria or lactobacilli (probiotics) or compounds that stimulate their growth (prebiotics).

Probiotics

Probiotics can be defined as viable microorganisms that exhibit a positive effect on the health of the host when they are ingested (119). In practise, probiotic strains are selected not only according to their functional properties, but also considering safety and technological aspects (115). Most commonly, probiotics are selected from strains of *Bifidobacterium* and *Lactobacillus*, while representatives of other microbial groups are rather exceptional, such as *Saccharomyces boulardii* or *Bacillus subtilis*. A myriad of health claims has been associated with probiotics (117), some of which have been substantiated for certain probiotic strains in clinical trials (Table 4). It must be emphasised that probiotic properties may be strain-dependent and can not be generalised to other probiotic strains even if they belong to the same species.

Prebiotics

Prebiotic compounds 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 (44). Most potential prebiotics are carbohydrates, either polysaccharides, such as inulin, or oligosaccharides including fructooligosaccharides (FOS) and galactooligosaccharides (GOS). The fermentation of carbohydrates by the intestinal microbiota results in end products that are beneficial to the host, in contrast to the potentially toxic protein fermentation end products and thus, the

metabolic activity of microbes on prebiotics may improve the host's health. Prebiotics are assumed to stimulate the growth of bifidobacteria, and possibly lactobacilli, that are generally considered beneficial, although this is not specified in the definition of prebiotics. Human milk contains N-acetylglucosamine containing oligosaccharides that have been shown to promote the growth of bifidobacteria (72). Numerous human feeding trials have been conducted to elucidate the effect of prebiotics on the gut microbiota (Table 5). The potential of prebiotics to modulate the gut microbiota was studied by assessing the shifts in the numbers of bifidobacteria and other bacterial groups usually by selective plating. The bifidogenic effect of fructose-based prebiotics inulin and FOS has been showed in several studies (22, 43, 67), whereas the results concerning GOS remain conflicting with some studies demonstrating a bifidogenic effect (21, 57) that was not confirmed by other studies (1, 3). Molecular monitoring of faecal bifidobacteria using the PCR-DGGE approach revealed that GOS administration (8 g per day for two weeks) did not have an effect on the qualitative composition of indigenous *Bifidobacterium* population (122).

Synbiotics

A synbiotic is a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare (44). The feasibility of the synbiotic concept to prolong the colonisation by a probiotic strain has been tested in several *in vivo* trials (1, 20, 69, 122) but only in one case the results suggested an improved survival of the probiotic strain due to a simultaneous prebiotic administration (69).

Table 4. Reported clinical effects of some probiotic strains.

Strain	Clinical effects in humans	References
Lactobacillus rhamnosus GG (ATCC 53103)	Reduction of antibiotic-associated diarrhoea in children, treatment and prevention of rotavirus and acute diarrhoea in children, treatment of relapsing *Clostridium difficile* diarrhoea, immune response modulation, alleviation of atopic dermatitis symptoms in children	6, 15, 63, 80, 81, 104, 107, 129
Lactobacillus johnsonii (*acidophilus*) LJ-1 (La1)	Modulation of intestinal flora, immune enhancement, adjuvant in *Helicobacter pylori* treatment	31, 75, 86, 92, 123
Bifidobacterium lactis Bb-12	Prevention of traveller's diarrhoea, treatment of viral diarrhoea including rotavirus diarrhoea, modulation of intestinal flora, modulation of immune response, alleviation of atopic dermatitis symptoms in children	17, 18, 63, 75, 116
Lactobacillus reuteri (BioGaia Biologics)	Shortening of rotavirus diarrhoea in children, treatment of acute diarrhoea in children, safe and well-tolerated in HIV-positive adult subjects	127, 128, 154, 155
Lactobacillus casei Shirota	Modulation of intestinal flora, positive effects on superficial bladder cancer and cervical cancer, no influence on the immune system of healthy subjects	7, 8, 102, 131, 139
Lactobacillus plantarum DSM9843 (299v)	Modulation of intestinal flora	59, 60
Saccharomyces boulardii	Prevention of antibiotic-associated diarrhoea, treatment of *Clostridium difficile* colitis, prevention of diarrhoea in critically ill tube-fed patients	19, 26, 91, 136

Adapted from Saarela *et al.*, 2000 (115).

Table 5. The gut microbiota modulating effects of some prebiotics

Prebiotic	Dose (g/d)	Supplement period (d)	Study volunteers (and No.)	Log increase in faecal bifidobacteria (statistical significance)	Significant decrease (-) or increase (+) in other groups	Reference
Inulin	15	15	adults (4)	0.9 ($P < 0.01$)	– gram-positive cocci ($P < 0.001$)	43
	20 and 40	8	elderly, constipated (10 in both groups)	0.9 and 1.3 ($P < 0.05$)	– enterococci with 40 g/d ($P < 0.01$)	67
FOS	5, 10 and 20	7	adults (8 in each group)	0.95 (not significant), 1.52 and 1.35 ($P < 0.05$)		22
	15	15	adults (8)	0.7 ($P < 0.01$)	– bacteroides, clostridia and fusobacteria ($P < 0.05$)	43
GOS	10	7, 14 and 21	adults (8)	1.1, 1.1 and 0.9 ($P < 0.05$)		21
	10	7	adults (12)	0.3 ($P < 0.001$)	+ lactobacilli 0.70 ($P < 0.05$)	57
	8.5 and 14.4	21	adults (13 and 14)	0.3 and 0.4 (not significant)		3
	8.1	14	adults (8)	0.2 (not significant)	– *Clostridium perfringensis* ($P < 0.05$)	1

FOS – fructooligosaccharide; GOS – galactooligosaccharide

In all studies the volunteers were healthy. Adults were 20 to 55 and elderly were 68-89 years of age.

Outline of the thesis

The aim of the research presented in this thesis was to develop and apply new molecular methods for the identification and detection of *Bifidobacterium* and *Lactobacillus* spp. in the human intestine.

Bifidobacteria are one of the major cultivable groups of bacteria in the intestine. Enumeration of bifidobacteria can be done in a straightforward way either using selective plating or FISH of 16S rRNA, while the assessment of species and/or strain diversity has necessitated the use of labour-intensive approaches, such as molecular fingerprinting of cultivated isolates. **Chapter 2** describes the development and validation of a method for the qualitative analysis of complex bifidobacterial populations based on genus-specific PCR on 16S rDNA and DGGE.

The *L. casei* -group of lactobacilli, which includes *L. casei, L. paracasei, L. rhamnosus*, and *L. zeae,* forms an important group of intestinal lactobacilli. The identification of the *L. casei* -group species is not reliable by phenotypic methods. In **Chapter 3** three molecular fingerprinting methods, including PFGE, RAPD and ribotyping were compared for the characterisation and identification of *L. casei* -group lactobacilli. Moreover, **Chapters 3 and 4** describe the development and validation of *L. rhamnosus* species-specific PCR.

The attempts to increase the potentially beneficial bifidobacteria and lactobacilli in the human gut include administration of probiotics and prebiotics. In **Chapters 4 and 5** the survival of two probiotic strains, *L. rhamnosus* GG and *B. lactis* Bb12, in the GI-tract was traced using the newly developed molecular methods. The identity of LGG colonies was verified using species-specific PCR (**Chapter 4**) and Bb12 was detected using the PCR-DGGE method modified for this purpose (**Chapter 5**). In addition, in **Chapter 5** the effect of Bb12 or/and galactooligosaccharide administration on the qualitative composition of the indigenous *Bifidobacterium* populations in healthy adults was studied by using *Bifidobacterium* genus-specific PCR-DGGE method.

Diet is generally considered one of the major factors determining the composition of the intestinal microbiota in infancy. In **Chapter 6** PCR-DGGE followed by the cloning and sequencing of amplified 16S rDNA fragments was

used to compare bifidobacterial and lactobacilli communities as well as the major bacterial groups in breast-fed and formula-fed infants. The summary and concluding remarks are presented in **Chapter 7**.

References

1. **Alander, M., J. Mättö, W. Kneifel, M. Johansson, B. Kögler, R. Crittenden, T. Mattila-Sandholm, and M. Saarela.** 2001. Effect of galacto-oligosaccharide supplementation on human faecal microflora and on survival and persistence of *Bifidobacterium lactis* Bb-12 in the gastrointestinal tract. J. Dairy Sci. 11:817–825.

2. **Alander, M., R. Satokari, R. Korpela, M. Saxelin, T. Vilpponen Salmela, T. Mattila Sandholm, and A. von Wright.** 1999. Persistence of colonization of human colonic mucosa by a probiotic strain, *Lactobacillus rhamnosus* GG, after oral consumption. Appl. Environ. Microbiol. **65:**351–354.

3. **Alles, M. S., R. Hartemink, S. Meyboom, J. L. Harryvan, K. M. Van Laere, F. M. Nagengast, and J. G. Hautvast.** 1999. Effect of transgalactooligosaccharides on the composition of the human intestinal microflora and on putative risk markers for colon cancer. Am. J. Clin. Nutr. **69:**980–991.

4. **Andersen, G. L.** 2001. Development of a high density microarray for the detection of bacteria in the environment. Abstract, p. 180. 9th International symposium on microbial ecology: Interactions in the microbial world, Amsterdam, The Netherlands.

5. **Anonymous.** 2001. Bacterial nomenclature up-to-date; *Lactobacillus*. Continuously updated by DSMZ – Deutsche Sammlung von Micro-organismen und Zellkulturen GmbH. Available from the internet at http://www.dsmz.de/bactnom/nam1537.htm.

6. **Arvola, T., K. Laiho, S. Torkkeli, H. Mykkänen, S. Salminen, L. Maunula, and E. Isolauri.** 1999. Prophylactic *Lactobacillus* GG reduces antibiotic-associated diarrhea in children with respiratory infections: a randomized study. Pediatrics. **104:**e64.

7. **Aso, Y., and H. Akazan.** 1992. Prophylactic effect of a *Lactobacillus casei* preparation on the recurrence of superficial bladder cancer. Urol. Int. **49:**125–129.

8. **Aso, Y., H. Akazan, T. Kotake, T. Tsukamoto, K. Imai, and S. Naito.** 1995. Preventive effect of a *Lactobacillus casei* preparation on the recurrence of superficial bladder cancer in a double-blind trial. Eur. Urol. **27:**104–109.

9. **Axelsson, L.** 1998. Lactic acid bacteria: classification and physiology, p. 1–72. *In* S. Salminen, and A. von Wright (eds), Lactic acid bacteria – Microbiology and functional aspects, 2nd ed. Marcel Dekker, Inc., New York.

10. **Ballongue, J.** 1998. Bifidobacteria and probiotic action, p. 519–587. *In* S. Salminen, and A. von Wright (eds), Lactic acid bacteria, Microbiology and functional aspects, 2nd ed. Marcel Dekkers, Inc, New York.

11. **Beerens, H.** 1990. An elective and selective isolation medium for *Bifidobacterium* spp. Lett. Appl. Microbiol. **11:**155–175.

12. **Beimfohr, C., A. Krause, R. Amann, W. Ludwig, and K.-H. Schleifer.** 1993. *In situ* identification of lactococci, enterococci and streptococci. System. Appl. Microbiol. **16:**450–456.

13. **Belkum, v. A., S. Scherer, L. van Alphen, and H. Verbrugh.** 1998. Short-sequence DNA repeats in prokaryotic genomes. Microbiol. Mol. Biol. Rev. **62:**275–293.

14. **Bengmark, S.** 2000. Bacteria for optimal health. Nutr. **16:**611-615.

15. **Bennett, R. G., S. L. Gorbach, B. R. Goldin, T.-W. Chang, B. E. Laughon, W. B. Greenough, and J. G. Bartlett.** 1996. Treatment of relapsing *Clostridium difficile* diarrhea with *Lactobacillus* GG. Nutr. Today. **31:**35S–38S.

16. **Björkroth, J., J. Ridell, and H. Korkeala.** 1996. Characterization of *Lactobacillus sake* strains associating with production of ropy slime by randomly amplified polymorphic DNA (RAPD) and pulsed-field gel electrophoresis (PFGE) patterns. Int. J. Food Microbiol. **31:**59–68.

17. **Black, F., K. Einarsson, A. Lidbeck, K. Orrhage, and C. E. Nord.** 1991. Effect of lactic acid producing bacteria on the human intestinal microflora during ampicillin treatment. Scand. J. Infect. Dis. **23:**247–254.

18. **Black, F. T., P. L. Andersen, J. Orskov, F. Orskov, K. Gaarslev, and S. Laulund.** 1989. Prophylactic efficacy of lactobacilli on traveller's diarrhoea. Travel Med. **333–335.**

19. **Bleichner, G., H. Blehaut, H. Mentec, and D. Moyse.** 1997. *Saccharomyces boulardii* prevents diarrhea in critically ill tube-fed patients. A multicenter, randomized, double-blind placebo-controlled trial. Intensive Care Med. **23:**517–523.

20. **Bouhnik, Y., B. Flourie, C. Andrieux, N. Bisetti, F. Briet, and J. C. Rambaud.** 1996. Effects of *Bifidobacterium* sp. fermented milk ingested with or without inulin on colonic bifidobacteria and enzymatic activities in healthy humans. Eur. J. Clin. Nutr. **50:**269–273.

21. **Bouhnik, Y., B. Flourie, L. D'Agay-Abensour, P. Pochart, G. Gramet, M. Durand, and J.-C. Rambaud.** 1997. Administration of transgalacto-oligosaccharides increases fecal bifidobacteria and modifies colonic fermentation metabolism in healthy humans. J. Nutr. **127**:444–448.

22. **Bouhnik, Y., K. Vahedi, L. Achour, A. Attar, J. Salfati, P. Pochart, P. Marteau, B. Flourie, F. Bornet, and J. C. Rambaud.** 1999. Short-chain fructo-oligosaccharide administration dose-dependently increases fecal bifidobacteria in healthy humans. J. Nutr. **129**:113–116.

23. **Bourget, N., J. M. Simonet, and B. Decaris.** 1993. Analysis of the genome of the five *Bifidobacterium breve* strains: plasmid content, pulsed-field gel electrophoresis genome size estimation and *rrn* loci number. FEMS Microbiol. Lett. **110**:11–20.

24. **Brigidi, P., B. Vitali, E. Swennen, L. Altomare, M. Rossi, and D. Matteuzzi.** 2000. Specific detection of *Bifidobacterium* strains in a pharmaceutical probiotic product and in human feces by polymerase chain reaction. System. Appl. Microbiol. **23**:391–399.

25. **Brosius, J., T. J. Dull, D. D. Sleeter, and H. F. Noller.** 1981. Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli.* J. Mol. Biol. **148**:107–127.

26. **Buts, J. P., G. Corthier, and M. Delmee.** 1993. *Saccharomyces boulardii* for *Clostridium difficile*-associated enteropathies in infants. J. Pediatr. Gastroenterol. Nutr. **16**:419–425.

27. **Chagnaud, P., K. Machinis, L. A. Coutte, A. Marecat, and A. Mercenier.** 2001. Rapid PCR-based procedure to identify lactic acid bacteria: application to six common *Lactobacillus* species. J. Microbiol. Meth. **44**:139–148.

28. **Chevallier, B., J. C. Hubert, and B. Kammerer.** 1994. Determination of chromosome size and number of *rrn* loci in *Lactobacillus plantarum* by pulsed field electrophoresis. FEMS Microbiol. Lett. **120**:51–56.

29. **Cooperstock, M. S., and A. J. Zedd.** 1983. Intestinal flora of infants., p. 79–99. *In* D. J. Hentges (ed.), Human intestinal microflora in health and disease. Academic Press, New York.

30. **Cummings, J. H.** 1995. Short chain fatty acids., p. 101–130. *In* G. R. Gibson, and G. T. Macfarlane (eds), Human colonic bacteria: role in nutrition, physiology, and pathology. CRC Press, Boca Raton.

31. **Donnet-Hughes, A., F. Rochat, P. Serrant, J. M. Aeschlimann, and J. E. Schiffrin.** 1999. Modulation of nonspecific mechanisms of defense by lactic acid bacteria: effective dose. J. Dairy Sci. **82**:863–869.

32. **Doré, J., G. Gramet, I. Goderel, and P. Pochart.** 1998. Culture-independent characterisation of human faecal flora using rRNA-targeted hybridisation probes. Genet. Sel. Evol. **30:**S287–S296.

33. **Drake, M., C. L. Small, K. D. Spence, and B. G. Swanson.** 1996. Rapid detection and identification of *Lactobacillus* spp. in dairy products using the polymerase chain reaction. J. Food Protection. **59:**1031–1036.

34. **Du Plessis, E. M., and L. M. T. Dicks.** 1995. Evaluation of random amplified polymorphic DNA (RAPD)-PCR as method to differentiate *Lactobacillus acidophilus, Lactobacillus crispatus, Lactobacillus amylovorus, Lactobacillus gallinarum, Lactobacillus gasseri* and *Lactobacillus johnsonii.* Curr. Microbiol. **31:**114–118.

35. **Ehrmann, M., W. Ludwig, and K. H. Schleifer.** 1994. Reverse dot blot hydridization: a useful method for the direct identification of lactic acid bacteria in fermented food. FEMS Microbiol. Lett. **117:**143–150.

36. **Favier, C., E. E. Vaughan, W. M. de Vos, and A. D. L. Akkermans.** Molecular monitoring of succession of bacterial communities in human neonates. Submitted for publication.

37. **Finegold, S. M., V. L. Sutter, and G. E. Mathisen.** 1983. Normal indigenous intestinal flora., p. 3–31. *In* D. J. Hentges (ed.), Human intestinal microflora in health and disease. Acamedic Press, Inc., London.

38. **Fodor, S. P. A., R. P. Rava, X. C. Huang, A. C. Pease, C. P. Holmes, and C. L. Adams.** 1993. Multiplex biochemical assays with biological chips. Nature. **364:**555–556.

39. **Franklin, R. B., D. R. Taylor, and A. L. Mills.** 1999. Characterization of microbial communities using randomly amplified polymorphic DNA (RAPD). J. Microbiol. Meth. **35:**225-235.

40. **Franks, A. H., H. J. M. Harmsen, G. C. Raangs, G. J. Jansen, F. Schut, and G. W. Welling.** 1998. Variations of bacterial populations in human feces measured by *fluorescent in situ* hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. Appl. Environ. Microbiol. **64:**3336–3345.

41. **Gavini, F., and H. Beerens** 1999. Origin and identification of bifidobacteria strains isolated from meat and meat products. Int. J. Food Microbiol. **46:**81–85.

42. **Gavini, F., A.-M. Pourcher, C. Neut, D. Monget, C. Romond, C. Oger, and D. Izard.** 1991. Phenotypic differentiation of bifidobacteria of human and animal origin. Int. J. Syst. Bacteriol. **41:**548–557.

43. **Gibson, G. R., E. R. Beatty, X. Wang, and J. H. Cummings.** 1995. Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin. Gastroenterol. **108:**975–982.

44. **Gibson, G. R., and M. B. Roberfroid.** 1995. Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. J. Nutr. **125:**1401–1412.

45. **Giraffa, G., P. De Vecchi, and L. Rossetti.** 1998. Identification of *Lactobacillus delbrueckii* subspecies *bulgaricus* and subspecies lactis dairy isolates by amplified rDNA restriction analysis. J. Appl. Microbiol. **85:**918–924.

46. **Giraffa, G., P. De Vecchi, P. Rossi, G. Nicastro, and M. G. Fortina.** 1998. Genotypic heterogeneity among *Lactobacillus helveticus* strains isolated from natural cheese starters. J. Appl. Microbiol. **85:**411–416.

47. **Harmsen, H. J., A. C. Wildeboer Veloo, G. C. Raangs, A. A. Wagendorp, N. Klijn, J. G. Bindels, and G. W. Welling.** 2000. Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. J. Pediatr. Gastroenterol. Nutr. **30:**61–67.

48. **Harmsen, H. J. M., P. Elfferich, F. Schut, and G. W. Welling.** 1999. A 16S rRNA-targeted probe for detection of lactobacilli and enterococci in faecal samples by fluorescent *in situ* hybridization. Microb. Ecol. Health Dis. **11:**3–12.

49. **Harmsen, H. J. M., A. C. M. Wildeboer-Veloo, J. Grijpstra, J. Knol, J. E. Degener, and G. W. Welling.** 2000. Development of 16S rRNA-based probes for the *Coriobacterium* group and the *Atopobium* cluster and their application for enumeration of *Coriobacteriaceae* in human feces from volunteers of different age groups. Appl. Environ. Microbiol. **66:**4523–4527.

50. **Hartemink, R., V. R. Domenech, and F. M. Rombouts.** 1997. LAMVAB – a new selective medium for the isolation of lactobacilli from faeces. J. Microbiol. Meth. **29:**77–84.

51. **Hartemink, R., B. J. Kok, G. H. Weenk, and F. M. Rombouts.** 1996. Raffinose-bifidobacterium (RB) agar, a new selective medium for bifidobacteria. J. Microbiol. Meth. **27:**33–43.

52. **Heilig, G. H. J., E. G. Zoetendal, E. E. Vaughan, P. Marteau, A. D. L. Akkermans, and W. M. de Vos.** Molecular diversity of *Lactobacillus* spp., and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. Appl. Environ. Microbiol. In press.

53. **Hensiek, R., G. Krupp, and E. Stackebrandt.** 1992. Development of diagnostic oligonucleotide probes for four *Lactobacillus* species occurring in the intestinal tract. System. Appl. Microbiol. **15:**123–128.

54. **Hertel, C., W. Ludwig, M. Obst, R. F. Vogel, W. P. Hammes, and K. H. Schleifer.** 1991. 23S rRNA-targeted oligonucleotide probes for the rapid identification of meat lactobacilli. System. Appl. Microbiol. **14:**173–177.

55. **Hertel, C., W. Ludwig, B. Pot, K. Kersters, and K. H. Schleifer.** 1993. Differentiation of lactobacilli occurring in fermented milk products by using oligonucleotide probes and electrophoretic protein profiles. System. Appl. Microbiol. **16:**463–467.

56. **Hooper, L. V., M. H. Wong, A. Thelin, L. Hansson, P. G. Falk, and J. I. Gordon.** 2001. Molecular analysis of commensal host-microbial relationships in the intestine. Science. **291:**881–884.

57. **Ito, M., Y. Deguchi, A. Miyamori, K. Matsumoto, H. Kikuchi, K. Matsumoto, Y. Kobayashi, T. Yajima, and T. Kan.** 1990. Effects of administration of galactooligosaccharides on the human faecal microflora, stool weight and abdominal sensation. Microb. Ecol. Health Dis. **3:**285–292.

58. **Jansen, G. J., A. C. M. Wildeboer-Veloo, R. H. J. Tonk, A. H. Franks, and G. W. Welling.** 1999. Development and validation of an automated, microscopy-based method for enumeration of groups of intestinal bacteria. J. Microbiol. Meth. **37:**215–221.

59. **Johansson, M.-L., G. Molin, B. Jeppson, S. Nobaek, S. Ahrné, and S. Bengmark.** 1993. Administration of different *Lactobacillus* strains in fermented oatmeal soup. *In vivo* colonization of human intestinal mucosa and effect on the indigenous flora. Appl. Environ. Microbiol. **59:**15–20.

60. **Johansson, M.-L., S. Nobaek, A. Berggren, M. Nyman, I. Björck, S. Ahrne, B. Jeppsson, and G. Molin.** 1998. Survival of *Lactobacillus plantarum* DSM 9843 (299v), and effect on the short-chain fatty acid content of faeces after ingestion of a rose-hip drink with fermented oats. Int. J. Food Microbiol. **42:**29–38.

61. **Johansson, M.-L., M. Quednau, G. Molin, and S. Ahrné.** 1995. Randomly amplified polymorphic DNA (RAPD) for rapid typing of *Lactobacillus plantarum* strains. Lett. Appl. Microbiol. **21:**155–159.

62. **Justesen, T., O. Haagen Nielsen, I. E. Jacobsen, J. Lave, and S. Norby Rasmussen.** 1984. The normal cultivable microflora in upper jejunal fluid in healthy adults. Scand. J. Gastroenterol. **19:**279–282.

63. **Kankaanpää, P., Y. Sutas, S. Salminen, and E. Isolauri.** 1998. Results on clinical demonstration of probiotics in children., p. 38–39. Functional food research in Europe, 3rd Workshop, FAIR CT96-1028, PROBDEMO, VTT Symposium 187. VTT, Haikko, Finland.

64. **Kaufmann, P., A. Pfefferkorn, M. Teuber, and L. Meile.** 1997. Identification and quantification of *Bifidobacterium* species isolated from food with genus-specific 16S rRNA-targeted probes by colony hybridization and PCR. Appl. Environ. Microbiol. **63:**1268–1273.

65. **Kimura, K., A. L. Mc Cartney, M. A. McConnell, and G. W. Tannock.** 1997. Analysis of populations of bifidobacteria and lactobacilli and investigation of the immunological responses of their human hosts to the predominant strains. Appl. Environ. Microbiol. **63:**3394–3398.

66. **Kleessen, B., E. Bezirtzoglou, and J. Mättö.** 2000. Culture-based knowledge on biodiversity, development and stability of human gastrointestinal microflora. Microb. Ecol. Health Dis. **Suppl. 2:**53–63.

67. **Kleessen, B., B. Sykura, H.-J. Zunft, and M. Blaut.** 1997. Effects of inulin and lactose on fecal microflora, microbial activity, and bowel habit in elderly constipated persons. Am. J. Clin. Nutr. **65:**1397–1402.

68. **Klein, G., A. Pack, C. Bonaparte, and G. Reuter.** 1998. Taxonomy and physiology of probiotic lactic acid bacteria. Int. J. Food Microbiol. **41:**103–125.

69. **Kontula, P.** 1999. PhD thesis. University of Helsinki.

70. **Kullen, M. J., M. M. Amann, O. M. J. Shaughnessy, O. D. J. Sullivan, F. F. Busta, and L. J. Brady.** 1997. Differentiation of ingested and endogenous bifidobacteria by DNA fingerprinting demonstrates the survival of an unmodified strain in the gastrointestinal tract of humans. J. Nutr. **127:**89–94.

71. **Kullen, M. J., L. J. Brady, and O. D. J. Sullivan.** 1997. Evaluation of using a short region of the *rec*A gene for rapid and sensitive speciation of dominant bifidobacteria in the human large intestine. FEMS Microbiol. Lett. **154:**377–383.

72. **Kunz, C., and S. Rudloff.** 1993. Biological functions of oligosaccharides in human milk. Acta Paediatr. **82:**903–912.

73. **Langendijk, P. S., F. Schut, G. J. Jansen, G. C. Raangs, G. R. Kamphuis, M. H. Wilkinson, and G. W. Welling.** 1995. Quantitative fluorescence *in situ* hybridization of *Bifidobacterium* spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. Appl. Environ. Microbiol. **61:**3069–3075.

74. **Leblond-Bourget, N., H. Philippe, I. Mangin, and B. Decaris.** 1996. 16S rRNA and 16S to 23S internal transcribed spacer sequence analyses reveal inter- and intraspecific *Bifidobacterium* phylogeny. Int. J. Syst. Bacteriol. **46:**102–111.

75. **Link-Amster, H., F. Rochat, K. Y. Saudan, O. Mignot, and J. M. Aeschlimann.** 1994. Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intake. FEMS Immunol. Med. Microbiol. **10:**55–63.

76. **Ludwig, W., and K. H. Schleifer.** 1994. Bacterial phylogeny based on 16S and 23S rRNA sequence analysis. FEMS Microbiol. Rev. **15:**155–173.

77. **Macfarlane, S., and G. T. Macfarlane.** 1995. Proteolysis and amino acid fermentation., p. 75–100. *In* G. R. Gibson, and G. T. Macfarlane (eds), Human colonic bacteria: Role in nutrition, physiology and pathology. CRC Press, Boca Raton.

78. **Mackowiak, M. D.** 1982. The normal microbial flora. N. Engl. J. Med. **307:**83–93.

79. **Maidak, B. L., J. R. Cole, C. T. J. Parker, G. M. Garrity, N. Larsen, B. Li, T. G. Lilburn, M. J. McCaughey, G. J. Olsen, R. Overbeek, S. Pramanik, T. M. Schmidt, J. M. Tiedje, and C. R. Woese.** 1999. A new version of the RDP (Ribosomal Database Project). Nucleic Acid Res. **27:**171–173.

80. **Majamaa, H., and E. Isolauri.** 1997. Probiotics: a novel approach in the management of food allergy. J. Allergy Clin. Immunol. **99:**179–185.

81. **Majamaa, H., E. Isolauri, M. Saxelin, and T. Vesikari.** 1995. Lactic acid bacteria in the treatment of acute rotavirus gastroenteritis. J. Pediatr. Gastroenterol. Nutr. **20:**333–338.

82. **Mangin, I., Y. Bouhnik, N. Bisetti, and B. Decaris.** 1999. Molecular monitoring of human intestinal *Bifidobacterium* strain diversity. Res. Microbiol. **150:**343–350.

83. **Mangin, I., N. Bourget, and B. Decaris.** 1996. Ribosomal DNA polymorphism in the genus *Bifidobacterium.* Res. Microbiol. **147:**183–192.

84. **Marsh, T. L.** 1999. Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. Curr. Opin. Microbiol. **2:**323–327.

85. **Marteau, P., P. Pochart, J. Dore, C. Bera-Maillet, A. Bernalier, and G. Corthier.** 2001. Comparative study of bacterial groups within the human cecal and fecal microbiota. Appl. Environ. Microbiol. **67:**4939–4942.

86. **Marteau, P., J. P. Vaerman, J. P. Dehennin, S. Bord, D. Brassart, P. Pochart, J. F. Desjeux, and J. C. Rambaud.** 1997. Effects of intrajejunal perfusion and chronic ingestion of *Lactobacillus johnsonii* strain La1 on serum concentrations and jejunal secretions of immunoglobulins and serum proteins in healthy humans. Gastroenterol. Clin. Biol. **21:**293–298.

87. **Matsuki, T., K. Watanabe, R. Tanaka, M. Fukuda, and H. Oyaizu.** 1999. Distribution of bifidobacterial species in human intestinal microflora examined with 16S rRNA-gene-targeted species-specific primers. Appl. Environ. Microbiol. **65:**4506–4512.

88. **Matsuki, T., K. Watanabe, R. Tanaka, and H. Oyaizu.** 1998. Rapid identification of human intestinal bifidobacteria by 16S rRNA-targeted species- and group-specific primers. FEMS Microbiol. Lett. **167:**113–121.

89. **McCartney, A. L., W. Wang, and G. W. Tannock.** 1996. Molecular analysis of the composition of the bifidobacterial and lactobacillus microflora of humans. Appl. Environ. Microbiol. **62:**4608–4613.

90. **McCracken, V. J., and H. R. Gaskins.** 1999. Probiotics and the immune system., p. 85–111. *In* G. W. Tannick (ed.), Probiotics – A critical review. Horizon Scientific Press, Wymondham.

91. **McFarland, L. V., C. M. Surawicz, and R. N. Greenberg.** 1994. A randomised placebo-controlled trial of *Saccharomyces boulardii* in combination with standard antibiotics for *Clostridium difficile* disease. JAMA. **271:**1913–1918.

92. **Michetti, P., G. Dorta, P. H. Wiesel, D. Brassart, E. Verdu, M. Herranz, C. Felley, N. Porta, M. Rouvet, A. L. Blum, and I. Corthesy-Theulaz.** 1999. Effect of whey-based culture supernatant of *Lactobacillus acidophilus* (*johnsonii*) La1 on *Helicobacter pylori* infection in humans. Digestion. **60:**203–209.

93. **Miyake, T., K. Watanabe, T. Watanabe, and H. Oyaizu.** 1998. Phylogenetic analysis of the genus *Bifidobacterium* and related genera based on 16S rDNA sequences. Microbiol. Immunol. **42:**661–667.

94. **Moore, W. E. C., and L. V. Holdeman.** 1974. Human fecal flora: The normal flora of 20 Japanese-Hawaiians. Appl. Microbiol. **27:**961–979.

95. **Morea, M., F. Baruzzi, F. Cappa, and P. S. Cocconelli.** 1998. Molecular characterization of the *Lactobacillus* community in traditional processing of Mozzarella cheese. Int. J. Food Microbiol. **43**:53–60.

96. **Morelli, L., C. Cesena, C. de Haën, and L. Gozzini.** 1998. Taxonomic *Lactobacillus* composition of feces from human newborns during the first few days. Microb. Ecol. **35**:205–212.

97. **Moschetti, G., G. Blaiotta, M. Aponte, G. Mauriello, F. Villani, and S. Coppola.** 1997. Genotyping of *Lactobacillus delbrueckii* subsp. *bulgaricus* and determination of the number and forms of *rrn* operons in *L. delbrueckii* and its subspecies. Res. Microbiol. **148**:501–510.

98. **Muyzer, G., E. C. de Waal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59**:695–700.

99. **Muyzer, G., and K. Smalla.** 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie van Leeuwenhoek. **73**:127–141.

100. **Nour, M.** 1998. 16S-23S and 23S-5S intergenic spacer regions of lactobacilli: nucleotide sequence, secondary structure and comparative analysis. Res. Microbiol. **149**:433–448.

101. **O' Riordan, K., and G. F. Fitzgerald.** 1997. Determination of genetic diversity within the genus *Bifidobacterium* and estimation of chromosomal size. FEMS Microbiol. Lett. **156**:259–264.

102. **Okawa, T., H. Niibe, T. Arai, K. Sekiba, K. Noda, S. Takeuchi, S. Hashimoto, and N. Ogawa.** 1993. Effect of LC9018 combined with radiation therapy on carcinoma of the uterine cervix. A phase III, multicenter, randomized, controlled study. Cancer. **72**:1949–1954.

103. **Ouwehand, A. C., P. V. Kirjavainen, C. Shortt, and S. Salminen.** 1999. Probiotics: mechanisms and established effects. Int. Dairy J. **9**:43–52.

104. **Pelto, L., E. Isolauri, E. M. Lilius, J. Nuutila, and S. Salminen.** 1998. Probiotic bacteria down-regulate the milk-induced inflammatory response in milk-hypersensitive subjects but have an immunostimulatory effect in healthy subjects. Clin. Exp. Allergy. **28**:1474–1479.

105. **Quiberoni, A., P. Tailliez, P. Quénée, V. Suárez, and J. Reinheimer.** 1998. Genetic (RAPD-PCR) and technological diversities among wild *Lactobacillus helveticus* strains. J. Appl. Microbiol. **85**:591–596.

106. **Quigley, M. E., and S. M. Kelly.** 1995. Structure, function, and metabolism of host mucus glycoproteins., p. 175–200. *In* G. R. Gibson, and G. T. Macfarlane (eds), Human colonic bacteria: role in nutrition, physiology, and pathology. CRC Press, Boca Raton.

107. **Rautanen, T., E. Isolauri, E. Salo, and T. Vesikari.** 1998. Management of acute diarrhoea with low osmolarity oral rehydration solution and *Lactobacillus* strain GG. Arch. Dis. Child. **79:**157–160.

108. **Reuter, G.** 1985. Elective and selective media for lactic acid bacteria. Int. J. Food Microbiol. **2:**55–68.

109. **Rodtong, S., and G. W. Tannock.** 1993. Differentiation of *Lactobacillus* strains by ribotyping. Appl. Environ. Microbiol. **59:**3480–3484.

110. **Roller, C., M. Wagner, R. Amann, W. Ludwig, and K.-H. Schleifer.** 1994. *In situ* probing of Gram-positive bacteria with high DNA G+C content using 23S rRNA-targeted oligonucleotides. Microbiol. **140:**2849–2858.

111. **Roussel, Y., C. Colmin, J. M. Simonet, and B. Decaris.** 1993. Strain characterization, genome size and plasmid content in the *Lactobacillus acidophilus* group (Hansen and Mocquot). J. Appl. Bacteriol. **74:**549–556.

112. **Roy, D., and S. Sirois.** 2000. Molecular differentiation of *Bifidobacterium* species with amplified ribosomal DNA restriction analysis and alignment of short regions of the *ldh* gene. FEMS Microbiol. Lett. **191:**17–24.

113. **Roy, D., P. Ward, and G. Champagne.** 1996. Differentiation of bifidobacteria by use of pulsed-field gel electrophoresis and polymerase chain reaction. Int. J. Food Microbiol. **29:**11–29.

114. **Roy, D., P. Ward, D. Vincent, and F. Mondou.** 2000. Molecular identification of potentially probiotic lactobacilli. Curr. Microbiol. **40:**40–46.

115. **Saarela, M., G. Mogensen, R. Fondén, J. Mättö, and T. Mattila-Sandholm.** 2000. Probiotic bacteria: safety, functional and technological properties. J. Biotechnol. **84:**197–215.

116. **Saavedra, J. M., N. A. Bauman, I. Oung, J. A. Perman, and R. H. Yolken.** 1994. Feeding of *Bifidobacterium bifidum* and *Streptococcus thermophilus* to infants in hospital for prevention of diarrhoea and shedding of rotavirus. Lancet. **344:**1046–1049.

117. **Salminen, S., M. A. Deighton, Y. Benno, and S. L. Gorbach.** 1998. Lactic Acid Bacteria in Health ad Disease., p. 211–253. *In* S. Salminen, and A. von Wright (eds), Lactic Acid Bacteria. Microbiology and Functional Aspects., 2nd ed. Marcel Dekker, Inc., New York.

118. **Salminen, S., E. Isolauri, and T. Onnela.** 1995. Gut flora in normal and disordered states. Chemotherapy. **41**:5–15.

119. **Salminen, S., A. von Wright, L. Morelli, P. Marteau, D. Brassart, W. M. de Vos, R. Fonden, M. Saxelin, K. Collins, G. Mogensen, S.-E. Birkeland, and T. Mattila-Sandholm.** 1998. Demonstration of safety of probiotics - a review. Int. J. Food Microbiol. **44**:93–106.

120. **Sanz, Y., M. Hernández, M. A. Ferrús, and J. Hernández.** 1998. Characterization of *Lactobacillus sake* isolates from dry-cured sausages by restriction fragment polymorphism analysis of the 16S rRNA gene. J. Appl. Microbiol. **84**:600–606.

121. **Satokari, R. M., E. E. Vaughan, A. D. L. Akkermans, M. Saarela, and W. M. de Vos.** 2001. Bifidobacterial diversity in human feces detected by genus-specific PCR and denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. **67**:504–513.

122. **Satokari, R. M., E. E. Vaughan, A. D. L. Akkermans, M. Saarela, and W. M. de Vos.** 2001. Polymerase chain reaction and denaturing gradient electrophoresis monitoring of fecal *Bifidobacterium* populations in a prebiotic and probiotic feeding trial. System. Appl. Microbiol. **24**:227–231.

123. **Schiffrin, E. J., F. Rochat, H. Link-Angler, J. M. Acschlimann, and A. Donnet-Hughes.** 1995. Immuno-modulation of human blood cells following the ingestion of lactic acid bacteria. J. Dairy Sci. **78**:491–497.

124. **Schwieger, F., and C. C. Tebbe.** 1998. A new approach to utilize PCR-single-strand-conformation polymorphism for 16S rRNA gene-based microbial community analysis. Appl. Environ. Microbiol. **64**:4870–4876.

125. **Sghir, A., D. Antonopoulos, and R. I. Mackie.** 1998. Design and evaluation of *Lactobacillus* group-specific ribosomal RNA-targeted hybridization probe and its application to the study of intestinal microecology in pigs. System. Appl. Microbiol. **21**:291–296.

126. **Sghir, A., G. Gramet, A. Suau, V. Rochet, P. Pochart, and J. Dore.** 2000. Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridization. Appl. Environ. Microbiol. **66**:2263–2266.

127. **Shornikova, A. V., I. Casas, E. Isolauri, H. Mykkänen, and T. Vesikari.** 1997. *Lactobacillus reuteri* as a therapeutic agent in acute diarrhea in young children. J. Pediatr. Gastroenterol. Nutr. **24:**399–404.

128. **Shornikova, A. V., I. A. Casas, H. Mykkänen, and T. Vesikari.** 1997. Bacteriotherapy with *Lactobacillus reuteri* in rotavirus gastroenteritis. Pediatr. Infect. Dis. **16:**1103–1107.

129. **Shornikova, A. V., E. Isolauri, L. Burkanova, S. Lukovnikova, and T. Vesikari.** 1997. A trial in Karelian republic of oral rehydration and *Lactobacillus* GG for treatment of acute diarrhoea. Acta Paediatr. **86:**460–465.

130. **Song, Y.-L., N. Kato, C.-X. Liu, Y. Matsumiya, H. Kato, and K. Watanabe.** 2000. Rapid identification of 11 human intestinal *Lactobacillus* species by multiplex PCR assays using group- and species-specific primers derived from the 16S-23S rRNA intergenic spacer region and its flanking 23S rRNA. FEMS Microbiol. Lett. **187:**167–173.

131. **Spanhaak, S., R. Havenaar, and G. Schaafsma.** 1998. The effect of consumption of milk fermented by *Lactobacillus casei* strain Shirota on the intestinal microflora and immune parameters in humans. Eur. J. Clin. Nutr. **52:**899–907.

132. **Stackebrandt, E., and B. M. Goebel.** 1994. Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. **44:**846–849.

133. **Stark, P. L., and A. Lee.** 1982. The microbial ecology of the large bowel of breast-fed and formula-fed infants during the first year of life. J. Med. Microbiol. **15:**189–203.

134. **Suau, A., R. Bonnet, M. Sutren, J. J. Godon, G. R. Gibson, M. D. Collins, and J. Dore.** 1999. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl. Environ. Microbiol. **65:**4799–4807.

135. **Suau, A., V. Rochet, A. Sghir, G. Gramet, S. Brewaeys, M. Sutren, L. Rigottier-Gois, and J. Dore.** 2001. *Fusobacterium prausnitzii* and related species represent a dominant group within the human fecal flora. System. Appl. Microbiol. **24:**139-145.

136. **Surawicz, C. M., G. W. Elmer, P. Speelman, L. McFarland, J. Chinn, and G. van Belle.** 1989. Prevention of antibiotic-associated diarrhea by *Saccharomyces boulardii.* A prospective study. Gastroenterol. **84:**981–988.

137. **Swift, S., E. E. Vaughan, and W. M. de Vos.** 2000. Quorom sensing within the gut ecosystem Microb. Ecol. Health Dis. **Suppl. 2:**81–92.

138. **Tajima, K., R. I. Aminov, T. Nagamine, H. Matsui, M. Nakamura, and Y. Benno.** 2001. Diet-dependent shifts in the bacterial populations of the rumen revealed with real-time PCR. Appl. Environ. Microbiol. **67:**2766–2774.

139. **Tanaka, R., and M. A. Ohwaki.** 1994. Controlled study on the ingestion of *Lactobacillus casei* fermented milk on the intestinal microflora, its microbiology and immune system in healthy adults. p. 85–104. XII Riken Symposium on Intestinal Flora., Tokyo, Japan.

140. **Tannock, G. W.** 1995. Normal microflora. An introduction to microbes inhabiting the human body. Chapman & Hall, London.

141. **Tannock, G. W., K. Munro, H. J. M. Harmsen, G. W. Welling, J. Smart, and P. K. Gopal.** 2000. Analysis of the fecal microflora of human subjects consuming a probiotic products containing *Lactobacillus rhamnosus* DR20. Appl. Environ. Microbiol. **66:**2578–2588.

142. **Tilsala-Timisjärvi, A., and T. Alatossava.** 1997. Development of oligonucleotide primers from the 16S-23S rRNA intergenic sequences for identifying different dairy and probiotic lactic acid bacteria by PCR. Int. J. Food Microbiol. **35:**49–56.

143. **Tyler, K. D., G. Wang, S. D. Tyler, and W. M. Johnson.** 1997. Guest commentary: Factors affecting reliability and reproducibility of amplification-based DNA fingerprinting of representative bacterial pathogens. J. Clin. Microbiol. **35:**339–346.

144. **Tynkkynen, S., R. Satokari, M. Saarela, T. Mattila Sandholm, and M. Saxelin.** 1999. Comparison of ribotyping, randomly amplified polymorphic DNA analysis, and pulsed-field gel electrophoresis in typing of *Lactobacillus rhamnosus* and *L. casei* strains. Appl. Environ. Microbiol. **65:**3908–3914.

145. **Ventura, M., R. Reniero, and R. Zink.** 2001. Specific identification and targeted characterization of *Bifidobacterium lactis* from different environmental isolates by combined multiplex-PCR approach. Appl. Environ. Microbiol. **67:**2760–2765.

146. **Vincent, D., D. Roy, F. Mondou, and C. Déry.** 1998. Characterization of bifidobacteria by random DNA amplification. Int. J. Food Microbiol. **43:**185–193.

147. **Vogel, R. F., G. Böcker, P. Stolz, M. Ehrmann, D. Fanta, W. Ludwig, B. Pot, K. Kersters, K. H. Schleifer, and W. P. Hammes.** 1994. Identification of lactobacilli from sourdough and description of *Lactobacillus pontis* sp. nov. Int. J. Syst. Bacteriol. **44:**223–229.

148. **Walter, J., C. Hertel, G. W. Tannock, C. M. Lis, K. Munro, and W. P. Hammes.** 2001. Detection of *Lactobacillus, Pediococcus, Leuconostoc,* and *Weissella* species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. **67:**2578–2585.

149. **Walter, J., G. W. Tannock, A. Tilsala-Timisjärvi, S. Rodtong, D. M. Loach, K. Munro, and T. Alatossava.** 2000. Detection and identification of gastrointestinal *Lactobacillus* species using denaturing gradient gel electrophoresis and species-specific PCR primers. Appl. Environ. Microbiol. **66:**297–303.

150. **Ward, L. J. H., and M. J. Timmins.** 1999. Differentiation of *Lactobacillus casei, Lactobacillus paracasei* and *Lactobacillus rhamnosus* by polymerase chain reaction. Lett. Appl. Microbiol. **29:**90–92.

151. **Wilson, K. H., and R. B. Blitchington.** 1996. Human colonic biota studied by ribosomal DNA sequence analysis. Appl. Environ. Microbiol. **62:**2273–2278.

152. **Wintzingerode, F. V., U. B. Göbel, and E. Stackebrandt.** 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol. Rev. **21:**213–229.

153. **Woese, C. R.** 1987. Bacterial evolution Microbiol. Rev. **51:**221–271.

154. **Wolf, B. W., D. G. Garleb, and I. Casas.** 1995. Safety and tolerance of *Lactobacillus reuteri* in healthy adult male subjects. Microb. Ecol. Health Dis. **8:**41–50.

155. **Wolf, B. W., K. B. Wheeler, D. G. Ataya, and K. A. Garleb.** 1998. Safety and tolerance of *Lactobacillus reuteri* supplementation to a population infected with human immunodeficiency virus. Food Chem. Toxicol. **36:**1085–1094.

156. **Yamamoto, T., M. Morotomi, and R. Tanaka.** 1992. Species-specific oligonucleotide probes for five *Bifidobacterium* species detected in human intestinal microflora. Appl. Environ. Microbiol. **58:**4076–4079.

157. **Yoon, J.-H., D. K. Yim, M. Goodfellow, and Y.-H. Park.** 1999. Sequence analysis of 16S rRNA genes amplified from two ribosomal RNA gene clusters of *Bifidobacterium bifidum.* Antonie van Leeuwenhoek. **75:**329–333.

158. **Zapparolli, G., S. Torriani, and F. Dellaglio.** 1998. Differentiation of *Lactobacillus sanfranciscensis* strains by randomly amplified polymorphic DNA and pulsed-field gel electrophoresis. FEMS Microbiol. Lett. **166:**325–332.

159. **Zoetendal, E., K. Ben-Amor, H. J. M. Harmsen, F. Schut, A. D. L. Akkermans, and W. M. de Vos.** Quantification of uncultured *Ruminococcus obeum*-like bacteria in human fecal samples with fluorescent *in situ* hybridization and flow cytometry using 16S ribosomal RNA targeted probes. Submitted for publication.

160. **Zoetendal, E. G., A. D. L. Akkermans, W. M. Akkermans-van Vliet, A. G. M. de Visser, and W. M. de Vos.** 2001. The host genotype affects the bacterial community in the human gastrointestinal tract Microb. Ecol. Health Dis. **13:**129–134.

161. **Zoetendal, E. G., A. D. L. Akkermans, and W. M. De Vos.** 1998. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl. Environ. Microbiol. **64:**3854–3859.

Chapter 2

Bifidobacterial diversity in human feces detected by genus-specific PCR and denaturing gradient gel electrophoresis

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Bifidobacterial Diversity in Human Feces Detected by Genus-Specific PCR and Denaturing Gradient Gel Electrophoresis

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We describe the development and validation of a method for the qualitative analysis of complex bifidobacterial communities based on PCR and denaturing gradient gel electrophoresis (DGGE). *Bifidobacterium* genus-specific primers were used to amplify an approximately 520-bp fragment from the 16S ribosomal DNA (rDNA), and the fragments were separated in a sequence-specific manner in DGGE. PCR products of the same length from different bifidobacterial species showed good separation upon DGGE. DGGE of fecal 16S rDNA amplicons from five adult individuals showed host-specific populations of bifidobacteria that were stable over a period of 4 weeks. Sequencing of fecal amplicons resulted in *Bifidobacterium*-like sequences, confirming that the profiles indeed represent the bifidobacterial population of feces. *Bifidobacterium adolescentis* was found to be the most common species in feces of the human adult subjects in this study. The methodological approach revealed intragenomic 16S rDNA heterogeneity in the type strain of *B. adolescentis*, E-981074. The strain was found to harbor five copies of 16S rDNA, two of which were sequenced. The two 16S rDNA sequences of *B. adolescentis* E-981074^T exhibited microheterogeneity differing in eight positions over almost the total length of the gene.

The human gastrointestinal (GI) tract hosts a rich and complex microbiota. Bifidobacteria are part of the normal microbiota of the human intestine, and they are considered to be important in maintaining well-balanced intestinal microbiota (4, 31). It has been postulated that *Bifidobacterium* spp. have several health-promoting effects, including the prevention of diarrhea and intestinal infections, alleviation of constipation, production of antimicrobials against harmful intestinal bacteria, and immunostimulation (4, 31). Therefore, many attempts have been made to increase the number of bifidobacteria in the intestine by administration of certain bifidobacterial strains (probiotics) or oligo- and polysaccharides that stimulate the growth of bifidobacteria (prebiotics) (2, 7, 10, 13). For the enumeration and isolation of bifidobacteria, several selective plating techniques have been developed (5, 14, 32). So far, 12 species have been associated with the human host: *Bifidobacterium adolescentis, Bifidobacterium infantis, Bifidobacterium longum, Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacterium catenulatum, Bifidobacterium pseudocatenulatum, Bifidobacterium angulatum, Bifidobacterium gallicum, Bifidobacterium inopinatum, Bifidobacterium dentium*, and *Bifidobacterium denticolens*, the last three being found primarily in the oral cavity (6, 11, 20, 23, 25).

Our present knowledge of the GI tract microbiota is largely based on cultivation studies, but according to recent estimates up to 85% of the entire microbial population in the human intestine might be uncultured (19, 36). Consequently, our picture of the intestinal microbiota has been biased in favor of the more easily cultured members of the community. Moreover, cultivation techniques are laborious and time-consuming, especially if bacterial isolates are to be identified. In order to overcome the limitations associated with culturing techniques, molecular biological methods are increasingly being applied to study the GI tract ecology (38). One of the most widely used approaches in ecological studies has been the use of rRNA and its encoding genes as target molecules (3). Specific PCR primers and probes can be designed based on the variable regions of this molecule to detect certain species or groups of bacteria. Numerous genus- and species-specific PCR primers and probes have been developed also for bifidobacteria (15, 19, 23, 24, 40). Species-specific primers and probes are excellent tools for targeting certain *Bifidobacterium* species in mixed populations, providing valuable help in identification, which is laborious and sometimes unreliable by phenotypic characterization. However, the use of specific primers and probes in ecological studies rules out the possibility of finding other than the target *Bifidobacterium* species possibly also present in the sample. On the other hand, genus-specific primers or probes can give a good overall picture of the bifidobacterial population, but no information is obtained about the species or strain composition.

Another way of utilizing the rRNA sequence heterogeneity in microbial ecology is to use universal bacterial PCR primers to amplify a fragment of rRNA or ribosomal DNA (rDNA) and then separate the obtained PCR products in a sequence-specific manner in temperature gradient gel electrophoresis (TGGE) or denaturing gradient gel electrophoresis (DGGE) (27, 28). The TGGE or DGGE profile thus obtained represents the prominent bacteria in the community. This technique has already been successfully applied to monitor the most

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TABLE 1. *Bifidobacterium* strains used in the present study

Species	Strain	Source	Code in other collection
B. adolescentis	E-981074^T	VTT Culture Collection	ATCC 15703
	E-991436	VTT Culture Collection	ATCC 15705
B. angulatum	CSCC 1925^T	CSCC	ATCC 27535
B. animalis	E-96663^T	VTT Culture Collection	ATCC 25527
B. bifidum	E-97795^T	VTT Culture Collection	ATCC 29521
	Bb-11	Chr. Hansen A/S	
B. breve	E-981075^T	VTT Culture Collection	ATCC 15700
B. catenulatum	CSCC 1967^T	CSCC	ATCC 27539
B. denticolens	E-991434^T	VTT Culture Collection	DSM 10105
B. dentium	E-991438^T	VTT Culture Collection	ATCC 27534
B. gallicum	CSCC 5492^T	CSCC	ATCC 49850
B. infantis	E-97796^T	VTT Culture Collection	ATCC 15697
	Bb-02	Chr. Hansen A/S	
B. lactis	E-97847^T	VTT Culture Collection	DSM 10140
	E-94508	VTT Culture Collection	Chr. Hansen A/S Bb-12
B. longum	E-96664^T	VTT Culture Collection	ATCC 15707
	E-94505	VTT Culture Collection	Chr. Hansen A/S Bb-46
	E-96702	VTT Culture Collection	
B. pseudocatenulatum	E-991439^T	VTT Culture Collection	ATCC 27919

predominant bacterial populations in human fecal samples (43).

In this study we describe the development and validation of a method that combines *Bifidobacterium* genus-specific PCR with DGGE that allowed us to analyze complex bifidobacterial communities. This approach was applied to study the bifidobacterial communities in the feces of adult subjects. The newly developed method also revealed intragenomic 16S rDNA heterogeneity in *B. adolescentis* E-981074^T that was demonstrated to contain at least two distinct copies of 16S rDNA.

MATERIALS AND METHODS

Strains and growth conditions. The 19 strains of bifidobacteria belonging to 13 different species used in this study are presented in Table 1. *Bifidobacterium lactis* and *Bifidobacterium animalis* species are often utilized in probiotic preparations for human and animal use whereas the other species listed in Table 1 are associated with the human host. Bacteria were obtained from the VTT Culture Collection (VTT Biotechnology, Espoo, Finland), Chr. Hansen A/S (Hørsholm, Denmark), and CSIRO Starter Culture Collection (CSCC) (Melbourne, Australia). The strains were grown in Man-Rogosa-Sharpe medium supplemented with 0.5 g of cysteine liter^{-1} in anaerobic jars with Anaerocult A-strips (Merck, Darmstadt, Germany) at 37°C.

Fecal samples. Fecal samples were collected from six Finnish individuals (subjects I to VI) of different ages (21 to 55 years) and sex (three women and three men). Samples were frozen at −70°C immediately after defecation. Bifidobacterial counts of fecal samples were determined by selective plating on Beerens agar (5) under anaerobic conditions in a Whitley Anaerobic Cabinet (model MK II; Don Whitley Scientific Ltd., Shipley, United Kingdom) with an atmosphere of N_2 (80%), CO_2 (10%), and H_2 (10%). The plates were incubated at 37°C for 4 days in anaerobic jars filled with mixed gas (85% N_2, 5% CO_2, and 10% H_2) by evacuation-replacement method (Anoxomat; Hart, Lichtenvoorde, The Netherlands).

Nucleic acid isolation. Isolation of chromosomal DNA from pure cultures was performed as described elsewhere (1). When necessary, the method was slightly modified by prolonging the time for enzymatic lysis from 1 h to 2 or 3 h. Methods previously described (43) were used to extract RNA from pure cultures and DNA from fecal samples.

Primers. All primers used in the study are listed in Table 2. *Bifidobacterium* genus-specific PCR was performed using 16S rDNA-targeted primers Bif164-f and Bif662-r or Im26-f and Im3-r, which produce approximately 520- or 1,420-bp PCR amplicons, respectively. For DGGE analysis of PCR products a 40-bp GC clamp was attached to the 5′ end of either Bif164-f or Bif662-r (Table 2).

TABLE 2. Primers used in the present study

Primer	Sequence (5′ to 3′)	Use	Specificity or target	Reference
Bif164-f	GGGTGGTAATGCCGGATG	PCR	*Bifidobacterium* 16S	17, 19
Bif662-r	CCACCGTTACACCGGGAA	PCR	*Bifidobacterium* 16S	17, 19
Bif164-GC-f	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCA CGGGGGG-GGGTGGTAATGCCGGATG	PCR	*Bifidobacterium* 16S	This study
Bif662-GC-r	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCA CGGGGGG-CCACCGTTACACCGGGAA	PCR	*Bifidobacterium* 16S	This study
Im26-f	GATTCTGGCTCAGGATGAACG	PCR	*Bifidobacterium* 16S	15
Im3-r	CGGGTGCTICCCACTTTCATG	PCR	*Bifidobacterium* 16S	15
7-f	AGAGTTTGAT(C/T)(A/C)TGGCTCAG	PCR	Eubacterial 16S	18
1510-r	ACGG(C/T)TACCTTGTTACGACTT	PCR	Eubacterial 16S	18
1401-r	CGGTGTGTACAAGACCC	RT-PCR	Eubacterial 16S	30
338-r	CTGCTGCCTCCCGTAGGAGT	Sequencing	Eubacterial 16S	18
338-f	CTCCTACGGGAGGCAGCAG	Sequencing	Eubacterial 16S	18
515-r	ATCGTATTACCGCGGCTGCTGGCAC	Sequencing	Eubacterial 16S	18
968-f	AACGCGAAGAACCTTA	Sequencing	Eubacterial 16S	18
1100-r	GGGTTGCGCTCGTTG	Sequencing	Eubacterial 16S	18
T7	TAATACGACTCACTATAGGG	Sequencing	pGEM^T	Promega
Sp6	GATTTAGGTGACACTATAG	Sequencing	pGEM^T	Promega

Complete 16S rDNA was amplified using primers 7-f and 1510-r. For reverse transcription of 16S rRNA to cDNA, primer 1401-r was used. Primers T7, Sp6, 338-r, 515-r, 1100-r, 338-f, and 968-f labeled with IRD800 were used for sequencing. All primers were purchased from MWG-Biotech (Ebersberg, Germany).

Reverse transcriptase PCR (RT-PCR) and PCR amplification. PCRs were performed using a *Taq* DNA polymerase kit from Life Technologies (Gaithersburg, Md.). The reaction mixture consisted of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate (dNTP), a 0.2 μM concentration of each primer, 1.25 U of *Taq* polymerase, and 1 μl of appropriately diluted template DNA in a final volume of 50 μl. In PCR with primers 7-f and 1510-r, the dNTP concentration was increased to 0.3 mM and the amount of *Taq* polymerase was increased to 1.5 U. The PCR thermocycling program with Bif164-f and Bif662-r primers was the following: 94°C for 5 min; 35 cycles of 94°C for 30 s, 62°C for 20 s, and 68°C 40 s; 62°C for 20 s; and 68°C for 7 min. The reactions were subsequently cooled to 4°C. For the amplification with primers 7-f and 1510-r the denaturation and elongation times were prolonged to 1 min 30 s and the annealing step was performed at 52°C for 30 s. The thermocycling program with primers Im26-f and Im3-r was: 94°C for 5 min; 30 cycles of 94°C for 30 s, 57°C for 30 s, and 68°C for 1 min 30 s; 57°C for 30 s; and 68°C for 7 min.

RT-PCR was performed with the Geneamp Thermostable r*Tth* Reverse Transcriptase RNA PCR kit (Perkin-Elmer, Norwalk, Conn.). Reverse transcription reaction mixtures (10 μl) consisted of 10 mM Tris-HCl (pH 8.3), 90 mM KCl, 1 mM $MnCl_2$, 0.25 mM dNTP, 0.75 μM primer 1401-r, 1.25 U of recombinant *Tth* DNA polymerase, and 1 μl of appropriately diluted RNA. The RT reaction was performed at 68°C for 30 min and followed by the addition of 40 μl of PCR mixture consisting of 5% glycerol, 10 mM Tris-HCl (pH 8.3), 100 mM KCl, 0.05% Tween 20, 0.75 mM EGTA, 3.75 mM $MgCl_2$, 0.2 mM dNTP, and a 0.25 μM concentration of each of the primers Bif164-f and Bif662-GC-r. The PCR thermocycling program was the same as described above for these primers.

The size and amounts of PCR products were estimated by analyzing 5-μl samples by 1.2% agarose gel (wt/vol) electrophoresis and ethidium bromide staining.

DGGE analysis of PCR products. DGGE analysis of PCR amplicons was performed essentially as described previously (27, 29) using the DCode or D GENE System apparatus (Bio-Rad, Hercules, Calif.). Polyacrylamide gels (8% [wt/vol] acrylamide–bisacrylamide [37.5:1]) in 0.5× Tris-acetic acid-EDTA buffer with a denaturing gradient were prepared with a gradient mixer and Econopump (Bio-Rad) using solutions containing 45 and 55% denaturant. A 100% denaturant corresponds to 7 M urea and 40% (vol/vol) 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 basically as described previously (35).

Cloning of the PCR products. The PCR products were purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and cloned in *E. coli* JM109 by using the pGEM-T vector system (Promega, Madison, Wis.). Colonies were picked and transferred into 20 μl of 10 mM Tris-HCl (pH 8.0)–1 mM EDTA and boiled for 15 min to lyse the cells, and the cell lysates were used to screen the transformants by PCR with Bif164-f and Bif662-GC-r primers followed by DGGE analysis. Plasmid DNA of selected transformants was isolated using a QIAprep spin miniprep kit (Qiagen).

Sequence analysis. Sequence analysis was carried out using purified plasmid DNA and sequencing primers T7 and Sp6 complementary to the adjacent sequences of the pGEM^T cloning site and other primers complementary to the 16S rDNA sequences (see Table 2). Sequencing was performed with the Sequenase sequencing kit (Amersham, Slough, United Kingdom) according to manufacturer's instructions. The sequences were analyzed with an automatic LI-COR (Lincoln, Nebr.) DNA sequencer 4000L and corrected manually. Pairwise and multiple sequence alignments and similarity comparisons between individual sequences were carried out using BCM services available on the Internet (http://www.hgsc.bcm.tmc.edu/SearchLaunher/) or from the DNASTAR (Madison, Wis.) program. Homology searches of 16S rDNA sequences derived from fecal clones and the DNA databases were carried out by using the BCM Nucleic acid sequence search service. In addition to the comparison with sequences in the databases, sequences of fecal clones were compared to the *B. adolescentis* E-981074^T sequences determined in this study, because the *B. adolescentis* 16S rDNA sequence deposited in the GenBank appears to contain many ambiguous bases.

Southern hybridization. Chromosomal DNA (2μg) was digested with *Eco*RI, *Eco*RV, or *Nru*I restriction enzymes (GibcoBRL, Paisley, United Kingdom) and the DNA fragments were separated by electrophoresis in 1% agarose. Fragments larger than 500 bp were transferred to Hybond-N+ membrane (Amersham, Aylesbury, United Kingdom) by vacuum blotting with a VacuGene XL vacuum blotting system (Pharmacia, Uppsala, Sweden), and hybridizations were carried out according to established protocols (34). The Bif164-f-to-Bif662-r PCR amplicon from strain *B. adolescentis* E-981074^T was labeled with [α-^{32}P]ATP by nick translation and used as a probe.

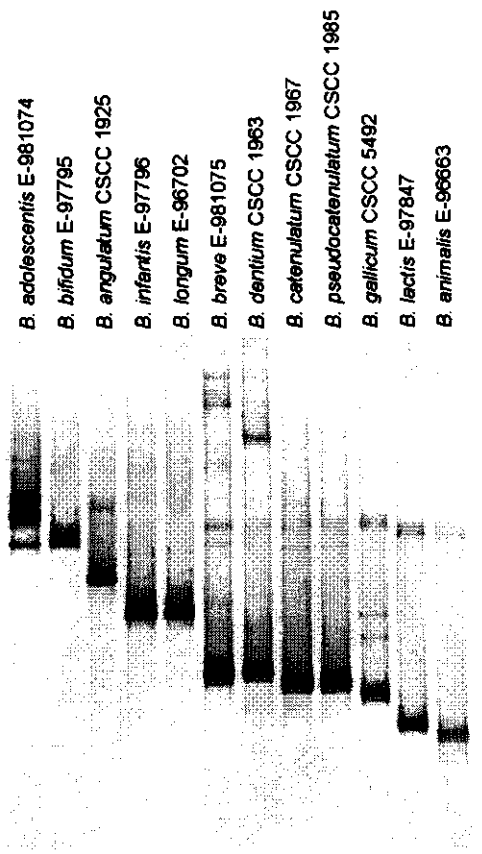

FIG. 1. Separation of PCR products from different *Bifidobacterium* species with genus-specific primers in 45 to 55% DGGE (increasing gradient of denaturant from top to bottom).

Extraction of chromosomal *Nru*I fragments from agarose gel. Chromosomal DNA (10 μg) was digested with *Nru*I restriction enzyme, and the DNA fragments were separated by agarose gel electrophoresis as mentioned above. DNA fragments of a size between 3 and 23 kb were recovered using Concert matrix gel extraction system (GibcoBRL) and checked for the presence of 16S rDNA by PCR with primers Bif164-f and Bif662-GC-r.

Nucleotide sequence accession numbers. The sequences of the two different 16S rDNA copies of *B. adolescentis* E-981074^T were deposited in the GenBank database and have been assigned accession numbers AF275881 (nru-1) and AF275882 (nru-5). The accession numbers of the fecal clones in GenBank are the following (clone code in parenthesis): AF275890 (7B), AF275891 (7G), AF275892 (9A), AF275893 (9B), AF275894 (9C), AF275884 (13D), AF275885 (15A), AF275883 (15B), AF275886 (15D), AF275887 (16B), AF275888 (16C), and AF275889 (16F).

RESULTS

Development of the DGGE method for separation of bifidobacteria. In order to set up the method based on genus-specific PCR and DGGE, primers that amplify a fragment that

TABLE 3. Bifidobacterial counts of the fecal samples

Subject	Bifidobacterial count (CFU g [wet wt]$^{-1}$) at wk:		
	0	3	4
I	9.0×10^8	2.2×10^9	1.3×10^9
II	1.8×10^8	3.4×10^{10}	2.5×10^9
III	2.2×10^9	2.9×10^9	7.3×10^9
IV	2.8×10^8	4.3×10^7	1.8×10^9
V	5.7×10^7	2.3×10^8	1.1×10^8
VI	4.1×10^4	$<10^4$	3.9×10^4

is separable by DGGE were selected and tested. The specificity of the previously described *Bifidobacterium* genus-specific primers Bif164-f and Bif662-r (17, 19) was confirmed using numerous bacterial species occurring in feces as the reference material. The primers showed good specificity for the genus *Bifidobacterium*, and the approximately 520-bp product was amplified exclusively from bifidobacteria (data not shown). In order to separate the bifidobacterial sequences by DGGE, a GC clamp was attached to either of the primers (Table 2). The use of primers Bif164-GC-f and Bif662-r to amplify bifidobacterial 16S rDNA resulted in PCR fragments from different species that showed very limited separation upon 45 to 55% DGGE (data not shown). In contrast, when the GC clamp was attached to the reverse primer (Bif662-GC-r) instead of the forward (Bif164-f), a good separation of different species in DGGE was obtained (Fig. 1). However, some closely related species could not be separated from each other by this approach. *B. longum* and *B. infantis* gave fragments in the same position in the gel as well as *B. catenulatum* and *B. pseudocatenulatum*. Also *B. breve* and *B. dentium* PCR fragments migrated to the same position. Different strains of the same species (Table 1) gave fragments in the same position upon DGGE. Two species, *B. breve* and *B. gallicum* gave diffuse fragments. An individual fragment from one strain was frequently observed as a doublet with two fragments very close to each other. This is very likely due to abortion of the elongation reaction during PCR caused by the GC clamp (hairpin formation), resulting in DNA molecules with slightly different migration behavior (30). However, *B. adolescentis* produced three strong fragments relatively far apart from each other (Fig. 1), and the cause for this was further examined in detail (see below).

Host-specific and stable DGGE patterns of bifidobacteria from human feces. The applicability of the DGGE method to monitoring complex bifidobacterial communities was first tested by using DNA from several *Bifidibacterium* species as the template in a competitive PCR. Fragments from all species were found in the DGGE profile, but in addition some extra fragments appeared above the single-stranded DNA in the DGGE profile (data not shown). These fragments are presumably heteroduplexes, which are more unstable and therefore, remain in the upper part of the DGGE gel.

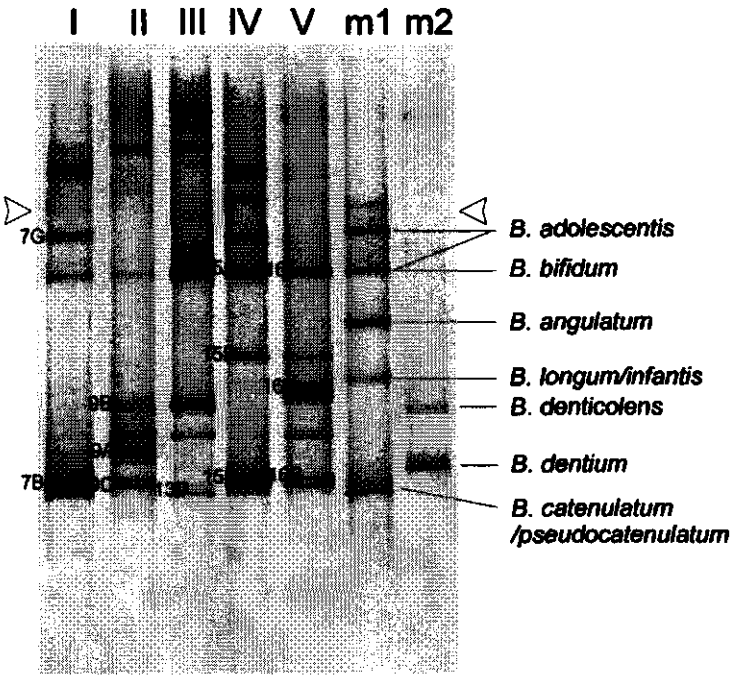

FIG. 2. DGGE of bifidobacterial PCR products of fecal samples from adult individuals (lanes I to V) and mixed PCR products from pure cultures (lanes m1 and m2). Single-stranded DNA and presumed heteroduplexes are above the line indicated with arrowheads. Indications 7B to 16F refer to the corresponding clones in Table 4.

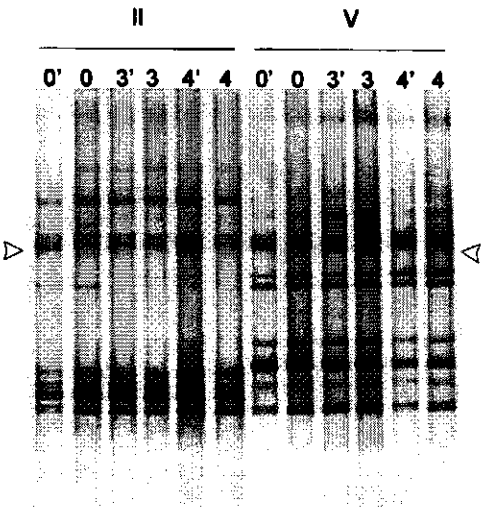

FIG. 3. DGGE of bifidobacterial PCR products of fecal samples from two adult individuals (II and V) from a 4-week period (samples from weeks 0, 3, and 4). In samples 0', 3', and 4' 10-fold-diluted DNA was used for PCR. Single-stranded DNA and presumed heteroduplexes are above the line indicated with arrowheads.

The bifidobacterial composition in fecal samples from six adult subjects (subjects I to VI) was studied. PCR products were obtained from samples I to V that had bifidobacterial counts reaching approximately 10^8 to 10^{10} CFU/g (wet weight) (Table 3), but no PCR product was obtained from subject VI, whose sample gave a low bifidobacterial count (approximately 10^4 CFU/g [wet weight]). The cultivation was performed from frozen samples, which is likely to have introduced a bias to the bifidobacterial counts, but allowed us to monitor fluctuations in the counts over time. DGGE analysis of bifidobacterial PCR products of fecal samples revealed complex host-specific patterns of bifidobacteria (Fig. 2, lanes I to V). In order to identify the *Bifidobacterium* species present in the feces, two samples consisting of a mixture of PCR products from identified species were run alongside the fecal samples (Fig. 2). However, most fragments of the fecal samples migrated to a different position than those of the culture collection strains and could not be identified in this way.

The stability of the bifidobacterial community over a period of 4 weeks was studied (Fig. 3). Analysis of three samples taken within this period showed stable bifidobacterial profiles, indicating that the composition of bifidobacterial community did not alter over this period despite some slight fluctuation in the bifidobacterial numbers (Table 3). Only subject II had a minor change in profile, where a faint fragment present in the first sample disappeared in the following samples (Fig. 3). DGGE profiles from undiluted and 10-fold-diluted fecal DNA samples were similar (Fig. 3), indicating that the template DNA from fecal samples can be diluted at least 10 times to avoid possible inhibition of the PCR without affecting the DGGE profile.

Validation of bifidobacterial profiles from feces. In order to identify some of the fragments in fecal profiles, a longer fragment of approximately 1,400 bp was amplified from the fecal samples with another set of *Bifidobacterium*-specific primers, lm26-f and lm3-r. The amplified fragments were cloned into *E. coli* JM109 by using pGEM-T, and transformants were amplified with primers Bif164-f and Bif662-GC-r. The mobility of these PCR products in DGGE was compared to the PCR pattern of the fecal sample obtained with the same primer set in order to determine which fragment they corresponded to. The plasmids from selected clones were purified, and the 16S rDNA insert was sequenced from both ends. The sequencing results confirmed that the DGGE profiles obtained with the primers Bif164-f and Bif662-GC-r indeed represent the bifidobacterial population in feces, since all sequenced fragments were derived from *Bifidobacterium* species. Clone sequences showed very high similarity to many *Bifidobacterium* species, and therefore could not be unambiguously identified to the species level (Table 4). All sequenced clones had the highest similarity to the sequence of *B. adolescentis* or its close phylogenetic relative species (*B. ruminantium* and *B. dentium*) or to *B. pseudocatenulatum*.

***B. adolescentis* shows 16S rDNA heterogeneity.** The *B. adolescentis* E-981074^T PCR product obtained with the primers Bif164-f and Bif662-GC-r appeared in the DGGE gel as three distinct fragments (bands A to C in Fig. 4). In order to study

TABLE 4. Sequencing of bifidobacterial clones from fecal samples

Subject no.	Clone	Sequence length (bp)	Ambiguity		Closest relatives (% sequence similarity[b])
			bp	%	
I	7B	1,267	16	1.3	*B. adolescentis* (98)[a], *B. ruminantium* (97), *B. dentium* (95)
I	7G	1,365	15	1.1	*B. adolescentis* (98)[a], *B. ruminantium* (97), *B. dentium* (96)
II	9A	1,346	11	0.8	*B. adolescentis* (97)[a], *B. ruminantium* (96), *B. dentium* (95)
II	9B	1,360	5	0.4	*B. pseudocatenulatum* (98), *B. angulatum* (97), *B. dentium* (96)
II	9C	1,267	8	0.6	*B. adolescentis* (98)[a], *B. ruminantium* (97), *B. dentium* (96)
III	13D	1,297	12	0.9	*B. pseudocatenulatum* (98), *B. ruminantium* (97), *B. adolescentis* (97)[a]
IV	15A	1,363	5	0.4	*B. adolescentis* (99)[a], *B. ruminantium* (98), *B. dentium* (97)
IV	15B	1,357	6	0.4	*B. adolescentis* (99)[a], *B. ruminantium* (98), *B. dentium* (96)
IV	15D	1,365	10	0.7	*B. adolescentis* (98)[a], *B. ruminantium* (97), *B. dentium* (96)
V	16B	1,268	18	1.4	*B. adolescentis* (97)[a], *B. ruminantium* (96), *B. dentium* (96)
V	16C	1,287	15	1.2	*B. ruminantium* (97), *B. adolescentis* (97)[a], *B. pseudocatenulatum* (96)
V	16F	1,362	17	1.3	*B. adolescentis* (98)[a], *B. ruminantium* (97), *B. dentium* (96)

[a] Compared to the 16S rDNA sequences determined in this study.
[b] Percent similarity values from pairwise sequence alignments.

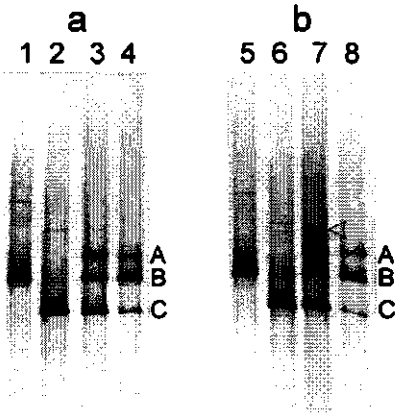

FIG. 4. DGGE profiles of *B. adolescentis* E-981074^T 16S PCR product and its derivative clones. (a) Heteroduplex formation experiment by PCR. Templates used in PCR are as follows: lane 1, plasmid from clone b1; lane 2, plasmid from clone c1; lane 3, plasmids from clones b1 and c2; lane 4, DNA from E-981074^T. (b) Heteroduplex formation experiment by melting PCR products. PCR products are as follows: lane 5, clone b1; lane 6, clone c1; lane 7, clones b1 and c1 heat denaturated together and cooled slowly to allow reannealing of complementary strands; lane 8, E-981074^T. Single-stranded DNA is indicated with an arrowhead.

the possible differences in sequences of the PCR fragments migrating to different positions, the PCR product was purified and cloned into *E. coli* JM109 using pGEM-T vector. The rDNA inserts of 40 clones were amplified by PCR with the primers Bif164-f and Bif662-GC-r, and analysis of their migration in DGGE showed that the majority of them produced 16S rRNA PCR amplicons that corresponded to either the middle fragment (B) or the lowest fragment (C) (clones b and c, respectively) but none that corresponded to the upper fragment (A). However, one clone (a1) was obtained that produced all three fragments, A, B, and C (data not shown). In order to obtain a clone corresponding to the single fragment A, we repeatedly colony purified the clone but only obtained colonies that upon PCR produced fragments corresponding to either B or C or all three fragments. Fragment A had the uppermost migration position in DGGE, indicating that it melts under weaker denaturing conditions than fragments B and C and thus is the most unstable fragment. We therefore came to the assumption that fragment A was a heteroduplex of fragments B and C formed during melting and reannealing of sequences in the PCR thermocycling. Presumably, clone a1 contained at least two plasmids carrying either fragment B or C and thereby produced all three fragments during PCR. Further evidence that fragment A was a heteroduplex of fragments B and C was obtained with the following experiments (Fig. 4). Firstly, purified plasmids from clones b1 and c1 were used separately and together as templates in PCR with the primers Bif164-f and Bif662-GC-r. Plasmid from clone b1 produced fragment B in DGGE, and similarly, clone c1 produced fragment C. When plasmids of b1 and c1 were both present in the PCR the three fragments (A, B, and C) could be observed after DGGE (lanes 1 to 3 in Fig. 4a). The same result was obtained following a nested-PCR amplification using fragments B and C separately and in combination as template in a subsequent PCR with the same primers (data not shown). Secondly, when PCR products B and C were mixed, heat denatured, and cooled to room temperature, analysis by DGGE showed the presence of all three fragments again (lanes 5 to 7 in Fig. 4b). Moreover, the inserts of four clones (b1, b2, c1, and c2) were sequenced. Sequence comparison revealed a minor difference in the sequences between clones b and c, i.e., a T deletion at position 219 (numbering begins at the 5′ end of the 16S rDNA) in clones c1 and c2 (Fig. 5).

Heterogeneity, complete sequence analysis, and expression of the 16S rRNA gene of *B. adolescentis* E-981074^T. The above observations prompted us to determine whether the microheterogeneity found in the PCR products resulted from sequence heterogeneity in the V2 region of different copies of 16S rDNA in the chromosome, thus ruling out the possibility of a PCR

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          76                                  105
nru-1     CGGCTGGAGCTTGCTCCGGCCGTGAGAGTG
nru-5     CC-CAGGAGCTTGCTCCTGG-GTGAGAGTG
M58729    CGGCTNGAGCTTGCTCCGGCTGTGAGAGTG

          210           225     391           405     451           465 466           480
nru-1     GAAAGATTCTATCGGT      GATGCAGCGACGCCG      AGCAAGCCCTTCGGG GCGAGTGTACCTTTC
nru-5     GAAAGATTC-ATCGGT      GATGCAGCGACGCCG      AGCAAGCCCTTCGGG GTGAGTGTACCTTTC
M58729    GAAAGATTCTATCGGT      AATGCAGCGACGCCG      AGCAAGCC-TTCGGG GTGAGTGTACCTTTC
b1        GAAAGATTCTATCGGT      GATGCAGCGACGCCG      AGCAAGCCCTTCGGG GTGAGTGTACCTTTC
b2        GAAAGATTCTATCGGT      GATGCAGCGACGCCG      AGCAAGCCCTTCGGG GTGAGTGTACCTTTC
c1        GAAAGATTC-ATCGGT      GATGCAGCGACGCCG      AGCAAGCCCTTCGGG GTGAGTGTACCTTTC
c2        GAAAGATTC-ATCGGT      GATGCAGCGACGCCG      AGCNAGCCCTTCGGG GTGAGTGTACCTTTC

          991           1005    1396          1410
nru-1     CCGACAG-CCCCAGA       TCAAGTCATGAAAGT
nru-5     CCGACAG-CCCCAGA       TCAAGTCATGAAAGT
M58729    CCGACAGGCCCCAGA       TCAAGCCATGAAAGT
```

FIG. 5. Sequence alignment of *B. adolescentis* E-981074^T clones with the *B. adolescentis* sequence from GenBank (M58729), showing sequence differences that were found (in boldface type). b1, b2, c1, nru-1, and nru-5 are double-stranded sequences, and c2 is a single-stranded sequence.

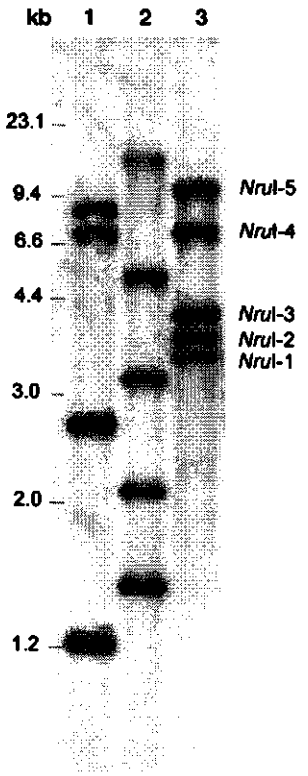

FIG. 6. Southern blot analysis of *rrn* operons of *B. adolescentis* E-981074^T. The genomic DNA cleaved with *Eco*RI (lane 1), *Eco*RV (lane 2), and *Nru*I (lane 3) and hybridized with 16S rDNA probe.

bias. Genomic DNA of *B. adolescentis* was prepared from a culture grown from a single colony and cleaved with restriction enzymes *Eco*RI, *Eco*RV, and *Nru*I. According to the GenBank sequence data (accession number M58729) (D. Yang and C. R. Woese, unpublished data) the first two enzymes cut the *B. adolescentis* 16S rDNA sequence only once at positions 660 and 691, respectively, while *Nru*I has no cleavage site within the 16S rDNA. Subsequent Southern hybridization was performed with a fragment homologous to the 16S rDNA sequence between bp 164 and 662 (Fig. 6). Hence, the number of fragments in *Eco*RI and *Eco*RV digests that hybridize with the probe correspond to the copy number of *rrn* operons in the chromosome. Four fragments were visible in the *Eco*RI digest, and five were visible in the *Eco*RV digest. The approximately 2.7-kb *Eco*RI fragment (lane 1 in Fig. 6) was relatively more intense than the other fragments and probably contains two fragments containing 16S rDNA sequences. From these results we concluded that *B. adolescentis* E-981074^T harbors five copies of *rrn* operon, a conclusion which is also supported by the observation that five *Nru*I fragments (*Nru*I-1 to *Nru*I-5) (Fig. 6), supposedly containing intact copies of the 16S rDNA, hybridized with the probe. The five *Nru*I fragments were isolated, and parts of the 16S rDNA sequences were amplified using primers Bif164-f and Bif662-GC-r. The resultant PCR products were analyzed by DGGE, and this showed that fragment B was produced from four of the 16S rDNA copies (*Nru*I-1 to *Nru*I-4 fragments) and that fragment C was produced from one copy (*Nru*I-5 fragment) (Fig. 6). Fragment A was not produced from any of the copies. Next, primers 7-f and 1510-r were used to amplify the full-length 16S rDNA from fragments *Nru*I-1 and *Nru*I-5, and the PCR products were cloned into *E. coli* JM109 using pGEM-T, generating clones nru-1 and nru-5. The heteroduplex formation experiments were repeated with the plasmids of these clones, and results analogous to those described above were obtained; i.e., clone nru-1 produced fragment B and nru-5 produced fragment C, but together they produced the additional fragment A.

The full-length 16S rDNA fragments from clones nru-1 and nru-5 were sequenced and compared. The two copies of 16S rDNA had a similarity of 99.4%, showing differences in eight positions. The difference previously described for the b and c clones was confirmed (Fig. 5); i.e., the T deletion in clones c1 and c2 at position 219 was also found in clone nru-5. In addition, clone nru-1 had a substitution of T to C at position 467. Thus, the deletion of one T at position 219 in clones c1, c2, and nru-5 changed the migration of the PCR fragment in DGGE, but the T-to-C substitution in clone nru-1 was not sufficient to alter its migration. The most prominent heterogeneity between the two chromosomal copies of 16S rDNA was, however, found outside the region that was amplified with primers Bif164-f and Bif662-r. Clones nru-1 and nru-5 differed in several base pairs between positions 77 to 80 and 93 to 96 (Fig. 5) in the V1 region of the 16S rDNA. The numerous ambiguous nucleotides present in the *B. adolescentis* 16S rDNA GenBank sequence (M58729) were determined in this study. Both sequences nru-1 and nru-5 are different from M58729 at positions 96, 391, 459, 998, and 1401, while nru-5 shows additional differences in the aforementioned positions in the V1 region.

In order to get a picture of the expression of the different 16S rDNA copies *in B. adolescentis*, an RT-PCR experiment was performed. First the 16S rRNA was transcribed to cDNA, which was then amplified in PCR with the primers Bif164-f and Bif662-GC-r. When the PCR product was analyzed by DGGE, the same pattern of three fragments, A, B, and C, was obtained. This showed that the 16S rDNA copy producing fragment C is transcribed together with the copies producing fragment B. As fragment B appeared significantly more intense than fragment C in DGGE, we estimated that more than one copy and possibly all four corresponding to fragment B are transcribed. Consistently, the intensity of the heteroduplex fragment A corresponded to that of fragment C.

DISCUSSION

In this study we describe the development and validation of a sensitive method for the qualitative analysis of complex bifidobacterial communities based on genus-specific PCR and DGGE. During the optimization of the method it was noticed that the location of the GC clamp in the DNA fragment greatly influenced the melting behavior and subsequently the migration of the fragment in the DGGE gel. Due to the use of different sequences in the GC clamps, direct comparison of the results obtained with the two primer pairs, Bif164-GC-f–

Bif662-r and Bif164-f–Bif662-GC-r, was not possible, but it is more than likely that the location of the GC clamp has more effect on the separation than its sequence. When the GC clamp was attached to the forward primer (Bif164-GC-f) the separation of different *Bifidobacterium* species by DGGE was not good, whereas an efficient separation was obtained when the GC clamp was attached to the reverse primer (Bif662-GC-r). Alignment of bifidobacterial sequences from GenBank showed considerable sequence heterogeneity close to the Bif164-f primer end (data not shown), which is apparently critical for the sequence-specific separation of the PCR products from different *Bifidobacterium* species.

The developed method based on genus-specific PCR and DGGE allows us to monitor the qualitative composition of the whole bifidobacterial population with merely a single PCR. In DGGE the PCR products from all culture collection strains of the same species migrated to the same position, but it was not possible to identify species in fecal samples by comparing the position of the fragment to those of the identified culture collection strains. Molecular typing methods such as ribotyping and pulsed-field gel electrophoresis have shown considerable genomic heterogeneity in strains of the same *Bifidobacterium* species (8, 22, 33). This heterogeneity is also present in the 16S-to-23S internally transcribed spacer sequences, but 16S rDNA sequences are very conserved among bifidobacteria and show 93% similarity between most of the species of the genus *Bifidobacterium* (21, 26). Even minor differences in the 16S rDNA sequence may, however, alter the migration behavior of a PCR fragment in DGGE, as shown in the case of *B. adolescentis* E-981074^T. This allows us to rapidly monitor changes occurring in the predominant members of the bifidobacterial community. The method may provide a valuable alternative to molecular typing techniques (22, 25) in rapidly monitoring qualitative changes in the bifidobacterial populations, although it does not allow definite discrimination or quantification of different strains. The DGGE method has an advantage of being independent of prior time-consuming culturing of the isolates on selective medium, which may favor the growth of some strains, thereby biasing the results. The PCR approach can also, however, lead to some distortions, because some sequences may amplify better than others, and heteroduplexes can be formed during PCR (39), as also observed in this study and further discussed below. In the PCR-DGGE approach identification of fragments can be done by subsequent cloning and sequencing of the PCR products, but it is hampered by the high similarity of 16S rDNA sequences between different *Bifidobacterium* species and the inadequate sequence data quality for many of the sequences in GenBank. We contributed to the construction of a more comprehensive database by depositing to the GenBank two accurate 16S sequences of *B. adolescentis* E-981074^T, which can be used for identification of new strains and phylogenetic studies.

The DGGE profiles of 16S PCR amplicons of bifidobacteria were found to be unique for each individual. This supports the results of previous studies that intestinal *Bifidobacterium* communities, like the dominant microbial populations, are host specific (16, 22, 25). The bifidobacterial populations were also found to be stable in composition during the 4-week study period. In general, the bifidobacterial population in the adult gut seems to be relatively stable for strain composition over several months or even a year, although some individual variations have also been detected (22, 25). In contrast, in the developing gut microbiota of infants bifidobacterial species change in time (C. Favier, E. E. Vaughan, W. M. de Vos, and A. D. L. Akkermans, unpublished data). Further studies with larger test groups are needed to make conclusions about development and the long-term stability of bifidobacterial communities.

Matsuki et al. (23) applied species- and group-specific PCR directly to fecal samples and found *B. catenulatum* group species (*B. catenulatum* and *B. pseudocatenulatum*) in 92% of adult fecal samples and *B. longum*, *B. adolescentis*, and *B. bifidum* in 65, 60, and 38% of the samples, respectively. Comparison of the species-specific PCR method with the classical culture method revealed that some species, most frequently *B. adolescentis*, were detected by the direct PCR method but not by culturing followed by specific PCR of the isolates (23). In these individuals *B. adolescentis* either was not among the most numerous bifidobacteria or it failed to grow on the selective media used. Our results indicate that *B. adolescentis* or closely related species are numerically the most prevailing bifidobacteria in some individuals, as it was most frequently found in the clone library. *B. adolescentis* was also the most widely distributed *Bifidobacterium* species among the subjects of the test group. Taking into account the possible heteroduplex formation and the fact that some species or strains may give more than one fragment in DGGE, it is difficult to give accurate estimates on the diversity of bifidobacteria in the fecal samples. The DGGE patterns show that the bifidobacterial diversity in individual samples is quite restricted, and according to sequence data some of these strains may belong to the same species. This result is in good agreement with previous studies showing that in most adults the bifidobacterial community is a combination of one to four species and that several distinct strains of the same species can coexist in one community (22, 23).

Our results show that *B. adolescentis* E-981074^T carries five rRNA gene clusters and exhibits intragenomic 16S rDNA sequence heterogeneity. Previously, *B. breve* has been found to have at least three *rrn* operons, and *B. bifidum* has been found to have two (8, 42). The two 16S rDNA copies of *B. bifidum* were sequenced, but no differences were found between the sequences (42). It is anticipated that the greater the number of rRNA operons is the higher the possibility of heterogeneity among rRNA genes within an organism is. Indeed, a *Paenibacillus polymyxa* strain that harbors at least twelve *rrn* operons was found to display a high degree of sequence diversity among its 16S rRNA genes (30). Intragenomic 16S rDNA heterogeneity, both microheterogeneities and larger changes, has been found also in other bacteria (9, 12, 37, 41). Microheterogeneities of a few base changes most likely result from mutations during DNA replication, whereas higher levels of sequence variation are considered to be a result of horizontal gene transfer (37, 41). The two 16S rDNA sequences of *B. adolescentis* E-981074^T differed in only eight bases over the almost total length of the gene and showed the highest similarity (99.4%) to each other over all the sequences available in GenBank. Therefore, we consider that the variability has resulted from mutations in one copy of the 16S rRNA gene. Intragenomic sequence variation has effects on the phylogeny of organisms

and biodiversity estimates. The heterogeneities may also interfere with analysis of denaturing gel patterns, and therefore some caution must be exercised when interpreting the results, especially when estimating strain and species numbers and diversity.

Probiotic and prebiotic research aims at developing functional food products that are able to modify the gut microbiota to a potentially more healthy one. A particular interest in these studies is to follow the marker organisms of well-balanced gut microbiota or the probiotic strains, often bifidobacteria and lactobacilli, and the changes of their proportions in the intestinal microbiota. Recently, genus-specific primers were designed for *Lactobacillus* spp. and also used successfully in combination with DGGE to analyze communities of lactobacilli (G. H. J. Heilig, E. G. Zoetendal, E. E. Vaughan, P. Marteau, A. D. L. Akkermans, and W. M. de Vos, unpublished data). In conclusion, this study demonstrates that the combination of genus- or group-specific PCR with DGGE is a powerful tool to study targeted microbial populations in the complex GI tract ecosystem. The approach opens new possibilities to follow the qualitative changes in the bifidobacterial and lactobacilli populations in response to probiotic or prebiotic administration as well as to study the effect of age, genetic background and other factors on the composition and diversity of these bacterial groups.

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REFERENCES

1. **Alander, M., R. Satokari, R. Korpela, M. Saxelin, T. Vilpponen-Salmela, T. Mattila-Sandholm, and A. von Wright.** 1999. Persistence of colonization of human colonic mucosa by a probiotic strain, *Lactobacillus rhamnosus* GG, after oral consumption. Appl. Environ. Microbiol. **65**:351–354.
2. **Alles, M. S., R. Hartemink, S. Meyboom, J. L. Harryvan, K. M. Van Laere, F. M. Nagengast, and J. G. Hautvast.** 1999. Effect of transgalactooligosaccharides on the composition of the human intestinal microflora and on putative risk markers for colon cancer. Am. J. Clin. Nutr. **69**:980–991.
3. **Amann, R. I., W. Ludwig, and K. H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59**:143–169.
4. **Ballongue, J.** 1998. Bifidobacteria and probiotic action, p. 519–587. *In* S. Salminen and A. von Wright (ed.), Lactic acid bacteria, microbiology and functional aspects, 2nd ed. Marcel Dekkers, Inc, New York, N.Y.
5. **Beerens, H.** 1990. An elective and selective isolation medium for *Bifidobacterium* spp. Lett. Appl. Microbiol. **11**:155–175.
6. **Biavati, B., B. Sgorbati, and V. Scardovi.** 1991. The genus *Bifidobacterium*, p. 816–833. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes, 2nd ed. Springer-Verlag, New York, N.Y.
7. **Bouhnik, Y., P. Pochart, P. Marteau, G. Arlet, L. Goderel, and J. C. Rambaud.** 1992. Fecal recovery in humans of viable *Bifidobacterium* sp ingested in fermented milk. Gastroenterology **102**:875–878.
8. **Bourget, N., J. M. Simonet, and B. Decaris.** 1993. Analysis of the genome of the five *Bifidobacterium breve* strains: plasmid content, pulsed-field gel electrophoresis genome size estimation and rrn loci number. FEMS Microbiol. Lett. **110**:11–20.
9. **Clayton, R. A., G. Sutton, P. S. J. Hinkle, C. Bult, and C. Fields.** 1995. Intraspecific variation in small-subunit rRNA sequences in GenBank: why single sequences may not adequately represent prokaryotic taxa. Int. J. Syst. Bacteriol. **45**:595–599.
10. **Collins, M. D., and G. R. Gibson.** 1999. Probiotics, prebiotics, and synbiotics: approaches for modulating the microbial ecology of the gut. Am. J. Clin. Nutr. **69**:1052s–1057s.
11. **Crociani, F., B. Biavati, A. Alessandrini, C. Chiarini, and V. Scardovi.** 1996. *Bifidobacterium inopinatum* sp. nov. and *Bifidobacterium denticolens* sp. nov., two new species isolated from human dental caries. Int. J. Syst. Bacteriol. **46**:564–571.
12. **Ebersbach, H., A. Breitenstein, and U. Lechner.** 2000. All species of *Desulfitobacterium* contain two types of 16S rRNA genes, p. 157. *In* A. Böck, J. Heesemann, K. H. Schleifer, and H. Wagner (ed.), Biospektrum. Proceedings of the Microbiology 2000 Congress, Munich, Germany. Spektrum Akademischer Verlag GmbH, Heidelberg, Germany.
13. **Gibson, G. R., E. R. Beatty, X. Wang, and J. H. Cummings.** 1995. Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin. Gastroenterology **108**:975–982.
14. **Hartemink, R., B. J. Kok, G. H. Weenk, and F. M. Rombouts.** 1996. Raffinose-bifidobacterium (RB) agar, a new selective medium for bifidobacteria. J. Microbiol. Methods **27**:33–43.
15. **Kaufmann, P., A. Pfefferkorn, M. Teuber, and L. Meile.** 1997. Identification and quantification of *Bifidobacterium* species isolated from food with genus-specific 16S rRNA-targeted probes by colony hybridization and PCR. Appl. Environ. Microbiol. **63**:1268–1273.
16. **Kimura, K., A. L. McCartney, M. A. McConnell, and G. W. Tannock.** 1997. Analysis of populations of bifidobacteria and lactobacilli and investigation of the immunological responses of their human hosts to the predominant strains. Appl. Environ. Microbiol. **63**:3394–3398.
17. **Kok, R. G., A. de Waal, F. Schut, G. W. Welling, G. Weenk, and K. J. Hellingwerf.** 1996. Specific detection and analysis of a probiotic *Bifidobacterium* strain in infant feces. Appl. Environ. Microbiol. **62**:3668–3672.
18. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115–175. *In* E. R. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley and Sons Ltd., Chichester, United Kingdom.
19. **Langendijk, P. S., F. Schut, G. J. Jansen, G. C. Raangs, G. R. Kamphuis, M. H. Wilkinson, and G. W. Welling.** 1995. Quantitative fluorescence in situ hybridization of *Bifidobacterium* spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. Appl. Environ. Microbiol. **61**: 3069–3075.
20. **Lauer, E.** 1990. *Bifidobacterium gallicum* sp. nov. isolated from human feces. Int. J. Syst. Bacteriol. **40**:100–102.
21. **Leblond-Bourget, N., H. Philippe, I. Mangin, and B. Decaris.** 1996. 16S rRNA and 16S to 23S internal transcribed spacer sequence analyses reveal inter- and intraspecific *Bifidobacterium* phylogeny. Int. J. Syst. Bacteriol. **46**:102–111.
22. **Mangin, I., Y. Bouhnik, N. Bisetti, and B. Decaris.** 1999. Molecular monitoring of human intestinal *Bifidobacterium* strain diversity. Res. Microbiol. **150**:343–350.
23. **Matsuki, T., K. Watanabe, R. Tanaka, M. Fukuda, and H. Oyaizu.** 1999. Distribution of bifidobacterial species in human intestinal microflora examined with 16S rRNA-gene-targeted species-specific primers. Appl. Environ. Microbiol. **65**:4506–4512.
24. **Matsuki, T., K. Watanabe, R. Tanaka, and H. Oyaizu.** 1998. Rapid identification of human intestinal bifidobacteria by 16S rRNA-targeted species- and group-specific primers. FEMS Microbiol. Lett. **167**:113–121.
25. **McCartney, A. L., W. Wang, and G. W. Tannock.** 1996. Molecular analysis of the composition of the bifidobacterial and lactobacillus microflora of humans. Appl. Environ. Microbiol. **62**:4608–4613.
26. **Miyake, T., K. Watanabe, T. Watanabe, and H. Oyaizu.** 1998. Phylogenetic analysis of the genus *Bifidobacterium* and related genera based on 16S rDNA sequences. Microbiol. Immunol. **42**:661–667.
27. **Muyzer, G., E. C. de Waal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59**:695–700.
28. **Muyzer, G., and K. Smalla.** 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Leeuwenhoek **73**:127–141.
29. **Myers, R. M., T. Maniatis, and L. S. Lerman.** 1987. Detection and localization of single base changes by denaturing gradient gel electrophoresis. Methods Enzymol. **155**:501–527.
30. **Nubel, U., B. Engelen, A. Felske, J. Snaidr, A. Wieshuber, R. I. Amann, W. Ludwig, and H. Backhaus.** 1996. Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. J. Bacteriol. **178**:5636–5643.
31. **O'Sullivan, D., and M. J. Kullen.** 1998. Tracking of probiotic bifidobacteria in the intestine. Int. Dairy J. **8**:513–525.
32. **Rowland, I. R., and R. Tanaka.** 1993. The effects of transgalactosylated oligosaccharides on gut flora metabolism in rats associated with a human faecal microflora. J. Appl. Bacteriol. **74**:667–674.
33. **Roy, D., P. Ward, and G. Champagne.** 1996. Differentiation of bifidobacteria by use of pulsed-field gel electrophoresis and polymerase chain reaction. Int. J. Food Microbiol. **29**:11–29.
34. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
35. **Sanguinetti, C. J., E. Dias Neto, and A. J. Simpson.** 1994. Rapid silver

staining and recovery of PCR products separated on polyacrylamide gels. BioTechniques 17:914–921.
36. **Suau, A., R. Bonnet, M. Sutren, J. J. Godon, G. R. Gibson, M. D. Collins, and J. Dore.** 1999. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl. Environ. Microbiol. 65:4799–4807.
37. **Ueda, K., T. Seki, T. Kudo, T. Yoshida, and M. Kataoka.** 1999. Two distinct mechanisms cause heterogeneity of 16S rRNA. J. Bacteriol. 181:78–82.
38. **Vaughan, E. E., F. Schut, H. G. H. J. Heilig, E. G. Zoetendaal, W. M. de Vos, and A. D. L. Akkermans.** 2000. A molecular view of the intestinal ecosystem. Curr. Issues Intest. Microbiol. 1:1–12.
39. **Wintzingerode, F. V., U. B. Göbel, and E. Stackebrandt.** 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol. Rev. 21:213–229.
40. **Yamamoto, T., M. Morotomi, and R. Tanaka.** 1992. Species-specific oligonucleotide probes for five *Bifidobacterium* species detected in human intestinal microflora. Appl. Environ. Microbiol. 58:4076–4079.
41. **Yap, W. H., Z. Zhang, and Y. Wang.** 1999. Distinct types of rRNA operons exist in the genome of the actinomycete *Thermomonospora chromogena* and evidence for horizontal transfer of an entire rRNA operon. J. Bacteriol. 181:5201–5209.
42. **Yoon, J.-H., D. K. Yim, M. Goodfellow, and Y.-H. Park.** 1999. Sequence analysis of 16S rRNA genes amplified from two ribosomal RNA gene clusters of *Bifidobacterium bifidum*. Antonie Leeuwenhoek 75:329–333.
43. **Zoetendal, E. G., A. D. L. Akkermans, and W. M. De Vos.** 1998. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl. Environ. Microbiol. 64:3854–3859.

Chapter 3

Comparison of ribotyping, randomly amplified polymorphic DNA analysis, and pulsed-field gel electrophoresis in typing of *Lactobacillus rhamnosus* and *L. casei* strains

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Comparison of Ribotyping, Randomly Amplified Polymorphic DNA Analysis, and Pulsed-Field Gel Electrophoresis in Typing of *Lactobacillus rhamnosus* and *L. casei* Strains

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A total of 24 strains, biochemically identified as members of the *Lactobacillus casei* group, were identified by PCR with species-specific primers. The same set of strains was typed by randomly amplified polymorphic DNA (RAPD) analysis, ribotyping, and pulsed-field gel electrophoresis (PFGE) in order to compare the discriminatory power of the methods. Species-specific primers for *L. rhamnosus* and *L. casei* identified the type strain *L. rhamnosus* ATCC 7469 and the neotype strain *L. casei* ATCC 334, respectively, but did not give any signal with the recently revived species *L. zeae*, which contains the type strain ATCC 15820 and the strain ATCC 393, which was previously classified as *L. casei*. Our results are in accordance with the suggested new classification of the *L. casei* group. Altogether, 21 of the 24 strains studied were identified with the species-specific primers. In strain typing, PFGE was the most discriminatory method, revealing 17 genotypes for the 24 strains studied. Ribotyping and RAPD analysis yielded 15 and 12 genotypes, respectively.

Lactobacilli have a worldwide industrial use as starters in the manufacturing of fermented milk products. Moreover, some *Lactobacillus* strains have probiotic characteristics and are therefore included in fresh fermented products or used in capsular health products, such as freeze-dried powder. The use of some *Lactobacillus* strains as probiotics is based on studies which show that these species belong to the normal intestinal flora and that the strains have beneficial effects on human and animal health (for reviews, see references 16 and 19). *Lactobacillus rhamnosus* and *L. casei* do not belong to the group of primary starters used in the dairy industry, but these species include many important probiotic strains, e.g., *L. casei* Shirota (26) and *L. rhamnosus* GG (20). These species are also naturally found in raw milk and in high numbers in cheese after it ripens (8, 15).

Traditionally, the identification of lactobacilli has been based mainly on fermentation of carbohydrates, morphology, and Gram staining, and these methods are still used. However, in recent years, the taxonomy has changed considerably with the increasing knowledge of the genomic structure and phylogenetic relationships between *Lactobacillus* spp. (14, 24, 30). The identification of some *Lactobacillus* species by biochemical methods alone is not reliable (6, 14, 22), as evidenced by the *L. casei* group (21, 32). The *L. casei* group includes *L. casei*, *L. paracasei*, *L. rhamnosus*, and *L. zeae*; the rejection of *L. paracasei* and its inclusion in *L. casei* has been proposed (7, 9, 10, 17).

Probiotic health products can contain, perhaps due to the lack of good identification methods, *Lactobacillus* species other than those declared on the product specifications (13, 14, 32). Difficulty in identification has also been reported for clinical isolates (21, 32). The need for rapid and reliable species-specific identification, e.g., by PCR, is obvious. Recently, species-specific oligonucleotide primers for *L. paracasei* and *L. rhamnosus* were described (1, 29).

The identification of lactobacilli at the strain level is important for their industrial use. The biotechnology industry needs tools to monitor, e.g., the use of patented strains or to distinguish probiotic strains from natural isolates in the host gastrointestinal tract. As for safety aspects, it is crucial to be able to compare clinical isolates and biotechnological strains and also to monitor the genetic stability of the strains (11, 14). Genotypic methods used for strain typing are typically PCR methods (e.g., randomly amplified polymorphic DNA [RAPD] analysis) or variations of restriction enzyme analysis (e.g., pulsed-field gel electrophoresis [PFGE] and ribotyping) (30). In RAPD analysis (31), short arbitrary sequences are used as primers in PCR, which yields strain-specific amplification product patterns. In PFGE and ribotyping analysis, genomic DNA is digested with restriction enzymes. In PFGE (23), rare-cutting enzymes are used and large genomic fragments are separated, while in ribotyping (25), rRNA genes and/or their spacer regions are used as probes that hybridize with genomic restriction fragments. These basic methodological differences may cause divergences in typing results.

The aims of this study were (i) to compare the identification of *L. casei* and *L. rhamnosus* strains by the API 50 CHL test and by species-specific PCR and (ii) to compare PFGE, RAPD analysis, and ribotyping techniques for the discrimination of closely related *L. casei* and *L. rhamnosus* strains.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used throughout the study are listed in Table 1. The strains were maintained at −80°C and subcultured in MRS broth or on MRS agar plates (LabM, Bury, England) anaerobically at 37°C. An API 50 CHL kit and APILAB Plus software using the API 50 CHL version 4.0 database (bioMérieux, Lyon, France) were used to identify strains biochemically.

***L. rhamnosus* and *L. paracasei* species-specific PCR.** Template DNA for the *L. rhamnosus* species-specific PCR was extracted as described previously (1) or, alternatively, PCR was performed with a fresh single colony grown overnight. The *L. rhamnosus* species-specific PCR assay described by Alander et al. (1) was used. The sequences of the primer pair (Table 2, RhaI) designed into the 16S

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TABLE 1. *Lactobacillus* strains used in the study

Bacterial strain	Identification by API 50 CHL (identification comment)[a]	Source
L. rhamnosus		
GG (ATCC 53103)	*L. rhamnosus* (doubtful)	Human isolate, Valio Ltd.[b]
VS 1030	*L. rhamnosus* (doubtful)	Human isolate, Valio Ltd.
VS 1031	*L. rhamnosus* (doubtful)	Human isolate, Valio Ltd.
VS 1032	*L. rhamnosus* (doubtful)	Human isolate, Valio Ltd.
VS 1033	*L. rhamnosus* (unacceptable)	Human isolate, Valio Ltd.
VS 1034	*L. rhamnosus* (good)	Human isolate, Valio Ltd.
E-78080	*L. rhamnosus* (very good)	Isolated from beer, VTT[c] Culture Collection
VS 872	*L. rhamnosus* (very good)	Isolated from milk, Valio Ltd.
E-97800	*L. rhamnosus* (doubtful)	Human isolate, VTT Culture Collection
Lactophilus	*L. rhamnosus* (excellent)	Isolated from Lactophilus[d] powder
VS 495	*L. rhamnosus* (good)	Isolated from cheese, Valio Ltd.
VS 1017	*L. rhamnosus* (doubtful)	Human isolate, Valio Ltd.
VS 1018	*L. rhamnosus* (unacceptable)	Human isolate, Valio Ltd.
VS 1019	*L. rhamnosus* (doubtful)	Human isolate, Valio Ltd.
VS 1020	*L. rhamnosus* (good)	Human isolate, Valio Ltd.
VS 1021	*L. rhamnosus* (good)	Human isolate, Valio Ltd.
VS 1022	*L. rhamnosus* (very good)	Human isolate, Valio Ltd.
ATCC 7469	*L. rhamnosus* (excellent)	American Type Culture Collection
ATCC 11443	*L. rhamnosus* (very good)	American Type Culture Collection
L. casei		
ATCC 393[e]	*L. rhamnosus* (good)	American Type Culture Collection
ATCC 334	*L. rhamnosus* (doubtful)	American Type Culture Collection
ATCC 4646	*L. paracasei* (very good)	American Type Culture Collection
L. paracasei VS 1023	*L. paracasei* (excellent)	Human isolate, Valio Ltd.
L. zeae ATCC 15820[f]	*L. paracasei* (unacceptable)	American Type Culture Collection

[a] Identification by the API 50 CHL kit and the profile status by APILAB Plus software using the API 50 CHL version 4.0 database. Identification comment given by APILAB Plus software: excellent, the percentage of identification (%ID) ≥ 99.9 and the T index (T) ≥ 0.75; very good, %ID ≥ 99.0 and T ≥ 0.5; good, %ID ≥ 90.0 and T ≥ 0.25; acceptable, %ID ≥ 80.0 and T ≥ 0; doubtful, several tests against identification (e.g., a rare biotype); unacceptable, below threshold value.
[b] Valio Ltd., Helsinki, Finland.
[c] VTT Biotechnology and Food Research, Espoo, Finland.
[d] Manufactured by Laboratoires Lyocentre, Aurillac, France.
[e] Recently proposed to belong to *L. zeae* (10, 17).
[f] *L. zeae* type strain (10, 17).

rRNA gene were 5′CTTGCATCTTGATTTAATTTTG3′ (forward) and 5′CCGTCAATTCCTTTGAGTTT3′ (reverse). The specificity of the primer pair was defined by the forward primer, and the expected PCR product size was 863 bp. The primers were made with a PCR Mate 391 DNA synthesizer (Perkin-Elmer Applied Biosystems, Foster City, Calif.) according to the manufacturer's instructions. *Taq* DNA polymerase and PCR buffer (final concentrations of 10 mM Tris-HCl, 1.5 mM $MgCl_2$, and 50 mM KCl [pH 8.3]) were obtained from Boehringer Mannheim (Mannheim, Germany). The amount of *Taq* DNA polymerase used was 2.0 U in a total reaction volume of 100 μl. The concentration of each primer was 0.5 μM, and that of each deoxynucleotide (Finnzymes Oy, Espoo, Finland) was 200 μM. The amount of template used was 1 μl of an appropriate dilution of the extracted DNA. A Gene Amp PCR System 9600 apparatus (Perkin-Elmer Applied Biosystems) was used for the PCR cycling. Initial denaturation was carried out at 94°C for 5 min, followed by a touch-down thermocycling program with 30 amplification cycles (annealing for 30 s at 62°C in cycles 1 to 10, 60°C in cycles 11 to 20, and 58°C in cycles 21 to 30; extension for 1 min at 72°C; and denaturation for 40 s at 94°C) and final extension for 10 min at 72°C. Reaction mixtures were subsequently cooled to 4°C. The PCR products were analyzed by agarose gel electrophoresis with 1% agarose in 0.5× Tris-borate-EDTA (10× is 89 mM Tris, 89 mM boric acid, and 25 mM EDTA [pH 8.0]) (TBE) buffer and ethidium bromide staining.

Other sets of species-specific primers, designed into the 16S-23S ribosomal DNA (rDNA) spacer region, were used to identify *L. rhamnosus* (Table 2, RhaII) and *L. paracasei* (Table 2, Cas) as described previously (29). Primer 5′CAGACTGAAAGTCTGACGG3′ was used with primers 5′GCGATGCGAATTTCTATTATT3′ and 5′GCGATGCGAATTTCTTTTC3′ to amplify *L. rhamnosus* and *L. paracasei* species-specific sequences, respectively. PCR amplification was performed with a DyNAzyme DNA polymerase kit (Finnzymes Oy) according to the instructions of the manufacturer. The PCR buffer contained 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, and 0.1% Triton X-100 (pH 8.8). The primers were used at 1 μM and deoxynucleotides were used at 200 μM. Initial denaturation was at 94°C for 2 min, and the thermocycling program was 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. With both the *L. rhamnosus* and *L. paracasei* primers, two PCR products of 350 and 185 bp were amplified.

RAPD genotyping. Template DNA for RAPD analysis was extracted from lactobacilli according to a modification of the method of Bollet et al. (4). Briefly, bacterial cells from a plate of a single-colony subculture of lactobacilli on MRS agar were harvested and transferred to Eppendorf tubes containing 100 μl of Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Tubes were vortexed well, 50 μl of 10% sodium dodecyl sulfate was added, and after vortexing, the tubes were incubated for 30 min at 65°C. The bacterial suspension was centrifuged (2,200 × *g* for 5 min), the supernatant was discarded, and the Eppendorf tubes containing the cells were heated in a microwave oven for 5 min at a power of 650 W. The pellets were dissolved in 500 μl of TE buffer, and a 1:100 dilution of cell lysate in water was used as a template in RAPD analysis. RAPD analysis was performed in a 50-μl reaction volume consisting of 200 μM deoxynucleoside triphosphate (Finnzymes Oy) a 0.4 μM concentration of random sequence primer 5′AGTCAGCCAC3′, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim), and 5 μl of template. The temperature profile in the Gene Amp PCR System 9600 thermocycler was 35 cycles as follows: 94°C for 1 min, 32°C for 2 min, and 72°C for 2 min. The initial denaturation was performed at 94°C for 5 min, and the final extension was done at 72°C for 5 min. Amplification products were analyzed electrophoretically in 1% (wt/vol) agarose gels containing ethidium bromide (0.5 μg/ml) and visualized under UV light. RAPD profiles of the strains were visually compared, and every clearly distinguishable profile was considered one RAPD genotype (A1, etc.)

Ribotyping. Ribotyping was performed by the automated ribotyping device RiboPrinter microbial characterization system (Qualicon, Wilmington, Del.). Standard reagents were used in all steps of the analysis. The method involves the release of DNA from cells, *Eco*RI digestion of chromosomal DNA, and the separation of the resulting fragments by agarose gel electrophoresis, followed by Southern hybridization probing with the *rrnB* rRNA operon from *Escherichia coli* (5) as a chemiluminescent probe. Images were acquired with a charge-coupled-

TABLE 2. Bacterial species detected by PCR with species-specific primer pairs

Bacterial strain	Result with primer pair		
	RhaI[a]	RhaII[b]	Cas[c]
L. rhamnosus			
GG	+	+	−
VS 1030	+	+	−
VS 1031	+	+	−
VS 1032	+	+	−
VS 1033	−	−	−
VS 1034	+	+	−
E-78080	+	+	−
VS 872	+	+	−
E-97800	+	+	−
Lactophilus	+	+	−
VS 495	+	+	−
VS 1017	+	+	−
VS 1018	+	+	−
VS 1019	+	+	−
VS 1020	+	+	−
VS 1021	+	+	−
VS 1022	+	+	−
ATCC 7469	+	+	−
ATCC 11443	+	+	−
L. casei			
ATCC 393[d]	−	−	−
ATCC 334	−	−	+
ATCC 4646	−	−	+
VS 1023	−	−	+
L. zeae ATCC 15820	−	−	−

[a] *L. rhamnosus* species-specific primers designed into the 16S rDNA gene (1).
[b] *L. rhamnosus* species-specific primers designed into the 16S-23S rDNA spacer region (29).
[c] *L. casei* species-specific primers designed into the 16S-23S rDNA spacer region (29).
[d] Recently proposed to belong to *L. zeae* (10, 17).

device camera and processed by RiboPrinter analysis software that normalizes fragment pattern data for band intensity and relative band position compared to the molecular weight marker. Similar fingerprint patterns (similarity of >0.95) were automatically clustered into ribogroups (R1, etc.). All strains were ribotyped at least twice to ensure the reproducibility of the fingerprint patterns.

PFGE. The preparation of genomic DNA in situ in agarose blocks was performed by a slight modification of the method of Tanskanen et al. (27). *Lactobacillus* strains were grown to an A_{600} of 0.6 in MRS broth containing 1% glycine to facilitate lysis. Chloramphenicol (100 μg/ml) was added, and incubation was continued for 1 to 2 h. Cells were harvested from 1.5 ml of culture, washed with 10 mM Tris–20 mM NaCl–50 mM EDTA (pH 7.2), and suspended in 300 μl of the same buffer. The suspension was heated in 50°C, and 300 μl of 2% agarose in 0.5× TBE buffer at the same temperature was added before solidifying the suspension in molds. The agarose blocks were incubated overnight at 37°C in lysis buffer, 6 mM Tris–1 M NaCl–100 mM EDTA–1% sarcosyl–0.2% deoxycholate (pH 7.6), containing 2.5 mg of lysozyme (Sigma, St. Louis, Mo.) per ml and 20 U of mutanolysin (Sigma) per ml. Proteinase K (1 mg/ml) treatment was performed in 100 mM EDTA–1% sarcosyl–0.2% deoxycholate buffer (pH 8.0) for 18 h at 50°C. The agarose blocks were washed four times for 1 h per wash with 20 mM Tris–50 mM EDTA (pH 8.0), the two first washes containing 1 mM phenylmethylsulfonyl fluoride (Sigma). Before restriction enzyme digestion, the agarose blocks were washed twice for 1 h per wash with TE buffer and then balanced for 1 h in an appropriate restriction enzyme buffer. Restriction enzyme digestions with *Not*I and *Sfi*I were performed overnight at 37°C. Electrophoresis was carried out with a CHEF DR II apparatus (Bio-Rad, Hercules, Calif.) in 1% PFGE certified agarose (Bio-Rad) with 0.5× TBE buffer. The pulse time was 1 to 15 s, the current was 5 V/cm, the temperature was 14°C, and the running time was 22 h. The agarose gel was stained with ethidium bromide (0.5 μg/ml) and visualized under UV light. The PFGE profiles of the strains were visually compared, and every clearly distinguishable profile was considered one *Not*I or *Sfi*I genotype. The final classification of PFGE genotypes (P1, etc.) combines the separate results obtained with these two restriction enzymes.

RESULTS

Identification of bacterial species. Biochemical identification of species was performed with an API 50 CHL kit. The identification results given by APILAB Plus software with the API 50 CHL version 4.0 database are shown in Table 1. For 13 strains, identification levels from good to excellent were obtained, and identification levels of 11 strains were considered doubtful or unacceptable due to atypical fermentation reactions.

The ribosomal intergenic regions are reported to be more variable between species than are the 16S or 23S RNA genes (2). Therefore, two sets of *L. rhamnosus* species-specific oligonucleotide primers were used to identify bacterial strains; the first pair of primers was designed into 16S rDNA (1) and the second into the 16S-23S rDNA spacer region (29). Both *L. rhamnosus* primer pairs gave PCR products of expected sizes with all strains except *L. zeae* ATCC 15820, *L. rhamnosus* VS 1033, *L. paracasei* VS 1023, and *L. casei* ATCC 393, ATCC 334, and ATCC 4646 (Table 2). The *L. paracasei* species-specific primers produced PCR products of expected sizes with *L. paracasei* VS 1023 and *L. casei* ATCC 334 and ATCC 4646. All three of these strains were classified as *L. casei* since the rejection of *L. paracasei* has been proposed (9, 10, 17); further, only the name *L. casei* is used. The *L. zeae* type strain, ATCC 15820, and *L. casei* ATCC 393, which was recently reclassified as *L. zeae* (10, 17), were not identified by either *L. rhamnosus*- or *L. casei*-specific primers. *L. rhamnosus* VS 1033 gave an API 50 CHL profile (Table 1) and was earlier identified as belonging to the *L. casei* group by 16S rRNA sequencing (unpublished results). It did not, however, give positive results with either of the *L. rhamnosus* or *L. casei* primers. This very likely indicates that this strain also belongs to *L. zeae*. PCR identifications of bacterial strains with the *L. rhamnosus* and *L. casei* species-specific oligonucleotide primers are in Table 2.

RAPD analysis. Twelve RAPD genotypes (A1 to A12) were detected among the 24 *Lactobacillus* strains. Genotypes A1 (Fig. 1, lanes 1 to 6), A2 (lanes 7 to 12), A3 (lanes 13 and 14), and A5 (lanes 15 and 18) were represented by six, six, two, and two strains, respectively, whereas the remaining eight strains each had a unique RAPD genotype (Fig. 1 and Table 3). All *L. rhamnosus* strains (Fig. 1, lanes 1 to 18) except for VS 1033 (Fig. 1, lane 20) produced a strong 1-kb amplification product that was either missing or weak in the *L. zeae* (Fig. 1, lanes 21 and 22) and *L. casei* (Fig. 1, lanes 19, 23, and 24) strains.

Ribotyping. Ribotyping with the *Eco*RI restriction enzyme produced 15 distinct fingerprint patterns for the 24 strains studied (Fig. 2 and Table 3). The triple band located between 4.8 and 6.2 kb seemed to be a feature typical of the *L. rhamnosus* fingerprint patterns; 16 of the 18 *L. rhamnosus* strains (identified by species-specific PCR) gave this type of fingerprint (Fig. 2, R1 to R4, R6, R7, and R9). *L. casei* VS 1023 (R11), ATCC 334 (R13), and ATCC 4646 (R14) (identified by species-specific PCR) shared bands of approximately 4.2 and 6.5 kb; in addition, strains VS 1023 (R11) and ATCC 334 (R13) shared bands of approximately 5 and 7 kb. The band pattern of *L. rhamnosus* VS 1030 (R8) resembled those of strains of both *L. rhamnosus* and *L. casei*. *L. zeae* ATCC 15820 (R15) and *L. casei* ATCC 393 (R12), which was proposed to belong to *L. zeae* (10, 17), had bands of approximately 1, 3.5, and 7 kb and a double band between 4.5 and 5.5 kb in common. VS 1033 (R10), which we suggest belongs to *L. zeae* according to the results of species-specific PCR, shared the bands of approximately 1 and 3.5 kb and the larger band of the double band between 4.5 and 5.5 kb with the *L. zeae* strains. The fingerprint of *L. rhamnosus* VS 1020 (R5) did not show simi-

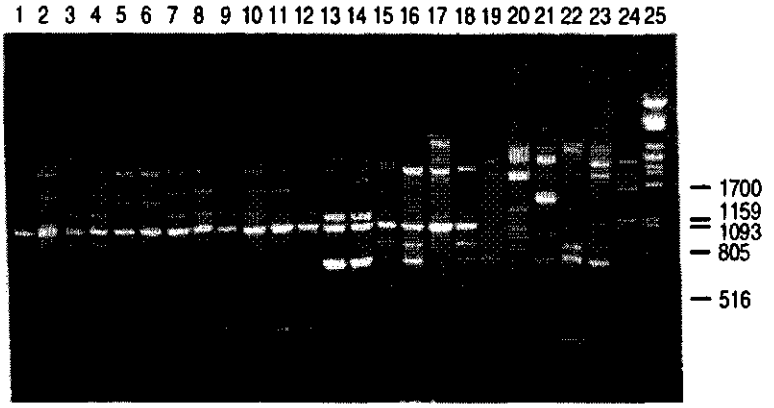

FIG. 1. RAPD patterns and genotypes (in parentheses) of the strains. Lanes: 1 to 18, *L. rhamnosus* GG (A1), VS 1031 (A1), VS 1032 (A1), VS 1034 (A1), VS 1017 (A1), VS 1018 (A1), ATCC 7469 (A2), ATCC 11443 (A2), E-78080 (A2), VS 872 (A2), VS 495 (A2), VS 1022 (A2), VS 1020 (A3), VS 1021 (A3), E-97800 (A4), VS 1030 (A5), Lactophilus (A6), and VS 1019 (A5), respectively; 19, *L. casei* VS 1023 (A7); 20, *L. rhamnosus* VS 1033 (A8); 21, *L. zeae* ATCC 15820 (A9); 22, *L. casei* ATCC 393 (A10); 23, *L. casei* ATCC 334 (A11); 24, *L. casei* ATCC 4646 (A12); 25, molecular weight marker (in kilobase pairs).

larity to any other fingerprints. Strains belonging to the same species were found to also share bands of >10 kb (Fig. 2). These bands are not listed individually because it was difficult to estimate the sizes of the bands with the coarse scale.

PFGE. *L. rhamnosus* genomic DNA digested with *Sfi*I and *Not*I yielded fragments of approximately 23 to 250 and 4 to 250 kb, respectively (Fig. 3 and 4). *Sfi*I revealed 16 (S1 to S16) and *Not*I revealed 15 (N1 to N15) distinct genotypes. Combining the results (Table 3), 17 distinct genotypes (P1 to P17) were found in the 24 *Lactobacillus* strains studied. Thirteen unique genotypes were found, and genotypes P1, P4, P5, and P8 were represented by four, three, two, and two strains, respectively (Table 3). All *L. rhamnosus* and *L. zeae* strains produced a typical double band (approximately 250 kb) and, possibly, additional bands with restriction enzyme *Sfi*I (Fig. 3a and b). *Not*I cut *L. rhamnosus* genomic DNA more often, and similar kinds of typical bands were not distinguishable (Fig. 4a and b). With the *L. casei* strains, a typical restriction pattern was not produced by either enzyme (Fig. 3c and 4c).

L. rhamnosus GG (Fig. 3a, lane 1, and 4a, lane 1), VS 1032 (Fig. 3b, lane 2, and 4b, lane 2), VS 1034 (Fig. 3b, lane 3, and 4b, lane 3), and VS 1018 (Fig. 3b, lane 7, and 4b, lane 7) had

TABLE 3. Abilities of RAPD analysis, ribotyping, and PFGE to differentiate *L. rhamnosus* and *L. casei* strains

Bacterial strain	Genotype by:		
	RAPD analysis	RiboPrint	PFGE[a]
L. rhamnosus			
GG	A1	R1	P1
VS 1032	A1	R1	P1
VS 1034	A1	R1	P1
VS 1018	A1	R1	P1
VS 1031	A1	R1	P2
VS 1017	A1	R1	P3
ATCC 7469	A2	R2	P4
ATCC 11443	A2	R2	P4
E-78080	A2	R2	P4
VS 872	A2	R3	P5
VS 1022	A2	R3	P5
VS 495	A2	R4	P6
VS 1020	A3	R5	P7
VS 1021	A3	R6	P7
E-97800	A4	R7	P8
VS 1019	A5	R7	P9
VS 1030	A5	R8	P10
Lactophilus	A6	R9	P11
VS 1033	A8	R10	P13
L. casei			
VS 1023	A7	R11	P12
ATCC 393[b]	A10	R12	P14
ATCC 334	A11	R13	P15
ATCC 4646	A12	R14	P16
L. zeae ATCC 15820	A9	R15	P17

[a] Combines the separate results obtained with *Sfi*I (genotypes S1 to S16) and *Not*I (genotypes N1 to N15).
[b] Recently proposed to belong to *L. zeae* (10, 17).

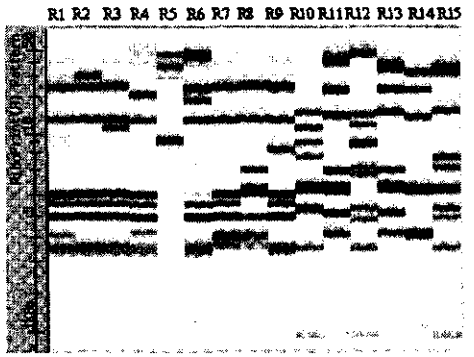

FIG. 2. RiboPrint patterns of *L. rhamnosus*, *L. casei*, and *L. zeae* strains. The patterns are composites of several individual patterns. Ribotypes: R1, *L. rhamnosus* GG, VS 1032, VS 1034, VS 1018, VS 1031, and VS 1017; R2, *L. rhamnosus* ATCC 7469, ATCC 11443, and E-78080; R3, *L. rhamnosus* VS 872 and VS 1022; R4, *L. rhamnosus* VS 495; R5, *L. rhamnosus* VS 1020; R6, *L. rhamnosus* VS 1021; R7, *L. rhamnosus* E-97800 and VS 1019; R8, *L. rhamnosus* VS 1030; R9, *L. rhamnosus* Lactophilus; R10, *L. rhamnosus* VS 1033; R11, *L. casei* VS 1023; R12, *L. casei* ATCC 393; R13, *L. casei* ATCC 334; R14, *L. casei* ATCC 4646; R15, *L. zeae* ATCC 15820.

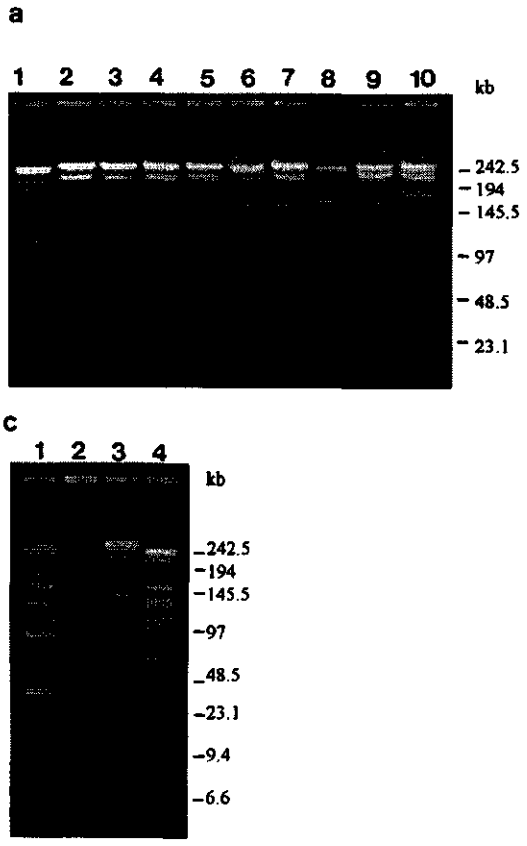

FIG. 3. PFGE profiles and genotypes (in parentheses) of the strains as determined with restriction enzyme *Sfi*I. (a) Lanes: 1 to 9, *L. rhamnosus* GG (S1), ATCC 7469 (S2), ATCC 11443 (S2), E-78080 (S2), VS 872 (S2), E-97800 (S3), VS 1030 (S4), VS 1033 (S5), and VS 1021 (S6), respectively; 10, *L. zeae* ATCC 15820 (S7). (b) Lanes: 1 to 10, *L. rhamnosus* VS 1031 (S8), VS 1032 (S1), VS 1034 (S1), Lactophilus (S9), VS 495 (S10), VS 1017 (S11), VS 1018 (S1), VS 1019 (S12), VS 1020 (S6), and VS 1022 (S2), respectively. (c) Lanes: 1 to 4, *L. casei* VS 1023 (S13), ATCC 393 (S14), ATCC 334 (S15), and ATCC 4646 (S16), respectively.

identical PFGE profiles with both enzymes and could not be distinguished from each other (Table 3, genotype P1). The *Sfi*I-produced profiles of *L. rhamnosus* VS 1017 (Fig. 3b, lane 6) and VS 1031 (Fig. 3b, lane 1) differed from those of the previous group by one and two extra bands, respectively. Another group with identical PFGE profiles (Table 3, genotype P4) with both enzymes consisted of *L. rhamnosus* ATCC 7469 (Fig. 3a, lane 2, and 4a, lane 2), ATCC 11443 (Fig. 3a, lane 3, and 4a, lane 3), and E-78080 (Fig. 3a, lane 4, and 4a, lane 4). The third group with identical PFGE patterns (Table 3, genotype P5) contained *L. rhamnosus* VS 872 (Fig. 3a, lane 5, and 4a, lane 5) and VS 1022 (Fig. 3b, lane 10, and 4b, lane 10), and the last group (Table 3, genotype P7) contained strains *L. rhamnosus* VS 1021 (Fig. 3a, lane 9, and 4a, lane 9) and VS 1020 (Fig. 3b, lane 9, and 4b, lane 9). All the other PFGE profiles of the *L. rhamnosus* strains were unique. The *L. casei* and *L. zeae* strains all had unique profiles.

DISCUSSION

Polyphasic taxonomy, which integrates phenotypic, genotypic, and phylogenetic information, has changed the classification of lactobacilli in recent years (for a review, see reference 30). Reliable identifications of some species are not obtained by traditional biochemical methods alone; genotypic methods are needed as well. This may cause problems for routine laboratories performing analyses if reliable and easy genetic methods, e.g., species-specific PCR, are not available.

We tested two pairs of recently published *L. rhamnosus* specific primers, one pair complementary to 16S rDNA and the other complementary to the spacer between 16S and 23S rDNA. Similar results were obtained with the primer pairs, and their specificity to the studied strains was good. No PCR signal was obtained with either *L. rhamnosus*- or *L. casei*-specific primers for *L. zeae* ATCC 15820 or *L. casei* ATCC 393, which was recently reclassified as *L. zeae*. Neotype strain *L. casei* ATCC 334 and the *L. rhamnosus* type strain, ATCC 7469, were correctly identified with their species-specific primers. Primers specific for *L. zeae* are needed for the complete identification of this bacterial group. All the strains studied were identified as belonging to the *L. casei* group, i.e., to *L. casei*, *L. rhamnosus*, or *L. zeae*, by the API 50 CHL test. However, the exact identifications of these closely related species were not reliable. Identifications of 11 strains were doubtful or unacceptable, and one strain, *L. casei* ATCC 393 (reclassified as *L. zeae*), was misidentified as *L. rhamnosus* with a good identification level.

At the species level, RAPD analysis yielded typical amplification products of 1 kb from all *L. rhamnosus* strains except for VS 1033, whose identification by the API 50 CHL test was unacceptable; we suggest that VS 1033 belongs to *L. zeae*, according to the results of species-specific PCR. The band representing the 1-kb amplification product was missing or weak with the *L. casei* and *L. zeae* strains. Ribotyping revealed a triple band (between 4.8 and 6.2 kb) which seems to be typical for most *L. rhamnosus* strains. In PFGE, all *L. rhamnosus* and *L. zeae* strains yielded a typical double band (over 250 kb) when cut with *Sfi*I, while no typical bands were distinguished by *Not*I. Typical bands in the fingerprints are very helpful but, of course, are not adequate alone for the identification of *L. rhamnosus*.

For strain typing, PFGE was the most discriminating method; it revealed 17 genotypes of the 24 strains studied, while 15

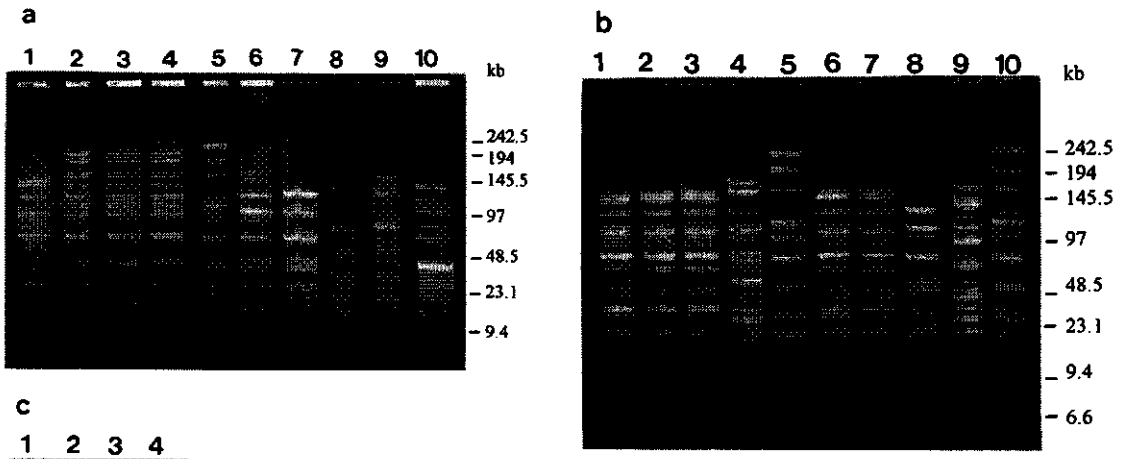

FIG. 4. PFGE profiles and genotypes (in parentheses) of the strains as determined with restriction enzyme *Not*I. (a) Lanes: 1 to 9, *L. rhamnosus* GG (N1), ATCC 7469 (N2), ATCC 11443 (N2), E-78080 (N2), VS 872 (N3), E-97800 (N4), VS 1030 (N5), VS 1033 (N6), and VS 1021 (N7), respectively; 10, *L. zeae* ATCC 15820 (N8). (b) Lanes: 1 to 10, *L. rhamnosus* VS 1031 (N1), VS 1032 (N1), VS 1034 (N1), Lactophilus (N9), VS 495 (N10), VS 1017 (N11), VS 1018 (N1), VS 1019 (N5), VS 1020 (N7), and VS 1022 (N3), respectively. (c) Lanes: 1 to 4, *L. casei* VS 1023 (N12), ATCC 393 (N13), ATCC 334 (N14), and ATCC 4646 (N15), respectively.

and 12 genotypes were distinguished by ribotyping and RAPD analysis, respectively. PFGE was performed with two enzymes, *Sfi*I and *Not*I, which increased its discrimination capability. However, even if the results obtained with *Sfi*I (which revealed 16 genotypes) or *Not*I (15 genotypes) are considered separately, PFGE remains the most discriminating or at least as discriminating as ribotyping. All non-*L. rhamnosus* strains (according to species-specific PCR) were distinguished from the *L. rhamnosus* strains by all three methods. The 18 *L. rhamnosus* strains were typed into 11 (10 genotypes by *Sfi*I and 9 by *Not*I), 9, and 6 genotypes by PFGE, ribotyping, and RAPD analysis, respectively. Table 3 shows that some *L. rhamnosus* strains were typed as belonging to the same genotype group by all three methods, which can be considered a very reliable identification. Based on our experience, PFGE analysis alone, performed with two or three appropriate enzymes, can be used for reliable strain typing. In several *Lactobacillus* studies, PFGE has been shown to be the most powerful method for strain typing (3, 12, 18), and it is also used in epidemiological studies (28). However, it is a laborious and expensive method; therefore, only a limited number of samples can be analyzed. Screening new primers in RAPD analysis and using other restriction enzymes in ribotyping could possibly increase their specificity for strain typing. Ribotyping can be done automatically (RiboPrinter) and is therefore easily applied, but the equipment is rather expensive. RAPD analysis is a rapid and cheap method, but careful optimization is needed to ensure the repeatability of the results.

To conclude, species-specific PCR, due to rapid and easy performance, is a very useful method for identifying species of the *L. casei* group. RAPD analysis, ribotyping, and PFGE are all primarily typing methods, but they do have the potential to also give species-specific information. Highly standardized and automated ribotyping could be suitable in forming large databases, giving rise to the possibility of using a typing method for identification purposes. Principal identification is still based on microbiological and biochemical methods, but for thorough analysis, conventional identification methods should be combined with genotypic methods.

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REFERENCES

1. **Alander, M., R. Satokari, R. Korpela, M. Saxelin, T. Vilpponen-Salmela, T. Mattila-Sandholm, and A. von Wright.** 1999. Persistence of colonization of human colonic mucosa by a probiotic strain, *Lactobacillus rhamnosus* GG, after oral consumption. Appl. Environ. Microbiol. 65:351–354.
2. **Barry, T., G. Colleran, M. Glennon, L. K. Dunican, and F. Gannon.** 1991. The 16S/23S ribosomal spacer region as a target for DNA probes to identify eubacteria. PCR Methods Appl. 1:51–56.
3. **Björkroth, J., J. Ridell, and H. Korkeala.** 1996. Characterization of *Lactobacillus sake* strains associating with production of ropy slime by randomly amplified polymorphic DNA (RAPD) and pulsed-field gel electrophoresis (PFGE) patterns. Int. J. Food Microbiol. 31:59–68.
4. **Bollet, C., M. J. Gevaudan, X. de Lamballerie, C. Zandotti, and P. de Micco.** 1991. A simple method for the isolation of chromosomal DNA from gram positive or acid fast bacteria. Nucleic Acids Res. 19:1955.
5. **Brosius, J., A. Ullrich, M. A. Raker, A. Gray, T. J. Dull, R. R. Gutell, and H. F. Noller.** 1981. Construction and fine mapping of recombinant plasmids containing the *rrn*B ribosomal RNA operon of *E. coli*. Plasmid 6:112–118.
6. **Charteris, W. P., P. M. Kelly, L. Morelli, and J. K. Collins.** 1997. Selective detection, enumeration and identification of potentially probiotic *Lactoba-*

cillus and *Bifidobacterium* species in mixed bacterial populations. Int. J. Food Microbiol. **35**:1–27.
7. **Collins, M. D., B. A. Phillips, and P. Zanoni.** 1989. Deoxyribonucleic acid homology studies of *Lactobacillus casei*, *Lactobacillus paracasei* sp. nov., subsp. *paracasei* and subsp. *tolerans*, and *Lactobacillus rhamnosus* sp. nov., comb. nov. Int. J. Syst. Bacteriol. **39**:105–108.
8. **Crow, V. L., T. Coolbear, P. K. Gopal, F. G. Martley, L. L. McKay, and H. Riepe.** 1995. The role of autolysis of lactic acid bacteria in the ripening of cheese. Int. Dairy J. **5**:855–875.
9. **Dellaglio, F., L. M. T. Dicks, M. du Toit, and S. Torriani.** 1991. Designation of ATCC 334 in place of ATCC 393 (NCDO 161) as the neotype strain of *Lactobacillus casei* subsp. *casei* and rejection of the name *Lactobacillus paracasei* (Collins et al., 1989). Int. J. Syst. Bacteriol. **41**:340–342.
10. **Dicks, L. M. T., E. M. du Plessis, F. Dellaglio, and E. Lauer.** 1996. Reclassification of *Lactobacillus casei* subsp. *casei* ATCC 393 and *Lactobacillus rhamnosus* ATCC 15820 as *Lactobacillus zeae* nom. rev., designation of ATCC 334 as the neotype of *L. casei* subsp. *casei*, and rejection of the name *Lactobacillus paracasei*. Int. J. Syst. Bacteriol. **46**:337–340.
11. **Donohue, D. C., and S. Salminen.** 1996. Safety of probiotic bacteria. Asia Pac. J. Clin. Nutr. **5**:25–28.
12. **Ferrero, M., C. Cesena, L. Morelli, G. Scolari, and M. Vescovo.** 1996. Molecular characterization of *Lactobacillus casei* strains. FEMS Microbiol. Lett. **140**:215–219.
13. **Holzapfel, W. H., P. Haberer, J. Snel, U. Schillinger, and J. H. J. Huis in't Veld.** 1998. Overview of gut flora and probiotics. Int. J. Food Microbiol. **41**:85–101.
14. **Klein, G., A. Pack, C. Bonaparte, and G. Reuter.** 1998. Taxonomy and physiology of probiotic lactic acid bacteria. Int. J. Food Microbiol. **41**:103–125.
15. **Lindberg, A.-M., A. Christiansson, E.-O. Rukke, T. Eklund, and G. Molin.** 1996. Bacterial flora of Norwegian and Swedish semi-hard cheese after ripening, with special reference to *Lactobacillus*. Neth. Milk Dairy J. **50**:563–572.
16. **Marteau, P., and J.-C. Rambaud.** 1993. Potential of using lactic acid bacteria for therapy and immunomodulation in man. FEMS Microbiol. Rev. **12**:207–220.
17. **Mori, K., K. Yamazaki, T. Ishiyama, M. Katsumata, K. Kobayashi, Y. Kawai, N. Inoue, and H. Shinano.** 1997. Comparative sequence analyses of the genes coding for 16S rRNA of *Lactobacillus casei*-related taxa. Int. J. Syst. Bacteriol. **47**:54–57.
18. **Roussel, Y., C. Colmin, J. M. Simonet, and B. Decaris.** 1993. Strain characterization, genome size and plasmid content in the *Lactobacillus acidophilus* group (Hansen and Mocquot). J. Appl. Bacteriol. **74**:549–556.
19. **Salminen, S., E. Isolauri, and E. Salminen.** 1996. Clinical uses of probiotics for stabilizing the gut mucosal barrier: successful strains and future challenges. Antonie Leeuwenhoek **70**:347–358.
20. **Saxelin, M.** 1997. *Lactobacillus* GG—a human probiotic strain with thorough clinical documentation. Food Rev. Int. **13**:293–313.
21. **Saxelin, M., H. Rautelin, S. Salminen, and P. H. Mäkelä.** 1996. Safety of commercial products with viable *Lactobacillus* strains. Infect. Dis. Clin. Pract. **5**:331–335.
22. **Schleifer, K.-H., M. Ehrmann, C. Beimfohr, E. Brockmann, W. Ludwig, and R. Amann.** 1995. Application of molecular methods for the classification and identification of lactic acid bacteria. Int. Dairy J. **5**:1081–1094.
23. **Schwartz, D. C., and C. R. Cantor.** 1984. Separation of yeast chromosome sized DNAs by pulsed-field gradient gel electrophoresis. Cell **37**:67–75.
24. **Stiles, M. E., and W. H. Holzapfel.** 1997. Lactic acid bacteria of foods and their current taxonomy. Int. J. Food Microbiol. **36**:1–29.
25. **Stull, T. L., J. J. LiPuma, and T. D. Edlind.** 1988. A broad-spectrum probe for molecular epidemiology of bacteria: ribosomal RNA. J. Infect. Dis. **157**:280–286.
26. **Sugita, T., and M. Togawa.** 1994. Efficacy of *Lactobacillus* preparation Biolactis powder in children with rotavirus enteritis. Jpn. J. Pediatr. **47**:2755–2762.
27. **Tanskanen, E. I., D. L. Tulloch, A. J. Hillier, and B. E. Davidson.** 1990. Pulsed-field gel electrophoresis of *Sma*I digests of lactococcal genomic DNA, a novel method of strain identification. Appl. Environ. Microbiol. **56**:3105–3111.
28. **Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. **33**:2233–2239.
29. **Tilsala-Timisjärvi, A., and T. Alatossava.** 1997. Development of oligonucleotide primers from 16S-23S rRNA intergenic sequences for identifying different dairy and probiotic lactic acid bacteria by PCR. Int. J. Food Microbiol. **35**:49–56.
30. **Vandamme, P., B. Pot, M. Gillis, P. De Vos, K. Kersters, and J. Swings.** 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol. Rev. **60**:407–438.
31. **Welsh, J., and M. McClelland.** 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. **18**:7213–7218.
32. **Zhong, W., K. Millsap, H. Bialkowska-Hobrzanska, and G. Reid.** 1998. Differentiation of *Lactobacillus* species by molecular typing. Appl. Environ. Microbiol. **64**:2418–2423.

Chapter 4

Persistence of colonization of human colonic mucosa by a probiotic strain, *Lactobacillus rhamnosus* GG, after oral consumption

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Persistence of Colonization of Human Colonic Mucosa by a Probiotic Strain, *Lactobacillus rhamnosus* GG, after Oral Consumption

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***Lactobacillus rhamnosus* GG is one of the most thoroughly studied probiotic strains. Its advantages in the treatment of gastrointestinal disorders are well documented. The aim of the present study was to demonstrate with colonic biopsies the attachment of strain GG to human intestinal mucosae and the persistence of the attachment after discontinuation of GG administration. A whey drink fermented with strain GG was fed to human volunteers for 12 days. Fecal samples were collected before, during, and after consumption. *L. rhamnosus* GG-like colonies were detected in both fecal and colonic biopsy samples. Strain GG was identified by its characteristic colony morphology, a lactose fermentation test, and PCR. This study showed that strain GG was able to attach in vivo to colonic mucosae and, although the attachment was temporary, to remain for more than a week after discontinuation of GG administration. The results demonstrate that the study of fecal samples alone is not sufficient in evaluating colonization by a probiotic strain.**

Oral consumption of health-promoting lactic acid bacteria or probiotics has been associated with the prevention, alleviation, or cure of diverse intestinal disorders such as lactose intolerance, viral and bacterial diarrhea, adverse effects of abdominal radiotherapy, constipation, inflammatory bowel disease, and food allergy (3, 5, 11). Much of the early evidence on the actual health effects of probiotics was anecdotal, but during the last few years data based on rigorous clinical studies indicating real health-promoting properties of certain well-characterized strains have started to accumulate (8).

Adhesion to the intestinal epithelium is one of the selection criteria for new probiotic strains (6). The adhesion properties have generally been deduced from in vitro experiments with intestinal cell lines, although, for example, rectal mucosal samples have been successfully used to demonstrate intestinal colonization by lactobacillar strains (7).

Lactobacillus rhamnosus GG (ATCC 53103) (previously known as *Lactobacillus casei* GG) is one of the most thoroughly studied probiotics (11). The reviewed beneficial effects (9, 12, 13) include prevention of antibiotic-associated diarrhea, treatment and prevention of rotavirus diarrhea, treatment of relapsing *Clostridium difficile* diarrhea, prevention of acute diarrhea, and enhancement of intestinal immunity. The ability of strain GG to survive passage through the gastrointestinal tract has been demonstrated in both adults and children by the use of fecal samples (4, 10, 14). Recently, adhesion of the strain to human colonic mucosae has been demonstrated with colonic biopsy samples (1). The aims of the present study were to confirm with colonic biopsy samples the attachment of *L. rhamnosus* GG to human intestinal mucosae and to evaluate the persistence of this attachment after discontinuation of strain GG administration.

Volunteers and *L. rhamnosus* GG administration. The three experimental groups in this study each consisted of six to eight adults undergoing routine diagnostic colonoscopy. The experimental protocol was designed to fit within the normal diagnostic schedule of the volunteers. Informed consent from all subjects was obtained before the experiment. With the exception of various gastric symptoms, all subjects considered themselves healthy. No antibiotic therapy was applied either during the trial or during the month immediately preceding the administration period. The volunteers had no immediate past history of consuming *L. rhamnosus* GG-containing products. For this study they took 100 ml of a commercial drink based on lactose-hydrolyzed whey fermented with strain GG and flavored with a peach-apricot concentrate (Gefilus; Valio Ltd., Kouvola Dairy, Kouvola, Finland) twice daily for 12 days. The daily dose of strain GG was approximately 6×10^{10} CFU. After administration of strain GG, the volunteers were divided into three groups (see Fig. 1): those having undergone colonoscopy immediately after the 12-day GG administration period (one male, five females, 34 to 78 years old), those having undergone colonoscopy 1 week after stopping GG administration (five males, three females, 42 to 68 years old), and those having undergone colonoscopy 2 weeks after stopping GG administration (four males, three females, 27 to 73 years old).

Colonoscopy and biopsies. In preparation for colonoscopy, evacuation of the colon was induced by three doses of a laxative (Pico-salax; Malmö, Sweden) consumed within 36 h. The instrument used for colonoscopy and sampling of biopsies was a Pentax ES-3801L (Tokyo, Japan). The diameter of the biopsies was approximately 3 mm. Three parallel biopsies were taken from the descending colon. This location was selected on the basis of previous results (1) showing preferential adhesion of *L. rhamnosus* GG to this part of the large intestine.

Cultivation of *L. rhamnosus* GG from fecal and biopsy samples. Fecal samples were collected as indicated in Fig. 1. The samples were immediately stored at about −20°C in the home freezers of the patients (for up to 3 weeks) and afterwards at −20°C in the laboratory until analysis (within 9 weeks after

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collection). Biopsy samples from the descending colon were immediately transferred into a thioglycolate medium (Difco, Detroit, Mich.) and stored at 4°C until analysis (within a day). The samples were homogenized for 30 s in a stomacher (Stomacher 400; Seward, London, United Kingdom) before dilution and cultivation on MRS agar (Merck, Darmstadt, Germany). The plates were incubated under anaerobic conditions (Anaerocult A; Merck) for 3 days at 37°C.

L. rhamnosus GG forms large, creamy, white colonies on MRS agar that are generally distinct from other lactic acid bacterial colonies. Strain GG is further distinguished from most other lactic acid bacteria by its inability to efficiently ferment lactose (4), which was tested by selecting one to four typical GG-like colonies from each fecal and biopsy sample and further cultivating them for 48 h in lactose MRS broth with indicator dye (bromocresol purple, 0.04 g/liter). One or two lactose-negative isolates per sample were further confirmed as *L. rhamnosus* by species-specific PCR.

PCR confirmation of *L. rhamnosus* isolates. Bacterial cells were collected from 1 ml of an overnight culture by centrifugation, washed with 50 mM Tris buffer (pH 8.0), and suspended in 100 μl of 50 mM Tris-EDTA buffer (pH 8.0). Lysozyme (100 μl, 20 mg/ml) (Sigma, St. Louis, Mo.) and mutanolysin (8 μl, 0.5 mg/ml) (Sigma) were added, and the mixture was incubated at 37°C for 1 h. The cells were lysed by addition of 20 μl of 20% sodium dodecyl sulfate and 12 μl of proteinase K solution (14.6 mg/ml) (Boehringer, Mannheim, Germany) followed by a 10-min incubation at 65°C. The volume was adjusted to 500 μl with sterile ultrapure water. Deproteinization was done by extraction with 1 volume of Tris-saturated phenol (Amresco, Solon, Ohio). The water phase was extracted once more with phenol-chloroform (1:1). Finally, DNA was precipitated by adding 0.1 volume of 3 M sodium acetate to the water phase followed by 2 volumes of 94% ethanol and incubating the mixture in an ice bath for 30 min. The DNA was collected by centrifugation at 13,000 rpm for 15 min, and the pellet was washed with 70% ethanol and finally dissolved in 20 μl of sterile ultrapure water.

The universal 16S rRNA gene forward and reverse primers (5′ to 3′) were AGAGTTTGATCCTGGCTCAGG and ACG GCAACCTTGTTACGAGTT, respectively. The species-specific primers (CTTGCATCTTGATTTAATTTTG, forward; CCGTCAATTCCTTTGAGTTT, reverse) were designed on the basis of the *L. rhamnosus* (previously *L. casei* subsp. *rhamnosus*) 16S ribosomal DNA sequence (GenBank accession no. M58815) specifying the 863-bp fragment between positions 91 and 953 in the gene. The primers were made with a PCR Mate EP 391 DNA synthesizer, model 391 (Applied Biosystems, Foster City, Calif.), according to the manufacturer's instructions.

Taq DNA polymerase and PCR buffer (final concentrations of 10 mM Tris-HCl, 1.5 mM $MgCl_2$ and 50 mM KCl [pH 8.3]) were obtained from Boehringer, and the deoxynucleotides were purchased from Sigma. The primer concentrations were 0.5 μM with specific primers and 0.25 μM with universal primers, and those of the deoxynucleotides were 200 μM. The amount of template was 1 μl of the DNA extracted from fecal isolates or 1 μl of an appropriate dilution of the DNA extracted from pure cultures. The amount of *Taq* DNA polymerase used was 2.0 U in a total reaction volume of 100 μl. A Gene Amp PCR System 9600 apparatus (Perkin-Elmer Cetus, Norwalk, Conn.) was used for PCR cycling. Initial denaturation was carried out at 94°C for 5 min followed by a touch-down thermocycling program with 30 amplification cycles (annealing for 30 s at 62°C in cycles 1 to 10, at 60°C in cycles 11 to 20, and at 58°C in cycles 21 to 30, with extension for 1 min at 72°C and denaturation for 40 s at 94°C) and a final extension for 10 min at 72°C. Reaction mixtures were subsequently cooled to 4°C. In the PCR with universal primers, the annealing temperature was 55°C.

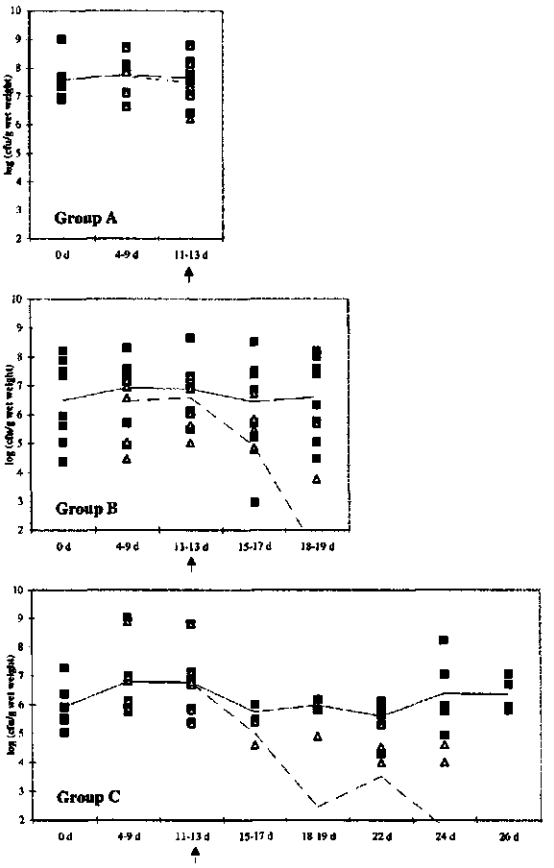

FIG. 1. Fecal counts of lactic acid bacteria (■) and *L. rhamnosus* GG-like colonies (△). The solid line shows the mean counts of lactic acid bacteria, and the dashed line shows the mean counts of strain GG. In this context, lactic acid bacteria are defined as colonies growing on MRS agar without further taxonomic characterization, with the exception of GG-like colonies. The end of *L. rhamnosus* GG administration is marked by a vertical arrow below the horizontal axis.

The specificity of the *L. rhamnosus* primers was confirmed with 8 different *L. rhamnosus* strains and 17 other lactobacillar species or strains as references (data not shown). To exclude the possibility of DNA extraction failure or the presence of inhibitors in samples, reference strains were subjected to PCR with universal primers prior to PCR with specific primers.

***L. rhamnosus* GG-like colonies in biopsy and fecal samples of different test groups.** The counts of total fecal lactic acid bacteria and strain GG-like colonies in the three experimental groups are presented in Fig. 1. The results of PCR (Fig. 2) were in good agreement (88%) with screening based on colony morphology and the lactose fermentation test, confirming the general reliability of identification of the strain. The counts of strain GG-like colonies decreased as a

FIG. 2. Detection of *L. rhamnosus* by PCR coupled with gel electrophoresis. Lanes: 1, molecular weight marker; 2 through 19, strain GG-like findings from fecal samples; 20, positive control (*L. rhamnosus* GG VTT E-96666); 21, control reaction with no template DNA.

function of time after discontinuation of GG administration. Strain GG was detected in biopsy specimens and final fecal samples of all volunteers in group A (Table 1). The counts of lactic acid bacteria in biopsy samples were 3×10^2 to 4×10^4 CFU per biopsy (mean, 6×10^3 CFU per biopsy). The corresponding counts of strain GG-like colonies were 6×10^1 to 4×10^4 CFU per biopsy.

In group B, *L. rhamnosus* GG-like colonies were detected in seven of eight biopsy samples (Tables 1 and 2), with counts varying between 2×10^3 and 1×10^6 CFU per biopsy. The total counts of lactic acid bacteria were 3×10^3 to 2×10^6 CFU per biopsy (mean, 1×10^5 CFU per biopsy). Only two of the eight subjects, however, had strain GG-like colonies at detectable levels in the final fecal samples; these counts were 6×10^3 and 5×10^5 CFU/g (wet weight). The individual counts of GG-like colonies in the biopsies and final fecal samples of the group B volunteers are presented in Table 2.

None of the seven subjects in group C had strain GG-like colonies in the final fecal samples (Table 1). However, GG-like colonies were detected in the biopsy samples of two of the seven volunteers at counts of 1×10^2 and 1×10^4 CFU per biopsy. The total counts of lactic acid bacteria in biopsies of group C were 6×10^2 to 2×10^5 CFU per biopsy (mean, 2×10^4 CFU per biopsy).

L. rhamnosus GG has been shown to adhere in vitro to the Caco-2 intestinal cell line (2) and in vivo to human colonic mucosae (1). The finding reported here that strain GG can persist in colonic mucosae even after its disappearance from fecal samples may have significance in the elucidation of the colonization mechanisms of probiotic strains. The fact that the strain GG counts observed in the biopsy samples from group B are rather similar to those obtained from group A is particularly interesting, since it indicates that GG can survive in high numbers in colonic mucosae despite its rapid turnover. This finding suggests that *L. rhamnosus* GG can multiply on the colonic surface at a rate that partially counterbalances its shedding. However, as can be seen from the results from group C, even an adherent strain can be gradually diluted out of the colon unless it is replenished with a fresh inoculum. The high counts of endogenous lactic acid bacteria associated with colonic biopsies mean that the probiotic strain faces strong competition when establishing itself. This may well be one of the reasons that permanent colonization by a probiotic strain seldom, if ever, occurs.

The present study confirms that *L. rhamnosus* GG is able to attach in vivo to colonic mucosae and to persist there for prolonged periods after discontinuation of administration of strain GG. In accounting for the findings reported here, the study of fecal samples alone may underestimate colonization by probiotic strains.

TABLE 1. Recovery of *L. rhamnosus* GG from colonic biopsy samples and final fecal samples[a]

Group	Day of colonoscopy	No. of subjects with the indicated characteristics/total no. of subjects			
		Both biopsy and feces positive	Biopsy positive, feces negative	Biopsy negative, feces positive	Both biopsy and feces negative
A	14	6/6	0/6	0/6	0/6
B	21	2/8	5/8	0/8	1/8
C	28	0/7	2/7	0/7	5/7

[a] Final fecal samples were obtained a day before evacuation and 2 days before colonoscopy.

TABLE 2. Counts of *L. rhamnosus* GG-like colonies in biopsy specimens and final fecal samples in group B

Volunteer	Strain GG-like colony count in:	
	Biopsy (CFU/biopsy)[a]	Final fecal sample (CFU/g [wet weight])[b]
1	—[c]	—
2	1.0×10^6	—
3	7.8×10^4	—
4	4.6×10^5	6.0×10^3
5	2.5×10^3	—
6	2.2×10^3	—
7	6.3×10^3	—
8	1.0×10^4	6.0×10^5

[a] Detection level, 10^2 CFU per biopsy. Biopsies were performed on day 21.
[b] Detection level, 10^3 CFU/g [wet weight]. Final fecal samples were obtained on day 18 or 19.
[c] —, below detection level.

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REFERENCES

1. **Alander, M., R. Korpela, M. Saxelin, T. Vilpponen-Salmela, T. Mattila-Sandholm, and A. von Wright.** 1997. Recovery of *Lactobacillus rhamnosus* GG from human colonic biopsies. Lett. Appl. Microbiol. **24**:361–364.
2. **Elo, S., M. Saxelin, and S. Salminen.** 1991. Attachment of *Lactobacillus casei* strain GG to human colon carcinoma cell line Caco-2: comparison with other dairy strains. Lett. Appl. Microbiol. **13**:154–156.
3. **Gilliland, S. E.** 1990. Health and nutritional benefits from lactic acid bacteria. FEMS Microbiol. Rev. **87**:175–188.
4. **Goldin, B. R., S. L. Gorbach, M. Saxelin, S. Barakat, L. Gualtieri, and S. Salminen.** 1992. Survival of *Lactobacillus* species (strain GG) in human gastrointestinal tract. Dig. Dis. Sci. **37**:121–128.
5. **Hammes, W. P., and P. S. Tichaczek.** 1994. The potential of lactic acid bacteria for the production of safe and wholesome food. Z. Lebensm.-Unters. -Forsch. **198**:193–201.
6. **Huis In't Veld, J. H. J., and C. Shortt.** 1996. Selection criteria for probiotic microorganisms. R. Soc. Med. Int. Congr. Symp. Ser. **219**:27–36.
7. **Johansson, M.-L., G. Molin, B. Jeppsson, S. Nobaek, S. Ahrné, and S. Bengmark.** 1993. Administration of different *Lactobacillus* strains in fermented oatmeal soup: in vivo colonization of human intestinal mucosa and effect on the indigenous flora. Appl. Environ. Microbiol. **59**:15–20.
8. **Lee, Y. K., and S. Salminen.** 1995. The coming age of probiotics. Trends Food Sci. Technol. **6**:241–245.
9. **Majamaa, H., and E. Isolauri.** 1997. Probiotics: a novel approach in the management of food allergy. J. Allergy Clin. Immunol. **99**:179–185.
10. **Millar, M. R., C. Bacon, S. L. Smith, V. Walker, and M. A. Hall.** 1993. Enteral feeding of premature infants with *Lactobacillus* GG. Arch. Dis. Child. **69**:483–487.

11. **Salminen, S., M. Deighton, Y. Benno, and S. Gorbach.** 1998. Lactic acid bacteria in health and disease, p. 211–253. *In* S. Salminen and A. von Wright (ed). Lactic acid bacteria. Microbiology and functional aspects. Marcel Dekker, New York, N.Y.
12. **Salminen, S., E. Isolauri, and E. Salminen.** 1996. Clinical uses of probiotics for stabilizing the gut mucosal barrier: successful strains and future challenges. Antonie Leeuwenhoek J. Microbiol. **70**:347–358.
13. **Saxelin, M.** 1997. *Lactobacillus* GG—a human probiotic strain with thorough clinical documentation. Food Rev. Int. **13**:293–313.
14. **Saxelin, M., M. Ahokas, and S. Salminen.** 1993. Dose response on the faecal colonisation of *Lactobacillus* strain GG administered in two different formulations. Microb. Ecol. Health Dis. **6**:119–122.

Chapter 5

Polymerase chain reaction and denaturing gradient gel electrophoresis monitoring of fecal *Bifidobacterium* populations in a prebiotic and probiotic feeding trial

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SYSTEMATIC AND APPLIED MICROBIOLOGY

Polymerase Chain Reaction and Denaturing Gradient Gel Electrophoresis Monitoring of Fecal *Bifidobacterium* Populations in a Prebiotic and Probiotic Feeding Trial

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Summary

A culture-independent approach based on genus-specific PCR and denaturing gradient gel electrophoresis (DGGE) was used to monitor qualitative changes in fecal bifidobacterial communities in a human feeding trial. DNA was extracted directly from feces and bifidobacterial 16S rDNA sequences were amplified using genus-specific PCR. The PCR fragments were subsequently separated in a sequence-specific manner by DGGE in order to obtain a profile of bifidobacterial fragments. The DGGE profiles revealed that in general, administration for two weeks of galactooligosaccharide and/or *Bifidobacterium lactis* Bb-12 (8 g and 3×10^{10} cfu per day, respectively) did not affect the qualitative composition of the indigenous *Bifidobacterium* population, while *B. lactis* Bb-12 transiently colonised the gut.

Key words: PCR – DGGE – *Bifidobacterium* spp. – probiotic – prebiotic – feeding trial

Introduction

It is generally recognised that the extremely complex and numerous intestinal microbiota has an impact on the hosts health and well-being. Bifidobacteria and lactobacilli are assumed to be beneficial to the human host (BALLONGUE, 1998; OUWEHAND et al., 1999). This potentially beneficial microbiota can be fortified by the ingestion of probiotics, prebiotics or synbiotics (COLLINS and GIBSON, 1999; FOOKS et al., 1999). Probiotics are viable microorganisms that exhibit a positive effect on the health of the host when they are ingested (SALMINEN et al., 1998). Prebiotics can be 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 bacterial species already resident in the colon, and thus attempt to improve host health (GIBSON and ROBERFROID, 1995). Prebiotics include carbohydrates such as inulin, fructo-oligosaccharides (FOS) and galactoligosaccharide (GOS) (COLLINS and GIBSON, 1999). Synbiotics are products that consist of both probiotics and prebiotics.

The effect of probiotics and prebiotics on the composition of the gut microbiota is commonly followed by enumeration of various bacterial groups by selective plating techniques. More recently, also fluorescent *in situ* hybridisation (FISH) techniques with specific probes for different phylogenetic groups have been used to monitor changes in bacterial numbers (HARMSEN et al., 2000; TANNOCK et al., 2000). Moreover, various molecular biological techniques have been developed to facilitate the identification of colonies of probiotic strains and to follow the fate of ingested probiotics through the gastro-intestinal tract (ALANDER et al., 1999; BRIGIDI et al., 2000; BUNTE et al., 2000; KULLEN et al., 1997; O'SULLIVAN and KULLEN, 1998; TANNOCK et al., 2000; VAUGHAN et al., 2000). Recently, we described a new method based on genus-specific PCR and denaturing gradient gel electrophoresis (DGGE) for the qualitative analysis of bifidobacterial populations in human feces (SATOKARI et al., 2001). DNA is extracted directly from feces without prior cultivation and used as template in *Bifidobacterium* genus-specific PCR. The PCR products of bifidobacterial 16S rDNA sequences are subsequently resolved in a sequence specific manner by DGGE and a characteristic profile of DNA-amplicons is obtained. Thus, the bifidobacterial community in feces can be monitored by a single PCR reaction.

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The aim of this study was to investigate the effect of the administration of galactooligosaccharide and/or the probiotic strain *Bifidobacterium lactis* Bb-12 (OUWEHAND et al., 1999) on the qualitative composition of indigenous *Bifidobacterium* population using genus-specific PCR combined with DGGE.

Materials and Methods

Volunteers and prebiotic and probiotic administration

The feeding trial is described in detail by ALANDER et al. (ALANDER et al., sin press). Briefly, 30 healthy Finnish adult volunteers (27 women, 3 men, mean age 32 years) participated in the study. They were randomly divided into three groups and volunteers of each group consumed Elix'or, a commercial food-grade syrup containing 60% galactooligosaccharide (GOS), 20% lactose, 19% glucose, 1% galactose in 75% dry matter (Borculo Whey Products Ltd., Borculo, The Netherlands) (group 1), probiotic bacteria *Bifidobacterium lactis* strain Bb-12 (Christian Hansen Ltd., Hørsholm, Denmark) (group 2) or a combination of these two (group 3). The study period was six weeks consisting of two weeks pre-feeding period, two weeks feeding period, and two weeks post-feeding period. During the pre-feeding period the volunteers consumed 125 ml of yoghurt twice a day and during the feeding-period 18 g Elix'or (8.1 g GOS per day) or freeze-dried Bb-12 (approximately 3×10^{10} cfu per day) or both were added to the yoghurt. During the post-feeding trial the volunteers had their normal diet. The volunteers were requested to reject products containing other probiotics or bifidobacteria during the study period but to retain otherwise a normal diet. Two volunteers from group 1 interrupted the study, one because of an antibiotic treatment and the other because of abdominal discomfort during the feeding period. Otherwise the products were well tolerated.

Fecal samples and DNA extraction

Fecal samples were collected at the end of the pre-feeding (sample 0) and feeding (sample 2) periods, and one and two weeks after the end of the administration (samples 3 and 4, respectively). Fecal samples from one volunteer in group 1 and one volunteer from group 2 could not be provided for this study, because the samples were first used for cultivation and not enough feces remained for DGGE analysis. Hence, fecal samples from 7 volunteers of group 1 (1-1 to 1-7), 10 volunteers of group 2 (2-1 to 2-10) and 9 volunteers of group 3 (3-1 to 3-9) were analysed in this study. DNA was extracted from fecal samples preserved at -70 °C essentially as described previously (ZOETENDAL et al., 1998).

PCR amplification and DGGE analysis

The qualitative analysis of intestinal bifidobacteria was carried out using PCR with primers Bif164-f and Bif662-GC-r (5' to 3' GGGTGGTAATGCCGGATG and CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-CCACCGTTACACCGGGAA, respectively) combined with DGGE as described previously (SATOKARI et al., 2001). The primers amplify an approximately 520 bp sequence of the 16S rDNA of bifidobacteria and show perfect match to the 16S rDNA sequences of all *Bifidobacterium* species found in the human intestine with the exception of *B. gallicum*, which has one base mismatch in the primer region. However, *B. gallicum* can be considered as an extremely rare intestinal species, since the type strain remains the only isolate described from human feces (LAUER, 1990).

The primer Bif164-f has one nucleotide mismatch to the 16S rDNA sequence of *B. lactis*, which is not considered to be a human intestinal species. In order to detect the probiotic strain *B. lactis* Bb-12, the forward primer was modified by one nucleotide (Bif164-mod-f, 5' to 3' GGGTGGTAATACCGGATG) to precisely match the *B. lactis* target sequence and to amplify the 16S rDNA fragment from this species more efficiently. PCR reaction conditions and thermocycling program with primers Bif164-f and Bif662-GC-r were described previously (SATOKARI et al., 2001). The same reaction conditions were used with primers Bif164-mod-f and Bif662-GC-r, but the annealing temperature was set at 58 °C. PCR products were resolved by DGGE (denaturing gradient of 45 - 55%, where 100% corresponds to 7 M urea and 40% formamide) (SATOKARI et al., 2001) and visualised by $AgNO_3$ staining (SANGUINETTI et al., 1994).

Sequence analysis

The PCR products were cloned in *E. coli* JM109 by using the pGEM-T vector system (Promega, Madison, Wis.) and sequenced using the Sequenase sequencing kit (Amersham, Slough, United Kingdom) according to manufacturer's instructions. The sequences were analyzed with automatic LI-COR DNA sequencer 4000L (Lincoln, Nebr.) and corrected manually. Homology searches, similarity comparisons, and sequence alignments were carried out using BCM services available in the internet (http://www.hgsc.bcm.tmc.edu/SearchLauncher/) or DNASTAR program (Madison, Wis.). The sequences of the 16S rDNA clones of fecal bifidobacteria were deposited in the GenBank database and have been assigned accession numbers (clone code in parenthesis): AY013814 (1-2A); AY013815 (1-2B), AY013816 (3-3A), AY013817 (3-4A), AY013818 (3-4B), AY013819 (2-3A), and AY013820 (1-5A).

Results and Discussion

The effect of Elix'or and/or Bb-12 administration on the bifidobacterial communities

In the feeding trial most changes in the bifidobacterial populations were expected to occur during the period from pre-feeding (0-sample) to the end of the feeding (2-sample) and, therefore, we first analysed the 0- and 2-samples from all volunteers. The 16S rDNA of intestinal bifidobacteria was amplified using primers Bif164-f and Bif662-GC-r. PCR products and profiles of bifidobacterial sequences in DGGE were obtained from all fecal samples 0 and 2 and compared (Table 1). Most volunteers showed no changes in the fecal bifidobacterial DGGE profiles between samples 0 and 2 (Table 1), and in these cases the follow-up samples 3 and 4 were not analysed. However, five volunteers out of 26 showed a change in fecal bifidobacterial DGGE profiles (Table 1) and these are presented in Fig. 1 together with six other volunteers where no changes occurred. There was no correlation between the change in the bifidobacterial count (MINNA ALANDER, personal communication) and the occurrence of qualitative changes in the bifidobacterial DGGE profile i.e. volunteers with DGGE profile changes did not have any greater differences in their bifidobacterial counts compared to the other volunteers (data not shown). Three volunteers (Fig. 1; 1-2, 3-3 and 3-4) who consumed Elix'or either alone or together with Bb-12 showed a change in the relative intensity of the amplicons in the profile. Further analysis of the follow-up samples 3 and 4 from these volunteers revealed that the profiles reverted to resemble the

Table 1. Changes in the bifidobacterial DGGE profile (other than the appearance of the Bb-12 amplicon) between fecal samples 0 and 2 (before and after the administration, respectively)

Group 1 (Elix'or)		Group 2 (Bb-12)		Group 3 (Elix'or +Bb-12)	
Volunteer	DGGE profile	Volunteer	DGGE profile	Volunteer	DGGE profile
1-1[1]	=	2-1	=	3-1	=
1-2[2]	# ←	2-2	=	3-2	=
1-3	=	2-3[1,2]	+2 →	3-3[2]	# ←
1-4	=	2-4	=	3-4[2]	# ←
1-5[2]	+2 →	2-5	=	3-5	=
1-6	=	2-6	=	3-6	=
1-7	=	2-7[1]	=	3-7	=
		2-8	=	3-8	=
		2-9	=	3-9	=
		2-10	=		

[1] male volunteer
[2] also fecal samples 3 and 4 (one and two weeks after the end of the administration, respectively) were analysed
= no change in the DGGE profile
change in the relative intensity of amplicons in the DGGE profile
+ appearance of a number of amplicons in the DGGE profile
← or → profile of the follow-up samples 3 and 4 reverted (←) or not reverted (→) to resemble the profile of 0-sample

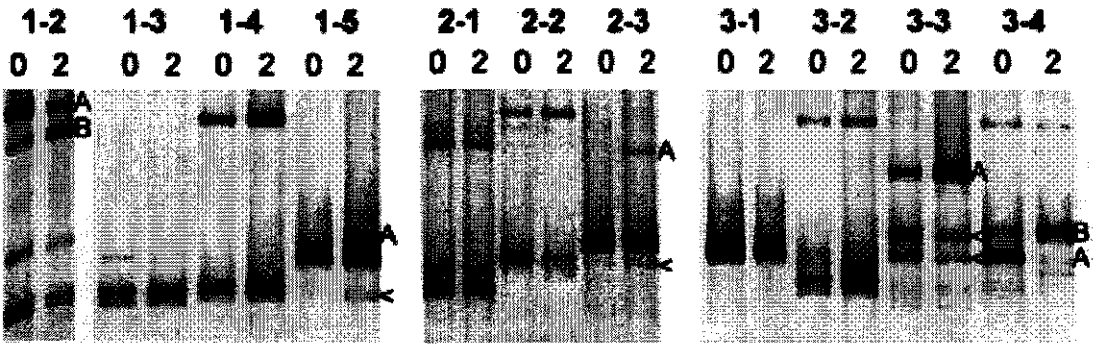

Fig 1. DGGE profiles of fecal bifidobacteria obtained by using primers Bif164-f and Bif662-GC-r from eleven volunteers (1-2 to 3-4, for test groups see Table 1) before (0) and after (2) the administration of Elix'or and/or Bb-12. Indications with letters A and B refer to the corresponding clones in Table 2. Unidentified changes are indicated with arrowheads (<).

original one (sample 0) after the end of the administration (data not shown). In two other cases (Fig.1; 1-5 and 2-3) new amplicons appeared in the profile and these did not disappear in the following samples 3 and 4, but remained present even after the end of the administration. The five amplicons that showed intensity changes and the two new amplicons appearing in the profiles were cloned and sequenced. The other newly emerging amplicons or amplicons with altered intensity could not be retrieved from the clone library with a reasonable screening because they were present in very low numbers, and hence, were not sequenced. Similarity comparison with sequences in the databases revealed that the sequenced 16S rDNA amplicons were derived from different *Bifidobacterium* species (Table 2). This result and the unchanging profiles of most volunteers indicated that Elix'or and/or Bb-12 did not selectively promote or suppress any particular *Bifidobacterium* species in the intestine.

Detection of the ingested probiotic strain B. lactis Bb-12

Bb-12 did not show up as a new amplicon in the bifidobacterial profiles produced with primers Bif164-f and Bif662-GC-r. *B. lactis* (GenBank accession number X89513) has one nucleotide mismatch to the Bif164-f primer sequence which is likely to have caused weaker amplification from Bb-12 compared to the intestinal species of bifidobacteria. Consequently, Bb-12 was not detected in the bifidobacterial profiles, although it was found to be present in relatively high numbers in the feces of the volunteers compared to the indigenous bifidobacteria (ALANDER et al., in press). Therefore, a modified forward primer (Bif164-f-mod) was used to monitor the presence of Bb-12 in the fecal samples from volunteers of groups 2 and 3. In order to identify the amplicon corresponding to Bb-12 in the fecal samples a PCR product

Table 2. Identification of amplicons in the fecal DGGE profiles by sequencing

Volunteer	Clone	Change	Sequence (bp)	No-N	%-N	% similarity to [1]
1-2	1-2A	↓	479	1	0.2	99.6 *B. adolescentis*
1-2	1-2B	↑	480	0	0	97.9 *B. ruminantium*
3-3	3-3A	↑	478	0	0	99.6 *B. angulatum*
3-4	3-4A	↓	475	7	1.5	98.1 *B. infantis*
3-4	3-4B	↑	481	8	1.7	97.1 *B. adolescentis*
2-3	2-3A	+	483	10	2.1	96.9 *B. adolescentis*
1-5	1-5A	+	478	3	0.6	99.0 *B. pseudocatenulatum*

N ambiguous bases
[1]%similarity from a pair-wise comparison
↓ and ↑ decreasing and increasing intensity in the DGGE profile from 0-sample to 2-sample
+ appearing amplicon in the DGGE profile

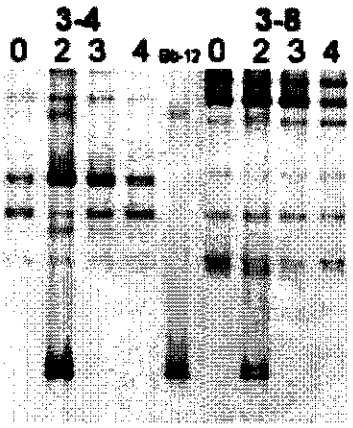

Fig. 2. Detection of *B. lactis* Bb-12 using primers Bif164-mod-f and Bif662-GC-r from the fecal samples of two volunteers (3-4 and 3-8) before (0) and after (2) two weeks administration of Elix'or and Bb-12, and one (3) and two (4) weeks after the end of the administration.

from Bb-12 was run alongside the fecal samples in DGGE (Fig. 2). One volunteer (2-1) was found to contain Bb-12-like bifidobacteria (amplicon in the same position in DGGE) in the feces before the feeding period, whereas all other volunteers of groups 2 and 3 were found to be Bb-12 negative in pre-feeding samples. A PCR amplicon corresponding to Bb-12 was detected in the fecal samples of all volunteers from groups 2 and 3 after two weeks of Bb-12 administration. All volunteers were again Bb-12 negative after one week after the end of the administration (sample 3), with the exception of one volunteer (2-10), who had the Bb-12 like amplicon in the 3-sample, but not in the 4-sample taken one week later. A Bb-12-like sequence was detected in the pre-feeding fecal sample of volunteer 2-1. Food products containing Bb-12 are on the market in Finland and volunteer 2-1 could have consumed a Bb-12 containing product previously, which had led to a long-term colonisation. Another explanation is that she accidentally consumed Bb-12-containing yoghurt already during the pre-feeding period. The latter explanation is more likely, since the subsequent fecal samples taken one and two weeks after the end of the administration were again Bb-12 negative.

In the present study, DGGE was found to be a convenient tool for large scale monitoring of fecal samples for qualitative changes in bifidobacterial communities, since the 16S rDNA sequence diversity of the whole target population could be monitored by a single PCR reaction. More detailed characterisation of the shifts in populations could be done by subsequent cloning and sequencing of the DNA amplicons as illustrated here for the most conspicuous alterations (Table 2).

The administration of Elix'or alone or in combination with *B. lactis* Bb-12 did not affect the qualitative composition of indigenous *Bifidobacterium* populations, although Bb-12 transiently colonised the gut. ALANDER et al. (ALANDER et al., in press) analysed the same feeding trial material using selective plating for the quantification of bifidobacteria and observed a significant increase in bifidobacterial numbers in both group 2 (Bb-12 alone) and group 3 (Elix'or and Bb-12). In group 1 (Elix'or alone) a slight increase was observed in the bifidobacterial counts, but this was statistically not significant. Although intestinal species of bifidobacteria generally utilise GOS *in vitro* (HOPKINS et al., 1998), it seems that no significant quantitative (ALANDER et al., in press; ALLES et al., 1999) or qualitative changes can be introduced to the intestinal *Bifidobacterium* population of healthy adult volunteers by the ingestion of GOS-containing Elix'or.

In previous trials it has been demonstrated that the consumption of significant amounts of probiotics have none or little effect on the dominant microbial communities of adults (VAUGHAN et al., 1999) which are very stable over time (ZOETENDAL, et al., 1998). Fecal bifidobacterial communities have also been shown to be relatively stable in most adults for a time period comparable to or longer than in this feeding trial (MCCARTNEY et al., 1996; MANGIN et al., 1999; SATOKARI et al., 2001). This is confirmed by the present study that shows fecal bifidobacterial communities to be unaffected by the passage of the probiotic strain Bb-12.

According to the DGGE analysis the simultaneous administration of Elix'or with Bb-12 (a synbiotic approach)

did not prolong the persistence of the probiotic strain in the gut, which is in agreement with the results obtained by ALANDER et al. Similar results were obtained in a previous synbiotic trial in which inulin was not found to improve the colonisation of an exogenous *Bifidobacterium* sp. in the human gut (BOUHNIK et al., 1996).

In conclusion, molecular monitoring by a combination of genus-specific 16S rDNA PCR and DGGE allow the qualitative analysis of fecal microbiota, in particular the screening of diversity and changes in the community structure. The method has a high throughput enabling the analysis of large numbers of fecal samples in feeding trials.

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References

ALANDER, M., MÄTTÖ, J., KNEIFEL, W., JOHANSSON, M., KÖGLER, B., CRITTENDEN, R., MATTILA-SANDHOLM, T., SAARELA, M.: Effect of galacto-oligosaccharide supplementation on human faecal microflora and on survival and persistence of Bifidobacterium lactis Bb-12 in the gastrointestinal tract. J. Dairy Sci. (in press).

ALANDER, M., SATOKARI, R., KORPELA, R., SAXELIN, M., VILPPONEN SALMELA, T., MATTILA SANDHOLM, T., VON WRIGHT, A.: Persistence of colonization of human colonic mucosa by a probiotic strain, Lactobacillus rhamnosus GG, after oral consumption. Appl. Environ. Microbiol. 65, 351–354 (1999).

ALLES, M. S., HARTEMINK, R., MEYBOOM, S., HARRYVAN, J. L., VAN LAERE, K. M., NAGENGAST, F. M., HAUTVAST, J. G.: Effect of transgalactooligosaccharides on the composition of the human intestinal microflora and on putative risk markers for colon cancer. Am. J. Clin. Nutr. 69, 980–991 (1999).

BALLONGUE, J.: Bifidobacteria and probiotic action, pp. 519 - 587. In: Lactic acid bacteria, microbiology and functional aspects (S. SALMINEN, A. VON WRIGHT, eds.) 2nd ed., New York, Marcel Dekkers, Inc. 1998.

BOUHNIK, Y., FLOURIE, B., ANDRIEUX, C., BISETTI, N., BRIET, F., RAMBAUD, J. C.: Effects of Bifidobacterium sp fermented milk ingested with or without inulin on colonic bifidobacteria and enzymatic activities in healthy humans. Eur. J. Clin. Nutr. 50, 269–273 (1996).

BRIGIDI, P., VITALI, B., SWENNEN, E., ALTOMARE, L., ROSSI, M., MATTEUZZI, D.: Specific detection of Bifidobacterium strains in a pharmaceutical probiotic product and in human feces by polymerase chain reaction. System. Appl. Microbiol. 23, 391–399 (2000).

BUNTE, C., HERTEL, C., HAMMES, W. P.: Monitoring and survival of Lactobacillus paracasei LTH 2579 in food and the human intestinal tract. System. Appl. Microbiol. 23, 260–266 (2000).

COLLINS, M. D., GIBSON, G. R.: Probiotics, prebiotics, and synbiotics: approaches for modulating the microbial ecology of the gut. Am. J. Clin. Nutr. 69, 1052s–1057s (1999).

FOOKS, L. J., FULLER, R., GIBSON, G. R.: Prebiotics, probiotics and human gut microbiology. Int. Dairy J. 9, 53–61 (1999).

GIBSON, G. R., ROBERFROID, M. B.: Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. J. Nutr. 125, 1401–1412 (1995).

HARMSEN, H. J., GIBSON, G. R., ELFFERICH, P., RAANGS, G. C., WILDEBOER VELOO, A. C., ARGAIZ, A., ROBERFROID, M. B., WELLING, G. W.: Comparison of viable cell counts and fluorescence in situ hybridization using specific rRNA-based probes for the quantification of human fecal bacteria. FEMS Microbiol. Lett. 183, 125–129 (2000).

HOPKINS, M. J., CUMMINGS, J. H., MACFARLANE, G. T.: Interspecies differences in maximum specific growth rates and cell yields of bifidobacteria cultured on oligosaccharides and other simple carbohydrate sources. J. Appl. Microbiol. 85, 381–386 (1998).

KULLEN, M. J., AMANN, M. M., SHHAUGHNESSY, O. M. J., SULLIVAN, O. D. J., BUSTA, F. F., BRADY, L. J.: Differentiation of ingested and endogenous bifidobacteria by DNA fingerprinting demonstrates the survival of an unmodified strain in the gastrointestinal tract of humans. J. Nutr. 127, 89–94 (1997).

LEUER, E.: Bifidobacterium gallicum sp. nov. isolated from human feces. Int. J. Syst. Bacteriol. 40, 100–102 (1990).

MANGIN, I., BOUHNIK, Y., BISETTI, N., DECARIS, B.: Molecular monitoring of human intestinal Bifidobacterium strain diversity. Res. Microbiol. 150, 343–350 (1999).

McCartney, A. L., W. Wang, G. W. Tannock. Molecular analysis of the composition of the bifidobacterial and lactobacillus microflora of humans. Appl. Environ. Microbiol. 62, 4608–4613 (1996).

O' SULLIVAN, D., KULLEN, M. J.: Tracking of probiotic bifidobacteria in the intestine. Int. Dairy J. 8, 513–525 (1998).

OUWEHAND, A. C., KIRJAVAINEN, P. V., SHORTT, C., SAALMINEN, S.: Probiotics: mechanisms and established effects. Int. Dairy J. 9, 43–52 (1999).

SALMINEN, S., VON WRIGHT, A., MORELLI, L., MARTEAU, P., BRASSART, D., DE VOS, W. M., FONDEN, R., SAXELIN, M., COLLINS, K., MOGENSEN, G., BIRKELAND, S.-E., MAATTILA-SANDHOLM, T.: Demonstration of safety of probiotics - a review. Int. J. Food Microbiol. 44, 93–106 (1998).

SANGUINETTI, C. J., DIAS NETO, E., SIMPSON, A. J.: Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. Biotechniques 17, 914–921 (1994).

SATOKARI, R. M., VAUGHAN, E. E., AKKERMANS, A. D. L., SAARELA, M., DE VOS, W. M.: Bifidobacterial diversity in human feces detected by genus-specific polymerase chain reaction and denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. 67, 505–513 (2001)

TANNOCK, G. W., MUNRO, K., HARMSEN, H. J. M., WELLING, G. W., SMART, J., GOPAL, P. K.: Analysis of the fecal microflora of human subjects consuming a probiotic products containing Lactobacillus rhamnosus DR20. Appl. Environ. Microbiol. 66, 2578–2588 (2000).

VAUGHAN, E. E., HEILIG, H. G. H. J., ZOETENDAL, E. G., SATOKARI, R., COLLINS, J. K., AKKERMANS, A. D. L., DE VOS, W. M.: Molecular approaches to study probiotic bacteria. Trends Food Sci. Technol.10, 400–404 (1999).

VAUGHAN, E. E., SCHUT, F., HEILIG, H. G. H. J., ZOETENDAL, E. G., DE VOS, W. M., AKKERMANS, A. D. L.: A molecular view of the intestinal ecosystem. Curr. Issues Intest. Microbiol. 1, 1–12 (2000).

ZOETENDAL, E. G., AKKERMANS, A. D. L., DE VOS, W. M.: Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl. Environ. Microbiol. 64, 3854–3859 (1998).

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Chapter 6

Diversity of *Bifidobacterium* and *Lactobacillus* spp. in breast-fed and formula-fed infants as assessed by 16S rDNA sequence differences

Modified version of this chapter has been submitted for publication.

Diversity of *Bifidobacterium* and *Lactobacillus* spp. in breast-fed and formula-fed infants as assessed by 16s rDNA sequence differences

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Abstract

A qualitative molecular monitoring approach based on PCR and denaturing gradient gel electrophoresis (DGGE) was used to study the diversity of dominant bacteria, bifidobacteria and lactobacilli in vaginally delivered full-term infants. Seven breast-fed and six formula-fed infants participated in the study. 16S rDNA targeted primers were used for the specific PCR amplification of fragments from bacteria, bifidobacteria and lactobacilli from faecal samples that were collected before and after weaning at the age of approximately one and seven months, respectively. The PCR fragments were subsequently resolved in a sequence-dependent manner by DGGE. In addition, cloning and sequence analysis of the PCR fragments was used to identify the species from which they originated. Based on the number of fragments in the DGGE profiles it was estimated that breast-fed and formula-fed infants harboured bacterial communities of equal complexity. There was no conspicuous difference in the distribution of *Bifidobacterium* or *Lactobacillus* species between breast-fed and formula-fed infants. The most frequently found representatives of these genera were *B. infantis* and species belonging to the *L. acidophilus* -group in both groups of infants. The predominant *Bifidobacterium* and *Lactobacillus* populations in most infants consisted of only one or two species.

Introduction

Microbial colonisation of the human GI-tract starts at birth when the newborn infant comes in contact with the microbes from the mother and surrounding environment. Colonisation proceeds in several stages until a complex, diverse and stable microbiota resembling that of an adult is developed (6, 17). The first one to two weeks of life are considered as the initial period of bacterial colonisation, during which diverse bacteria transit through infants GI-tract and can be found in the feces. Facultative anaerobic bacteria such as *Escherichia coli* and streptococci colonise the gut during this period. During the next stage anaerobic bacteria such bifidobacteria, *Bacteroides* and clostridia are found in the feces of infants. In breast-fed infants bifidobacteria predominate, whereas in formula-fed infants a more diverse microbiota develops (6, 17). Once dietary supplementation begins, the difference in the faecal microbiota between breast-fed and formula-fed infants disappears. The last stage represents the period of conversion to adult microbiota after the weaning is completed. Factors that influence the microbial succession and colonisation include both host-related (36) and external factors such as the mode of delivery (vaginal or caesarean), microbial load of the surrounding environment (hygienic conditions), type of feeding (breast- or formula-feeding) and possible antibiotic therapy of the infant (17, 24).

Early culture-based and microscopic investigations showed that bifidobacteria were the predominant microorganisms in feces of breast-fed infants but not in formula-fed infants (5, 32). However, several other culturing studies have shown that both groups of infants have equal frequency and level of bifidobacterial colonisation by the age of approximately one month or earlier (3, 12, 16). Conflicting results have also been obtained regarding other groups of bacteria such as *Bacteroides*, clostridia, enterococci, lactobacilli and enterobacteria in breast-fed and formula-fed infants. Culture-based studies have shown a more diverse microbiota in formula-fed infants in comparison to those breast-fed (1, 32). Moreover, in some studies formula-fed infants were found to have more complex faecal short-chain fatty acid (SCFA) profiles than breast-fed infants, which indicates a more complex microbiota in the aforementioned group (23, 30). In contrast, earlier studies based on cultivation showed no difference in

the composition of anaerobic and facultative anaerobic bacteria between breast-fed and formula-fed infants (16, 31).

New microbiological, biochemical and molecular biological methods for detection of different bacterial groups and species have been developed in order to describe the microbial communities more accurately (17, 35). Modern molecular ecology techniques based on direct detection and sequence comparison of nucleic acids (DNA or RNA) have been applied in numerous studies to characterise adult human GI-tract microbiota (29, 33, 35), but only in a few to describe infant microbiota (7, 10). A widely used approach is to detect the target molecules 16S rDNA or rRNA by using fluorescent *in situ* hybridisation (FISH), dot blot hybridisation, and PCR-based techniques (10, 29, 33, 35). Recently, culture-independent study based on FISH was reported that supported the predominance of bifidobacteria in breast-fed infants in comparison to formula-fed infants and suggested a more diverse microbiota in the latter group (10).

Combination of PCR amplification of 16S rDNA and rRNA fragments with temperature or denaturing gradient gel electrophoresis (TGGE and DGGE, respectively) can be used to achieve sequence-specific separation of PCR-fragments obtained from bacterial communities in gastrointestinal samples (26, 37). This approach has been used to follow the colonisation of new-born infants in considerable detail (7). It is also possible to target separate genera or groups of bacteria with specific PCR primers in order to obtain DGGE community-fingerprints of certain bacterial populations (11, 26). In this study we used a culture-independent molecular approach based on DGGE of 16S rDNA amplicons to compare the diversity of the microbiota in breast-fed and formula-fed infants before and after weaning. Cloning and sequencing of 16S rDNA derived PCR fragments was used to identify the predominant *Bifidobacterium* and *Lactobacillus* species present in feces of breast-fed and formula-fed infants.

Materials and methods

Infants, faecal samples and DNA extraction. Thirteen vaginally delivered infants (seven breast-fed and six formula-fed) were recruited to the study (Table 1). Two infants, S and D are Dutch, whereas all other infants are Scottish. All

infants were in general good health and were not treated with antibiotics during the sampling period. The weaning of infants (introduction of solid food) took place from weeks 12 to 21 after birth. The infants continued taking breast or formula milk (Milumil for infant 49 and Aptamil for all others, Milupa, GmbH & Co.KG, Friedrichdorf, Germany) after weaning during the whole sampling period with the exception of infants S and D from whom breast-milk was withdrawn at 1,5 and 6,5 months, respectively. Faecal samples were collected before and after weaning, at the age of approximately one month and seven months and kept frozen at –20 °C until the analysis. Isolation of DNA from faecal samples was performed as described elsewhere (37).

Table 1. Infants and faecal samples.

		Breast-fed				Formula-fed	
Code	Sex	Weaning age (weeks)	Sampling times (month)	Code	Sex	Weaning age (weeks)	Sampling times (month)
1	F	15	1 and 7	9	F	12	1 and 7
2	M	13	1 and 8	13	F	12	1 and 7
33	F	21	1 and 6	24	F	16	1 and 7
40	M	14	1,5 and 8,5	28	F	14	1 and 7
52	F	12	1 and 7	49	F	13	1 and 6
S	F	17	1 and 7	54	F	12	3 weeks and 7
D	M	19	1 and 7				

Primers. All primers used in the study are targeted on the 16S rRNA gene (Table 2). Bacterial PCR products were produced with primers 968-GC-f and 1401-r, *Bifidobacterium* genus-specific PCR was performed with primers Bif164-f and Bif662-GC-r and a nested PCR with primer sets 7-f and 0677lab-r, followed by 124-GC-f and 515-r was used to detect lactobacilli and related bacteria. A 40 bp GC-clamp was attached to the 5' end of Bif662-GC-r, 124-GC-f and 968-GC-f primers (see Table 2) in order to facilitate the analysis of the PCR products by DGGE. Primers T7 and Sp6 labelled with IRD800 were used for sequencing. All primers were purchased from MWG-Biotech (Ebersberg, Germany).

Table 2. Primers.

Primer	Sequence 5'to 3'	Use	Target	Ref
968-GC-f	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCAC GGGGGG-AACGCGAAGAACCTTA	PCR	bacteria	13
1401-r	CGGTGTGTACAAGACCC	PCR	bacteria	13
Bif-164-f	GGGTGGTAATGCCGGATG	PCR	bifidobacteria	14, 26
Bif-662-GC-r	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCAC GGGGGG-CCACCGTTACACCGGGAA	PCR	bifidobacteria	"
7-f	AGAGTTTGAT C/T A/C TGG CTCAG	PCR	lactobacilli	11,13
0677-lab	CACCGCTACACATGGAG	PCR	lactobacilli	11
124-GC-f	CGCCGGGGGCGCGCCCCGGGCGGGGCGGGGGCAC GGGGGGCACGG-ATCCGGACGGGTGAGTAACACG	PCR / nested	lactobacilli	11,13
515-r	ATCGTATTACCGCGGCTGCTGGCAC	PCR / nested	lactobacilli	11,13
Sp6	GATTTAGGTGACACTATAG	Sequencing	pGEM-^{T}	Promega
T7	TAATACGACTCACTATAGGG	Sequencing	pGEM-^{T}	Promega

PCR amplification. PCR reactions were performed as described previously (11, 26, 37) using *Taq* DNA polymerase kit from Life Technologies (Gaithersburg, Md., US). The reaction mixture consisted of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM of each primer, 1.25 U of *Taq* polymerase and 1 μl of appropriately diluted template DNA in a final volume of 50 μl. The PCR thermocycling program with primers 968-GC-f and 1401-r was the following: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 56 °C for 20 s, 68 °C 40 s; and 68 °C for 7 min. The reactions were subsequently cooled to 4 °C. The annealing temperature was set at 62 °C with primers Bif164-f and Bif662-GC-r and at 66 °C with primers 7-f and 0677-lab-r. For the nested PCR, the *Lactobacillus*-like 7-f to 0677-lab-r PCR products were purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and eluted into 50 μl of milliQ water (autoclaved, sterile-filtrated and UV-treated). One microliter of the resulting eluent was used as a template in the subsequent PCR with primers 124-GC-f and 515-r and using the same thermocycling program as described above for primers 968-GC-f and 1401-r. The size and amounts of PCR products were estimated by analysing 5 μl

samples by 1.2 % agarose gel (w/v) electrophoresis and ethidium bromide staining.

DGGE analysis of PCR products. DGGE analysis of PCR amplicons was performed essentially as described previously (21, 22) using the DCode or DGENE System apparatus (BioRad, Hercules, CA, US). Polyacrylamide gels (8% w/v, acrylamide : bisacrylamide – 37.5 : 1) in 0.5 x TAE with a denaturing gradient were prepared with a gradient mixer and Econo-pump (BioRad). The following denaturing gradients were used: 40 to 50 % for bacterial, 45 to 60 % for bifidobacterial and 30 to 60 % for lactobacilli PCR products, respectively. A 100 % denaturant corresponds to 7 M urea and 40 % (vol/vol) 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 visualised by $AgNO_3$ staining and developing as described previously (25).

Analysis of the DGGE gels. DGGE gels were scanned at 400 dpi and analysed using the Molecular Analyst 1.12 software (BioRad). Similarity indexes of compared profiles were calculated from the densitometric curves of DGGE profiles by using the Pearsons product-moment correlation coefficient. For the assessment of the diversity of 16S rDNA sequences in DGGE, a manual check was performed to ensure that all fragments in a gel track were found by the software. The surface area of fragments was calculated and the fragments constituting less than 1% of the total surface area of all fragments were omitted from the account of fragments for the diversity assessment. Student's t-tests were used for the statistical analysis of the data.

Cloning of the PCR products. The PCR products were purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to manufacturer's instructions and cloned in *E. coli* JM109 by using the pGEM-^{T} vector system (Promega, Madison, Wis., US). Colonies were picked and transferred into 20 µl of TE, boiled for 15 min to lyse the cells and the cell lysates were used to screen the transformants by the *Bifidobacterium*- or *Lactobacillus*- specific PCR followed by DGGE analysis. Clones for subsequent sequence analysis were selected according to the migration position of the clone

PCR fragment in DGGE in comparison to the fragments in the original sample DGGE profile. Plasmid DNA of selected transformants was isolated using QIAprep spin miniprep kit (Qiagen).

Sequence analysis. Sequencing of the cloned PCR fragments was carried out using purified plasmid DNA and sequencing primers T7 and Sp6 complementary to the adjacent sequences of the pGEM-^{T} cloning site. Sequencing reactions were performed with the Sequenase sequencing kit (Amersham, Slough, United Kingdom) according to manufacturer's instructions. The sequences were analysed with automatic LI-COR DNA sequencer 4000L (Lincoln, Nebr., US) and corrected manually. Sequence alignment of the complementary strands was carried out using DNASTAR SeqMan program (Madison, Wis., US). Similarity searches of 16S rDNA sequences derived from faecal clones were performed using the BCM Nucleic acid sequence search service available on the internet (http://www.searchlauncher.bcm.tmc.edu).

Results

DGGE profiles of predominant bacterial communities

In this study the diversity of the microbiota in breast-fed and formula-fed infants before and after weaning was compared. The faecal samples were collected at one and seven months of age. In order to assess the diversity and changes of the bacterial populations in the infant faecal samples, PCR fragments generated with primers 968-GC-f and 1401-r were analysed by DGGE (Fig. 1). Differences seen in the faecal DGGE profiles between the samples taken before and after weaning reflected changes occurring in the faecal microbiota. The changes varied in both groups of infants from a complete or almost complete change of the fragments in the profile (infants 24 and 33, Fig. 1) to appearance and disappearance of some fragments in the profile (infants 13 and 40, Fig. 1). The similarity indexes between the DGGE profiles of pre- and post-weaning samples from each infant were calculated. The similarity indexes of the breast-fed infants (mean 51.3; SD 26.0) and the formula-fed infants (mean 46.2; SD 23.8) did not differ

significantly (P = 0.72) showing an equal extent of profile changes in both groups.

In order to compare the relative diversity of the predominant bacterial populations in breast-fed and formula-fed infants, the numbers of predominant individual fragments in the DGGE profiles were compared. No significant difference was found in the number of fragments in the DGGE profiles between breast-fed and formula-fed infants at approximately one month or seven months of age (P values 0.98 and 0.95, respectively). These results suggest that the diversity in predominant species or strains do not differ significantly between the two groups of infants before or after weaning.

The DGGE profiles from different infants had some fragments in common (Fig. 1). The uppermost fragment (a) in common to the infants was cloned and sequenced from the one month samples of infants 1, 9 and 13. The sequences of these fragments showed highest similarity (96 to 98 %) to that of *Ruminococcus gnavus* 16S rDNA sequence. The lowest fragments (c) from the one month samples of infants 9 and 13 were identified as *Bifidobacterium* spp. sequences (96 and 97 % similarity). The common fragment appearing in the middle part of the gel (b) was cloned and sequenced from the one month sample of infant 1 and it appeared to have originated from *Escherichia coli* (similarity 99 %).

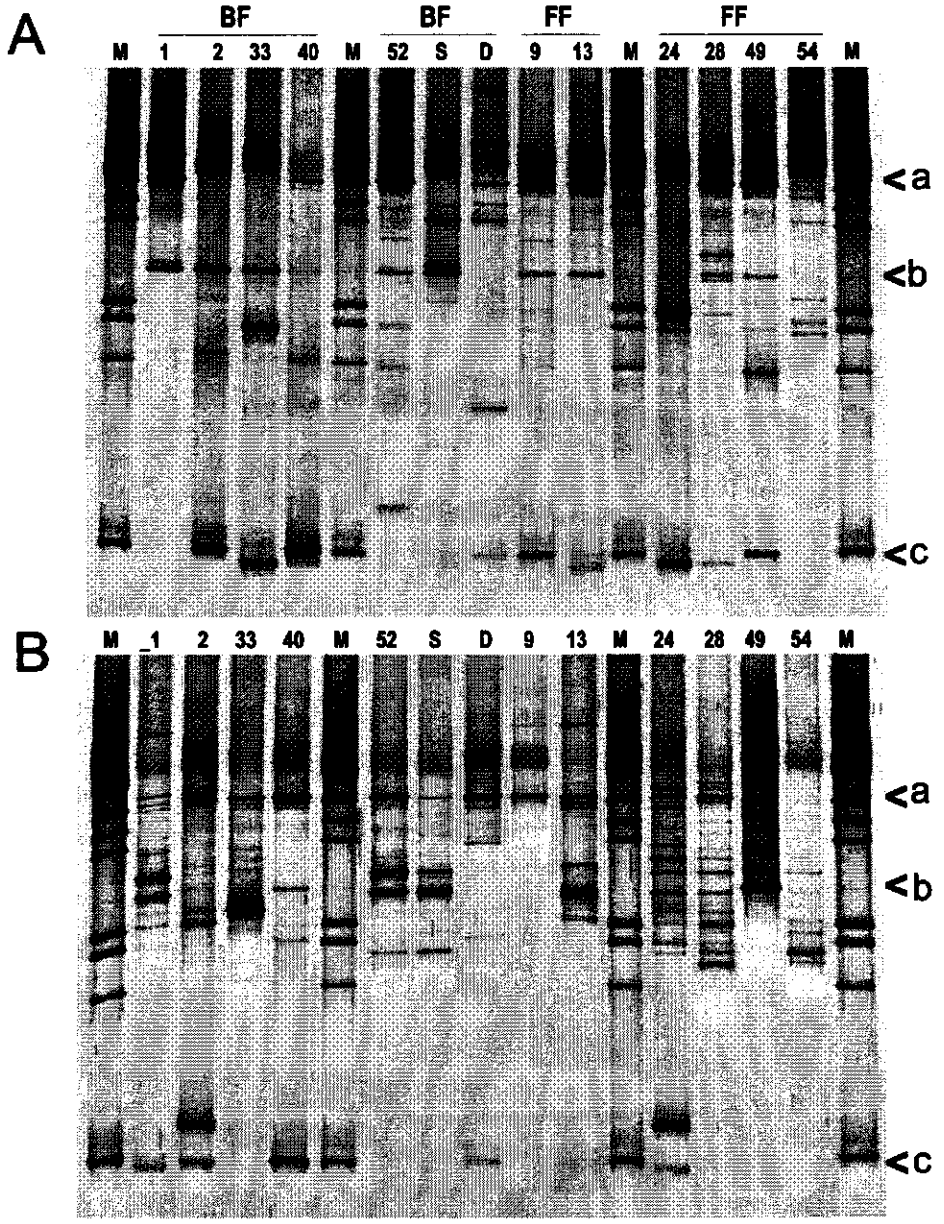

Figure 1. Bacterial DGGE profiles of faecal samples from breast-fed (BF) and formula-fed (FF) infants at approximately one month (A) and seven months (B) of age. Lanes are marked with the code of the infant (see also Table 1). The lane containing the marker is indicated with M. Positions where fragments were found in the majority of samples are labelled with a, b and c, and marked with arrowheads.

Detection and identification of bifidobacteria

In order to find out whether the changes detected in the dominant bacterial population were also reflected to the bifidobacterial populations, the samples were analysed using genus-specific PCR combined to DGGE and cloning and sequencing of the specific PCR fragments. Bifidobacteria were detected in five out of seven breast-fed infants and in all six formula-fed infants at one month of age. At seven months of age bifidobacteria were detected in six out of seven breast-fed and five out of six formula-fed infants. One breast-fed infant (coded 52) lacked bifidobacteria at both sampling times. The bifidobacterial DGGE profiles from all positive samples are shown in Fig. 2. Sequence-specific separation of the amplified bifidobacterial 16S rDNA fragments revealed that in most infants the predominant *Bifidobacterium* population consisted of one or two dominant species (Fig. 2). Comparison of bifidobacterial profiles before and after weaning was possible in the case of ten infants when PCR products were obtained from both one and seven month faecal samples. Three breast-fed (33, S and D; Fig. 2) and three formula-fed (9, 24 and 54; Fig. 2) infants showed no changes in their bifidobacterial profiles before and after weaning. Three infants (2, 13 and 28; Fig. 2) showed some changes in their *Bifidobacterium* DGGE profiles, whereas one infant (40; Fig. 2) showed a complete change in the composition of his *Bifidobacterium* population. Although the DGGE profiles of dominant bacterial populations changed in all infants during the weaning at least to some extent (Fig. 1), the specific bifidobacterial profiles remained unaltered in half of the infants (Fig. 2).

The predominant *Bifidobacterium* species of most samples were identified by subsequent sequence analysis of the 16S rDNA PCR fragments. The PCR fragments were first cloned into *E. coli* and clones with an insert that produced a PCR fragment corresponding to a predominant fragment in the original DGGE profile of the faecal sample were selected for sequencing. Comparative sequence analysis with the databases revealed that all fragments had high similarity, (97 % or higher) to the 16S rDNA sequences of known *Bifidobacterium* species (Fig. 2). Most of the sequenced fragments resembled typical infant *Bifidobacterium* species, such as *B. infantis, B. bifidum, B. breve,* and *B. dentium.* Four infants were also found to harbour *B. pseudocatenulatum* -like species, which is found

more typically in adults. In general, no prominent difference was found in the distribution of bifidobacterial species between breast-fed and formula-fed infants in the faecal samples from either one month or seven months. Based on sequencing of the PCR amplicons, *B. infantis* was the most frequently found species in both groups of infants (Fig 2). *B. breve* was detected in two of the breast-fed infants, but not in the formula-fed infants, and *B. dentium* was detected in one formula-fed infant. *B. ruminantium*, which is not considered to be a human-associated *Bifidobacterium* species was found in a faecal sample from one breast-fed infant. However, it remains debatable whether the amplified 16S rDNA fragment derived from *B. ruminantium* or its closely related species *B. dentium* or *B. adolescentis* because an unequivocal species identification can not be obtained based only on the 16S rDNA sequence data.

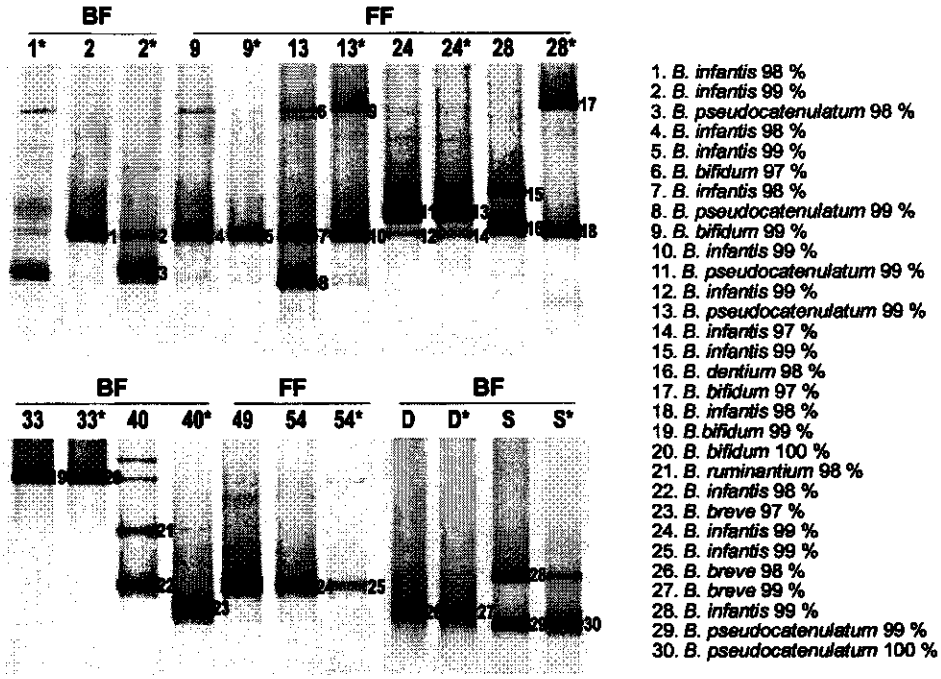

Figure 2. Bifidobacterial DGGE profiles of feces and PCR fragments identified by cloning and sequencing from breast-fed (BF) and formula-fed (FF) infants at approximately one month and seven months of age. Lanes are marked with the code of the infant (see also Table 1) and the approximate 7 month sample is indicated with an asterisk.

Detection and identification of lactobacilli

Lactobacilli form a well-studied bacterial group and are found in variable but usually low numbers in infants (10, 17). To analyse the effect of diet and time on the diversity of lactobacilli, a similar approach as for bifidobacteria was used consisting of a group-specific PCR combined to DGGE and cloning and sequencing of the specific PCR fragments (11). Lactobacilli were detected by PCR in eight of the thirteen infants, five breast-fed and three formula-fed (Fig. 3). Subsequent DGGE analysis revealed that the predominant *Lactobacillus* population, like the predominant *Bifidobacterium* population, consisted of one or two dominant species or strains (Fig. 3). *Lactobacillus*-specific PCR products were obtained from both the one- and seven-month samples from four infants, 28, 40, D and S (Fig. 3). In the two infants 28 and 40 (Fig. 3), the major *Lactobacillus* fragment in the one-month sample was replaced with another fragment in the seven-month sample. It is noteworthy, that for the same infants complete changes of the bifidobacterial DGGE profiles were observed (Fig 2). In the other two infants, D and S (Fig. 3), the *Lactobacillus* population appeared more stable; the same predominant fragment was present in both one and seven-month samples, but also some fragments appeared or disappeared in the seven-month sample as compared to the one-month sample. Infants D and S did not have any major changes in their *Bifidobacterium* DGGE profiles either.

The most commonly detected *Lactobacillus* species was *L. acidophilus* (*sensu lato*). The species *L. acidophilus*, *L. johnsonii* and *L. gasseri* share 16S rDNA sequences with high similarity, thus, the PCR fragments with highest similarity to *L. acidophilus* may have originated from any of these three species. Other species found with the *Lactobacillus*-group specific are listed in Fig. 3. In general, the *Lactobacillus*-group primers had a good specificity for the target group, which includes *Lactobacillus*, *Pediococcus*, *Weissella* and *Leuconostoc*, but occasionally PCR fragments from other bacteria such as streptococci and enterococci were also amplified (Fig. 3).

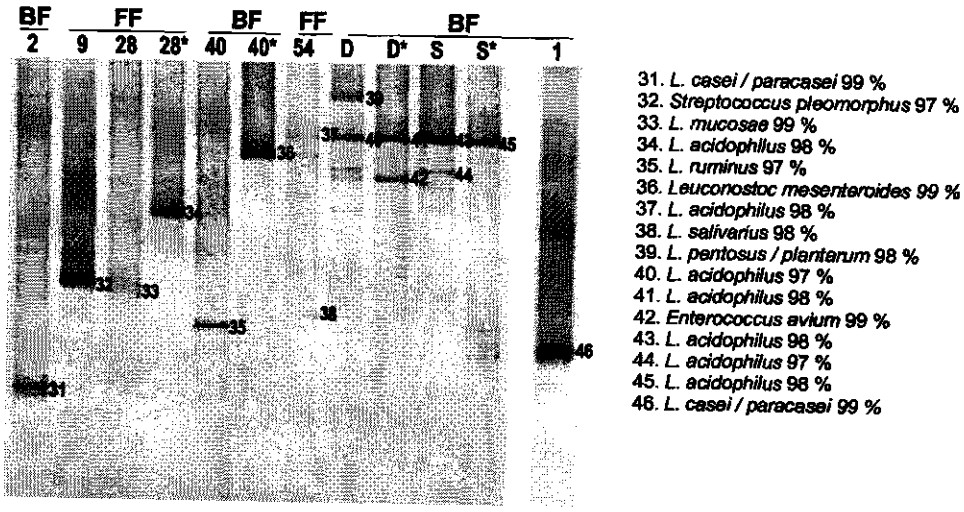

Figure 3. Lactobacilli DGGE profiles of feces and PCR fragments identified by cloning and sequencing from breast-fed (BF) and formula-fed (FF) infants at approximately one and seven months of age. Lanes are marked with the code of the infant (see also Table 1) and the approximate 7 month sample is indicated with an asterisk.

Discussion

In this study the diversity of dominant bacteria was monitored by amplifying 16S rDNA fragments from bacteria and subsequently separating the amplicons in a sequence-specific manner by DGGE. The abundance of fragments in DGGE profiles representing different 16S rDNA sequences reflects the bacterial species diversity. The diversity of fragments in DGGE profiles of 13 infants indicated that there was no significant difference in the complexity of the predominant bacterial community between breast-fed and formula-fed infants before or after weaning. Tannock (34) and Orrhage & Nord (24) compared the results from numerous original cultivation studies and concluded that differences in the infants' intestinal microbiota due to breast-feeding or formula-feeding are minor and apply mainly to clostridia that are found more frequently and in higher numbers in formula-fed infants. The results of our molecular study are in agreement with this observation. This deviation from the original idea that

bifidobacteria are found mainly in breast-fed infants may be due to numerous factors. Firstly, the composition of infant formulae has been improved over the years (15, 34), which is likely to have led to a more breast-fed-like colonisation pattern in formula-fed infants. Secondly, delivery with few exposures to microbes due to the use of vaginal antiseptics at the time of birth or caesarean section can lead to an altered intestinal colonisation in infancy (8, 9, 16). Finally, there are significant geographical differences in the composition of intestinal microbiota of infants (28, 31). It is noteworthy that a fragment in the DGGE profile may include different sequences with the same mobility and in some cases one species can produce more than one fragment in DGGE (26). Nevertheless, PCR-DGGE can give a reasonable indication on the bacterial diversity.

Ruminococcus gnavus-like sequences corresponding to an upper dominant band in the DGGE profiles were detected in several of the faecal samples and appears to be one of the major species at least in some infants. This fragment was previously cloned and sequenced also from infant D and was tentatively identified to have derived from *Ruminococcus gnavus* (96 % similarity) (7). The use of alternative universal 16S rRNA primers with modification of the denaturing gradient resulted in different migration for some of the upper bands (data not shown) suggesting that species other than ruminococci may be responsible for some of these dominant bands. Occasionally, fragments from different species migrate to the same position in DGGE (26), so identity confirmation by cloning and sequencing or hybridisation is necessary.

The changes in bacterial communities between one and seven months were comparable in both feeding groups. This disagrees with cultivation studies indicating that breast-fed infants have more intense shifts in their bacterial communities due to weaning (32). The intestinal microbiota in infants of both groups seem to undergo equally intense changes, at least at the level of bacterial species and strains. Interestingly, the bifidobacterial communities remained stable in many infants despite the weaning period. The *Bifidobacterium* species typically found in adults are different from those in infants (18). According to our results, the shifts in the species composition of the bifidobacterial populations do not occur directly in response to weaning, i.e., the species / strain

composition of the *Bifidobacterium* populations was not generally affected by the introduction of solid food to the infants diet.

The colonisation frequency of infants by bifidobacteria, as detected by genus-specific PCR, was comparable in both feeding groups or even higher in the formula-fed group. This result is in contrast with the classical idea that breast-fed infants are more frequently colonised by bifidobacteria. Some of the faecal samples in this study have also been subjected to quantitative 16S rDNA dot blot analysis with specific probes for different bacterial groups or genera (27). The dot blot analysis confirmed our finding that not all breast-fed infants were colonised by bifidobacteria. Our results are in line with several studies that show equal, either high (3, 12) or low (9, 16, 31), frequency of bifidobacterial colonisation in both groups. Previous observations that the bifidobacterial populations of infants generally consist of one to three species (4, 18, 19) are confirmed by our findings. The most commonly found *Bifidobacterium* species in infants vary from one study to another (2, 3, 4, 18, 19). This may reflect divergent distribution of *Bifidobacterium* species in various geographical regions or ethnic groups. It can not be excluded that variation in the predominant species between different studies may also be due to differences in the identification procedures (phenotypic or genotypic methods). However, in most studies there was no difference in the species distribution between breast-fed and formula-fed babies (3, 4, 18). In this study, *B. infantis* was the most frequently found *Bifidobacterium* species in both feeding groups. Concerning lactobacilli, our molecular results support the findings of previous culture-based studies that *L. acidophilus* (*sensu lato*) is the most common *Lactobacillus* species in infants irrespective of the type of feeding (3, 20).

In conclusion, PCR-DGGE is a useful method for comparison of the microbial diversity in individual samples and following shifts in populations in time. The possibilities for comprehensive analysis of intestinal microbiota can be further improved by developing of PCR-DGGE methods for other bacterial groups and by using the qualitative tools in combination with quantitative molecular tools such as FISH.

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References

1. **Balmer, S. E., and B. A. Wharton.** 1989. Diet and faecal flora in the newborn: breast milk and infant formula. Arch. Dis. Child. **64:**1672–1677.
2. **Beerens, H., C. Romond, and C. Neut.** 1980. Influence of breast-feeding on the bifid flora of the newborn intestine. Am. J. Clin. Nutr. **33:**2434–2439.
3. **Benno, Y., K. Sawada, and T. Mitsuoka.** 1984. The intestinal microflora of infants: composition of fecal flora in breast-fed and bottle-fed infants. Microbiol. Immunol. **28:**975–986.
4. **Biavati, B., P. Castagnoli, F. Crociani, and L. D. Trovatelli.** 1984. Species of the Bifidobacterium in the feces of infants. Microbiologica. **7:**341–345.
5. **Bullen, C. L., P. V. Tearle, and M. G. Stewart.** 1977. The effect of "humanised" milks and supplemented breast feeding on the faecal flora of infants. J. Med. Microbiol. **10:**403–413.
6. **Cooperstock, M. S., and A. J. Zedd.** 1983. Intestinal flora of infants, p. 79–99. *In* D. J. Hentges (ed.), Human intestinal microflora in health and disease. Academic Press, New York.
7. **Favier, C., E. E. Vaughan, W. M. de Vos, and A. D. L. Akkermans.** Molecular monitoring of succession of bacterial communities in human neonates. Submitted for publication.
8. **Grönlund, M.-M., O.-P. Lehtonen, E. Eerola, and P. Kero.** 1999. Fecal microflora in healthy infants born by different methods of delivery: permanent changes in intstinal flora after cesarean delivery. J. Pediatr. Gastroenterol. Nutr. **28:**19–25.

9. **Hall, M. A., C. B. Cole, S. L. Smith, R. Fuller, and C. J. Rolles.** 1990. Factors influencing the presence of faecal lactobacilli in early infancy. Arch. Dis. Child. **65:**185–188.

10. **Harmsen, H. J., A. C. Wildeboer Veloo, G. C. Raangs, A. A. Wagendorp, N. Klijn, J. G. Bindels, and G. W. Welling.** 2000. Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. J. Pediatr. Gastroenterol. Nutr. **30:**61–67.

11. **Heilig, G. H. J., E. G. Zoetendal, E. E. Vaughan, P. Marteau, A. D. L. Akkermans, and W. M. de Vos.** Molecular diversity of *Lactobacillus* spp., and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. Appl. Environ. Microbiol. In press.

12. **Kleessen, B., H. Bunke, K. Tovar, J. Noack, and G. Sawatzki.** 1995. Influence of two infant formulas and human milk on the development of the faecal flora in newborn infants. Acta Paediatr. **84:**1347–1356.

13. **Lane, D. J.** 1991. 16S / 23S rRNA sequencing, p. 115–175. *In* E. R. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley and Sons Ltd, Chichester, UK.

14. **Langendijk, P. S., F. Schut, G. J. Jansen, G. C. Raangs, G. R. Kamphuis, M. H. Wilkinson, and G. W. Welling.** 1995. Quantitative fluorescence in situ hybridization of *Bifidobacterium* spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. Appl. Environ. Microbiol. **61:**3069–3075.

15. **Lo, C. W., and R. E. Kleinman.** 1996. Infant formula, past and future: opportunities for improvement. Am. J. Clin. Nutr. **63:**646S–650S.

16. **Lundequist, B., C. E. Nord, and J. Winberg.** 1985. The composition of the faecal microflora in breastfed and bottle fed infants from birth to eight weeks. Acta Paediatr. Scand. **74:**45–51.

17. **Mackie, R. I., A. Sghir, and H. R. Gaskins.** 1999. Developmental microbial ecology of the neonatal gastrointestinal tract. Am. J. Clin. Nutr. **69:**1035S–1045S.

18. **Matsuki, T., K. Watanabe, R. Tanaka, M. Fukuda, and H. Oyaizu.** 1999. Distribution of bifidobacterial species in human intestinal microflora examined with 16S rRNA-gene-targeted species-specific primers. Appl. Environ. Microbiol. **65:**4506–4512.

19. **Mevissen-Verhage, E. A., J. H. Marcelis, M. N. de Vos, W. C. M. Harmsen-van Amerongen, and J. Verhoef.** 1987. *Bifidobacterium, Bacteroides*, and *Clostridium* spp. in fecal samples from breast-fed and bottle-fed infants with and without iron supplement. J. Clin. Microbiol. **25:**285–289.

20. **Morelli, L., C. Cesena, C. de Haën, and L. Gozzini.** 1998. Taxonomic *Lactobacillus* composition of feces from human newborns during the first few days. Microb. Ecol. **35:**205–212.

21. **Muyzer, G., E. C. de Waal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59:**695–700.

22. **Myers, R. M., T. Maniatis, and L. S. Lerman.** 1987. Detection and localization of single base changes by denaturing gradient gel electrophoresis. Meth. Enzymol. **155:**501–527.

23. **Ogawa, K., R. A. Ben, S. Pons, M. I. L. de Paolo, and L. B. Fernandez.** 1992. Volatile fatty acids, lactic acid, and pH in the stools of breast-fed and bottle-fed infants. J. Pediatr. Gastroenterol. Nutr. **15:**248–252.

24. **Orrhage, K., and C. E. Nord.** 1999. Factors controlling the bacterial colonization of the intestine in breastfed infants. Acta Paediatr. Suppl. **430:**47–57.

25. **Sanguinetti, C. J., E. Dias Neto, and A. J. Simpson.** 1994. Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. Biotechniques. **17:**914–921.

26. **Satokari, R. M., E. E. Vaughan, A. D. L. Akkermans, M. Saarela, and W. M. de Vos.** 2001. Bifidobacterial diversity in human feces detected by genus-specific PCR and denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. **67:**504–513.

27. **Savage, S. A. H., A. M. Parret, A. Fletcher, F. Martin, G. Gramet, J. Dore, and C. A. Edwards.** Faecal bacteria and short chain fatty acids in breast fed and formula fed infants in the first year of life - a longitudinal study. Manuscript in preparation.

28. **Sepp, E., K. Julge, M. Vasar, P. Naaber, B. Björksten, and M. Mikelsaar.** 1997. Intestinal microflora of Estonian and Swedish infants. Acta Paediatr. **86:**956–961.

29. **Sghir, A., G. Gramet, A. Suau, V. Rochet, P. Pochart, and J. Dore.** 2000. Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridization. Appl. Environ. Microbiol. **66:**2263–2266.

30. **Siigur, U., A. Ormisson, and A. Tamm.** 1993. Faecal short-chain fatty acids in breast-fed and bottle-fed infants. Acta Paediatr. **82:**536–538.

31. **Simhon, A., J. R. Douglas, B. S. Drasar, and J. F. Soothill.** 1982. Effect of feeding on infants' faecal flora. Arch. Dis. Childhood. **57:**54–58.

32. **Stark, P. L., and A. Lee.** 1982. The microbial ecology of the large bowel of breast-fed and formula-fed infants during the first year of life. J. Med. Microbiol. **15:**189–203.

33. **Suau, A., R. Bonnet, M. Sutren, J. J. Godon, G. R. Gibson, M. D. Collins, and J. Dore.** 1999. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl. Environ. Microbiol. **65:**4799–807.

34. **Tannock, G. W.** 1994. The acquisition of the normal microflora of the gastrointestinal tract, p. 1–16, Human health: the contribution of micro-organisms. Springer-Verlag, London.

35. **Vaughan, E. E., H. G. H. J. Heilig, E. G. Zoetendal, R. Satokari, J. K. Collins, A. D. L. Akkermans, and W. M. de Vos.** 1999. Molecular approaches to study probiotic bacteria. Trends Food Sci. Technol. **10:**400–404.

36. **Zoetendal, E. G., A. D. L. Akkermans, W. M. Akkermans-van Vliet, A. G. M. de Visser, and W. M. de Vos.** The host genotype affects the bacterial community in the human gastrointestinal tract. Microb. Ecol. Health Dis. 13: 129–134.

37. **Zoetendal, E. G., A. D. L. Akkermans, and W. M. De Vos.** 1998. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl. Environ. Microbiol. **64:**3854–3859.

Chapter 7

Summary and concluding remarks

Summary and concluding remarks

Bifidobacteria and lactobacilli are considered to be members of the beneficial microbiota in the human gastrointestinal (GI) -tract. Traditionally bifidobacteria and lactobacilli are detected in intestinal samples, most commonly faeces, by cultivation on selective nutrient media. Bifidobacteria are strictly anaerobic bacteria and anaerobic practise has to be followed for their cultivation, which hampers the sample collection and handling. In many cases phenotypic characterisation is not enough to identify *Bifidobacterium* and *Lactobacillus* strains at the species level. The studies described in this thesis involve the development and validation of new molecular methods for the detection and analysis of bifidobacteria and lactobacilli (**Chapters 2, 3** and **4**) and the application of new methodologies to trace ingested probiotic strains (**Chapters 4** and **5**). The indigenous *Bifidobacterium* and *Lactobacillus* populations in the human intestine were also investigated using these techniques (**Chapters 2, 5,** and **6**).

Methodological considerations

Molecular fingerprinting methods are highly discriminatory on the strain level, which allows a comprehensive diversity assessment of isolates recovered from cultivation plates. The main drawback of this approach to study the diversity is its labour-intensity. **Chapter 2** describes the development and validation of a method based on genus-specific PCR of 16S rDNA and denaturing gradient gel electrophoresis (DGGE) for profiling *Bifidobacterium* populations in human faeces. The PCR-DGGE method is a qualitative tool for assessing species and/or strain composition of complex communities by a single PCR reaction and subsequent resolution of the amplification products by DGGE in a sequence-dependent manner. The approach greatly facilitates the monitoring of faecal samples from large numbers of subjects to reveal bifidobacterial diversity and shifts occurring in it, either spontaneous or by introduction of an ingested probiotic strain to the ecosystem (**Chapters 2, 5 and 6**). The identification of

DGGE fragments can be done by subsequent cloning and sequencing of the PCR products, but ultimate species identification is hampered by the high similarity of 16S rDNA sequences between closely related *Bifidobacterium* species.

The PCR-DGGE method revealed intragenomic 16S rDNA sequence heterogeneity in *B. adolescentis* E-981074^T **(Chapter 2)**. The strain was found to harbour five copies of 16S rDNA, two of which were sequenced and found to differ in eight positions over almost the total length of the gene. The sequenced copies showed highest similarity (>99 %) to each other over all the sequences available in the public databases and it was concluded that the variability resulted from mutations in one copy of the gene. The intragenomic 16S rDNA heterogeneity in bacteria seems to be rather exceptional, but nevertheless it has an important impact on the phylogeny of organisms and molecular studies that apply these sequences such as biodiversity estimates. The genetic diversity of rRNA sequences in an organism can cause identification problems, especially if the sequences differ greatly as a result of horizontal gene transfer (31). Therefore, definite species identification still rely on polyphasic taxonomy, which integrates phenotypic, genotypic, and phylogenetic information (30).

The *L. casei* -group lactobacilli including *L. casei, L. paracasei, L. rhamnosus*, and *L. zeae* form an important group of intestinal lactobacilli and several strains of these species are used in probiotic preparations. The identification of *L. casei* -group lactobacilli is not reliable by phenotypic methods, which prompted the development of genotypic methods for their identification and characterisation **(Chapter 3).** Moreover, a *L. rhamnosus* species-specific PCR was developed and validated. Specific PCR primers are now available for all members of the *L. casei*-group and they provide a practical means, due to rapid and easy performance, for identifying species of this group.

Species-specific PCRs and three genetic fingerprinting methods, pulsed field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD) and ribotyping were used to identify and characterise 24 strains of the *L. casei* -group lactobacilli **(Chapter 3)**. The obtained results supported the proposed new classification of the *L. casei* -group, namely the reclassification *L. casei* type strain (ATCC 393) as *L. zeae* and the rejection of *L. paracasei* and its inclusion in *L. casei*. Furthermore, the discriminatory power of the three fingerprinting techniques was compared. All three techniques were highly

effective in differentiating strains below the species level and they can be placed in the following order with respect to their discriminatory power: PFGE > ribotyping > RAPD. This finding is in agreement with several other studies that have compared two or more molecular methods for typing of lactobacilli (4, 11, 18, 21, 23, 26, 27, 32). All aforementioned fingerprinting methods have higher discriminatory power than amplified ribosomal DNA restriction analysis (ARDRA).

Molecular typing methods are accurate tools for assessing the genetic diversity in a population, tracing probiotic strains, and they can also be applied for identification purposes. The choice of a typing method depends on its application. For example in tracing of a probiotic strain in the GI-tract good discrimination power and high throughput capacity are needed in order to screen a large number of isolates, whereas for species identification discrimination at the species level is adequate and the reproducibility of the technique should be emphasised. The identification of bacterial species by typing methods necessitates the construction of a fingerprint library of well-characterised and correctly identified strains and an appropriate image analysis system and software for the efficient comparison of fingerprints. The more discriminative the technique is, the greater the variety of fingerprints produced by strains of one species, and consequently larger numbers of strains are needed to construct a comprehensive identification library.

The molecular techniques available today provide a powerful toolkit for a comprehensive analysis of different microbial groups in the GI-tract. This is optimally achieved by combined use of different methods that complement each other. For example, cells labelled by fluorescent *in situ* hybridisation (FISH) can be enumerated using flow cytometry and simultaneously sorted and collected. Subsequent diversity screening is then possible by other methods such as PCR combined with DGGE or 16S rDNA sequence analysis. Further improvements in molecular monitoring of intestinal microbiota can be expected when new high-throughput techniques such as DNA-microarray technology will become applicable for GI-tract molecular ecology studies.

Survival of probiotic strains in the GI-tract

Newly developed molecular methods were used to trace ingested probiotic strains *L. rhamnosus* GG (LGG) and *B. lactis* Bb12 in the GI-tract. The identity of LGG colonies was verified using a species-specific PCR (**Chapter 4**) and Bb12 was detected using the PCR-DGGE method modified for this purpose (**Chapter 5**). Both probiotic strains colonised the gut transiently and they were no longer detected in the faeces one week after the end of the administration in most subjects. The synbiotic approach with galactooligosaccharide (GOS) did not prolong the persistence of Bb12. In the LGG trial biopsy samples were also examined. The study confirmed the *in vivo* attachment of LGG to human colonic mucosa (1), which correlates with the *in vitro* adherence of LGG to the Caco-2 intestinal cell line (8). Furthermore, LGG was found to persist in colonic mucosa for one to two weeks after the end of the administration, i.e. longer than it could be detected in the faeces suggesting that the study of faecal samples alone may underestimate the persistence of a probiotic strain.

Survival of the probiotic strain in the GI-tract is usually a prerequisite for its functionality. Adhesion to intestinal epithelium is considered important for its persistence and certain probiotic functions such as immune stimulation (7). The comparison of the *in vitro* and *in vivo* properties of probiotic strains is relevant in order to evaluate the present *in vitro* screening procedures and to improve them further for a more rational selection of new probiotic strains (7). The successful introduction of exogenous bifidobacteria and lactobacilli into the GI-tract and their adhesion to the intestinal epithelium opens new possibilities for their use as vehicles to deliver therapeutically active compounds such as antigenic epitopes (vaccines) or enzymes to defined locations in the GI-tract (28). Strains of lactic acid bacteria have already been genetically modified to express foreign antigens and successfully used to vaccinate mice (22). Likewise a genetically modified *Lactococcus lactis* strain has been used to deliver active interleukin-10 (IL-10) in the gut lumen of mice with inflammatory bowel disease (IBD). The therapeutic effect was comparable with the conventional treatment, but due to the local delivery lower amounts of IL-10 were required (29). In designing such treatments for humans and selecting strains for particular

applications prior knowledge about the preferential adhesion sites and the persistence of different strains in the gut may prove valuable.

The effect of diet on indigenous *Bifidobacterium* and *Lactobacillus* populations

In **Chapter 5** qualitative changes in adult faecal *Bifidobacterium* populations in response to *B. lactis* Bb12 and/or GOS administration were monitored using PCR-DGGE method. In most subjects two weeks administration of Bb12 and/or GOS (3×10^{10} cfu and 8 g per day, respectively) did not affect the qualitative composition of indigenous bifidobacterial populations, while Bb12 transiently colonised the gut. Previously, it was demonstrated that GOS administration (8 to 14 g per day for at least two weeks) had no effect on the faecal counts of bifidobacteria (2, 3). It can be hypothesised that in general, the composition of the intestinal microbiota in healthy adult subjects is affected more by the host-derived nutrients (mucin, dead epithelial cells) and other host factors (such as immune response) than the diet. Several observations support this possibility. Firstly, no major differences were found in the numbers of major microbial groups when people with different diets were compared by using cultivation (5, 9). Secondly, molecular community fingerprinting revealed that the predominant faecal microbiota was stable in two healthy adult individuals with usual unrestricted diet over a six to seven months study period (34). Furthermore, molecular studies showed that the host genotype or/and colonisation history has more impact on the composition of the dominant bacterial community than diet or environment (33). Thus, in healthy adults the fully developed complex and diverse microbiota is not prone to notable fluctuations, and in general, the potential of probiotics and prebiotics to modulate the intestinal microbiota of healthy adults seems fairly marginal. It still remains speculative, whether even minor changes would have significant effect on the host health and whether probiotics could play a role in the prophylaxis of various illnesses (25). In summary, healthy adults are a group of choice to study the tolerance to prebiotics and probiotics and the survival and colonisation of probiotics, but it is difficult to prove the efficacy of pre- and probiotics (prevention of disorders) in this target population.

The intestinal microbiota in infants, elderly and people with different GI-tract disorders such as IBD can be affected more readily in healthy adults. The administration of exogenous bifidobacteria resulted in an enormous increase of approximately four logarithmic units in the initially low faecal counts of bifidobacteria in patients with IBD, either ulcerative colitis or pouchitis (6). Many successful applications of probiotics as therapeutic agents to alleviate clinical symptoms of various disorders have been reported, but more clinical trials are still needed to confirm the efficacy (20, 24, 25). Another major task of probiotic research in future is to establish the mechanisms of action on how bifidobacteria and lactobacilli influence the human host.

Chapter 6 describes a qualitative molecular analysis of the bacterial, bifidobacterial and lactobacilli populations in faeces of breast-fed and formula-fed infants before and after weaning at approximately one and seven months of age. Genus and group-specific PCRs combined with DGGE and subsequent sequencing of the amplified 16S rDNA fragments revealed no difference in the prevalence or species distribution of *Bifidobacterium* and *Lactobacillus* between the two groups of infants. In general, DGGE patterns of 16S rDNA showed equal complexity of bacterial communities in breast-fed and formula-fed infants. Equally intensive changes occurred in the faecal microbiota in infants of both groups due to weaning. Human milk contains various bifidogenic factors (10) and bifidobacteria are thought to be the most characteristic microbial group in breast-fed infants. Our results showed that breast-feeding does not ensure, however, the development of bifidobacterial population, since bifidobacteria were not found in two of the seven breast-fed infants examined at the age of one month. On the other hand, current infant formulas (supplemented with bifidogenic factors) also support the growth of bifidobacteria. This and several other studies (13, 14, 19) emphasise the importance of other factors than the type of feeding in the microbial community development in infants. An overly hygienic life-style with few exposures to microbes or cesarean delivery can lead to an altered intestinal colonisation in infancy (13, 14, 19). The development of the microbial community likely proceeds through a complex regulatory network of host-microbe and microbe-microbe interactions dependent on intra- and interspecies communication systems in order to efficiently exploit and develop suitable nutrient sources (15). Studies with mice have shown that the host GI-

tract during its development influences the microbiota, and *vice versa,* the microbiota has an impact on host tissue development (16). Thus, the introduction of each microbe into the GI-tract or the absence of certain microbes may have consequences in the chain reaction of development of both the microbiota and the host. Indeed, the latest studies with human neonates suggest that the composition of the intestinal microbiota during infancy has a crucial role in the maturation of the immune system (12, 17). It is not well known how bifidobacterial and lactobacilli populations in particular influence the postnatal development, and whether healthy infants lacking bifidobacteria or lactobacilli would benefit from inoculation with probiotic preparations.

In conclusion, new molecular techniques provide efficient tools to assess the composition and fluctuations in bifidobacterial and lactobacilli communities in the GI-tract, which will facilitate a more comprehensive description of these bacterial groups in different human populations. This will give us a deeper understanding about the GI-tract ecology of bifidobacteria and lactobacilli, and consequently, about the possibilities to modulate the intestinal microbiota to the benefit of host health.

References

1. **Alander, M., R. Korpela, M. Saxelin, T. Vilpponen-Salmela, T. Mattila-Sandholm, and A. von Wright.** 1997. Recovery of *Lactobacillus rhamnosus* GG from human colonic biopsies. Lett. Appl. Microbiol. **24**:361–364.

2. **Alander, M., J. Mättö, W. Kneifel, M. Johansson, B. Kögler, R. Crittenden, T. Mattila-Sandholm, and M. Saarela.** 2001. Effect of galacto-oligosaccharide supplementation on human faecal microflora and on survival and persistence of *Bifidobacterium lactis* Bb-12 in the gastrointestinal tract. J. Dairy Sci. **11**:817–825.

3. **Alles, M. S., R. Hartemink, S. Meyboom, J. L. Harryvan, K. M. Van Laere, F. M. Nagengast, and J. G. Hautvast.** 1999. Effect of transgalactooligosaccharides on the composition of the human intestinal microflora and on putative risk markers for colon cancer. Am. J. Clin. Nutr. **69**:980–991.

4. **Björkroth, J., J. Ridell, and H. Korkeala.** 1996. Characterization of *Lactobacillus sake* strains associating with production of ropy slime by randomly amplified polymorphic DNA (RAPD) and pulsed-field gel electrophoresis (PFGE) patterns. Int. J. Food Microbiol. **31:**59–68.

5. **Bornside, G. H.** 1978. Stability of human fecal flora. Am. J. Clin. Nutr. **31:**S141–S144.

6. **Brigidi, P., B. Vitali, E. Swennen, L. Altomare, M. Rossi, and D. Matteuzzi.** 2000. Specific detection of *Bifidobacterium* strains in a pharmaceutical probiotic product and in human feces by polymerase chain reaction. System. Appl. Microbiol. **23:**391–399.

7. **Dunne, C., L. O'Mahony, L. Murphy, G. Thornton, D. Morrisey, S. O'Halloran, M. Feeney, S. Flynn, G. Fitzgerald, C. Daly, B. Kiely, G. C. O'Sullivan, F. Shanahan, and J. K. Collins.** 2001. *In vitro* selection criteria for probiotic bacteria of human origin: correlation with *in vivo* findings. Am. J. Clin. Nutr. **73:**386S–392S.

8. **Elo, S., M. Saxelin, and S. Salminen.** 1991. Attachment of *Lactobacillus casei* strain GG to human colon carcinoma cell line Caco-2: comparison with other dairy strains. Lett. Appl. Microbiol. **13:**154–156.

9. **Finegold, S. M., V. L. Sutter, and G. E. Mathisen.** 1983. Normal indigenous intestinal flora, p. 3–31. *In* D. J. Hentges (ed.), Human intestinal microflora in health and disease. Acamedic Press, Inc., London.

10. **Gil, A., and R. Rueda.** 2000. Modulation of intestinal microflora by specific dietary components. Microb. Ecol. Health Dis. **Suppl. 2:**31–39.

11. **Giraffa, G., P. De Vecchi, P. Rossi, G. Nicastro, and M. G. Fortina.** 1998. Genotypic heterogeneity among *Lactobacillus helveticus* strains isolated from natural cheese starters. J. Appl. Microbiol. **85:**411–416.

12. **Grönlund, M.-M., H. Arvolommi, P. Kero, O.-P. Lehtonen, and E. Isolauri.** 2000. Importance of intestinal colonization in the maturation of humoral immunity in early infancy: a prospective follow up study of healthy infants aged 0–6 months. Arch. Dis. Child. Fetal. Neonatal Ed. **83:**F186–F192.

13. **Grönlund, M.-M., O.-P. Lehtonen, E. Eerola, and P. Kero.** 1999. Fecal microflora in healthy infants born by different methods of delivery: permanent changes in intestinal flora after cesarean delivery. J. Pediatr. Gastroenterol. Nutr. **28:**19–25.

14. **Hall, M. A., C. B. Cole, S. L. Smith, R. Fuller, and C. J. Rolles.** 1990. Factors influencing the presence of faecal lactobacilli in early infancy. Arch. Dis. Childhood. **65:**185–188.

15. **Hooper, L. V., and J. I. Gordon.** 2001. Commensal host-bacterial relationships in the gut. Science **292:**1115–1118.

16. **Hooper, L. V., M. H. Wong, A. Thelin, L. Hansson, P. G. Falk, and J. I. Gordon.** 2001. Molecular analysis of commensal host-microbial relationships in the intestine. Science **291:**881–884.

17. **Kalliomäki, M., P. Kirjavainen, E. Eerola, P. Kero, S. Salminen, and E. Isolauri.** 2001. Distinct patterns of neonatal gut microflora in infants in whom atopy was and was not developing. J. Allergy Clin. Immunol. **107:**129–134.

18. **Kimura, K., A. L. Mc Cartney, M. A. McConnell, and G. W. Tannock.** 1997. Analysis of populations of bifidobacteria and lactobacilli and investigation of the immunological responses of their human hosts to the predominant strains. Appl. Environ. Microbiol. **63:**3394–3398.

19. **Lundequist, B., C. E. Nord, and J. Winberg.** 1985. The composition of the faecal microflora in breastfed and bottle fed infants from birth to eight weeks. Acta Paediatr. Scand. **74:**45–51.

20. **Marteau, P.** 2000. Role of the intestinal flora in gastrointestinal diseases. Lancet. **356:**S28.

21. **McCartney, A. L., W. Wang, and G. W. Tannock.** 1996. Molecular analysis of the composition of the bifidobacterial and lactobacillus microflora of humans. Appl. Environ. Microbiol. **62:**4608–4613.

22. **Mercenier, A.** 1999. Lactic acid bacteria as live vaccines, p. 113–127. *In* G. W. Tannock (ed.), Probiotics – A critical review. Horizon Scientific Press, Wymondham, UK.

23. **Moschetti, G., G. Blaiotta, M. Aponte, G. Mauriello, F. Villani, and S. Coppola.** 1997. Genotyping of *Lactobacillus delbrueckii* subsp. *bulgaricus* and determination of the number and forms of *rrn* operons in *L. delbrueckii* and its subspecies. Res. Microbiol. **148:**501–510.

24. **Pathmakanthan, S., S. Meance, and C. A. Edwards.** 2000. Prebiotics: A review of human studies to date and methodological approaches. Microb. Ecol. Health Dis. **Suppl. 2:**10–30.

25. **Roos, de. N. M., and M. B. Katan.** 2000. Effects of probiotic bacteria on diarrhea, lipid metabolism, and carcinogenesis: a review of papers published between 1988 and 1998. Am. J. Clin. Nutr. **71:**401–411.

26. **Roussel, Y., C. Colmin, J. M. Simonet, and B. Decaris.** 1993. Strain characterization, genome size and plasmid content in the *Lactobacillus acidophilus* group (Hansen and Mocquot). J. Appl. Bacteriol. **74:**549–556.

27. **Sanz, Y., M. Hernández, M. A. Ferrús, and J. Hernández.** 1998. Characterization of *Lactobacillus sake* isolates from dry-cured sausages by restriction fragment polymorphism analysis of the 16S rRNA gene. J. Appl. Microbiol. **84:**600–606.

28. **Shanahan, F.** 2001. Turbo probiotics for IBD. Gastroenterol. **120:**1297–1303.

29. **Steidler, L., W. Hans, L. Schotte, S. Neirynck, F. Obermeier, W. Falk, W. Fiers, and E. Remaut.** 2000. Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. Science **289:**1352–1355.

30. **Vandamme, P., B. Pot, M. Gillis, P. de Vos, K. Kersters, and J. Swings.** 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol. Rev. **60:**407–438.

31. **Yap, W. H., Z. Zhang, and Y. Wang.** 1999. Distinct types of rRNA operons exist in the genome of the actinomycete *Thermomonospora chromogena* and evidence for horizontal transfer of an entire rRNA operon. J. Bacteriol. **181:**5201–5209.

32. **Zapparolli, G., S. Torriani, and F. Dellaglio.** 1998. Differentiation of *Lactobacillus sanfranciscensis* strains by randomly amplified polymorphic DNA and pulsed-field gel electrophoresis. FEMS Microbiol. Lett. **166:**325–332.

33. **Zoetendal, E. G., A. D. L. Akkermans, W. M. Akkermans-van Vliet, A. G. M. de Visser, and W. M. de Vos.** 2001. The host genotype affects the bacterial community in the human gastrointestinal tract. Microb. Ecol. Health. Dis. **13:**129–134.

34. **Zoetendal, E. G., A. D. L. Akkermans, and W. M. De Vos.** 1998. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl. Environ. Microbiol. **64:**3854–3859.

Samenvatting

De humane darm herbergt een diverse gemeenschap van talrijke micro-organismen die algemeen wordt aangeduid als microbiota. Deze microbiota bestaat deels uit potentieel gevaarlijke en gunstige micro-organismen. Bifidobacteriën en lactobacillen zijn leden van de darmmicrobiota, waarvan voor de mens een gunstige werking wordt veronderstelt. Van beiden wordt veronderstelt dat ze een gezonde, gebalanceerde microbiële gemeenschap in de darm in stand houden en dat daarnaast de totale lichamelijke gezondheid van de gastheer wordt gestimuleerd. De mogelijkheid dat lactobacillen en bifidobacteriën gunstig zijn voor de mens heeft geleid tot pogingen hun aantallen in de darm te verhogen door het toedienen van levende bifidobacteriën of lactobacillen (probiotica) of stoffen welke de groei bevorderen (prebiotica). Prebiotica zijn meestal koolwaterstoffen zoals inuline, fructo- en galactooligosacchariden.

Van oudsher worden, door het gebruik van selectieve media, bifidobacteriën en lactobacillen gedetecteerd in darmmonsters, veelal feces. Bifidobacteriën zijn strikt anaëroob, waardoor het toepassen van anaërobe technieken noodzakelijk is voor cultivatie. Praktisch gezien bemoeilijkt dit het verzamelen en verwerken van dit soort monsters. De identificatie van *Bifidobacterium* en *Lactobacillus* stammen op soortniveau, is in hoge mate gebaseerd op fenotypische kenmerken zoals celmorfologie en suikerfermentatie eigenschappen. Vaak is dit echter niet voldoende voor een betrouwbare soortidentificatie. In de laatste jaren is een toenemend aantal moleculair-biologische technieken ontwikkeld en toegepast voor de identificatie en differentiatie van geïsoleerde *Bifidobacterium* en *Lactobacillus* stammen en de directe detectie, zonder cultivatie, van deze micro-organismen in fecale monsters.

De studies welke beschreven staan in dit proefschrift hebben betrekking op de ontwikkeling en validatie van nieuwe moleculaire methoden voor bifidobacteriën en lactobacillen (hoofdstuk 2, 3 en 4). Verder wordt beschreven hoe de nieuwe methoden is gebruikt om probiotische bacteriën te volgen na inname (hoofdstuk 4 en 5) en de studie van endemische *Bifidobacterium* en *Lactobacillus* populaties in de humane darm (hoofdstuk 2, 5 en 6).

De microbiële diversiteit in een monster wordt vaak vastgesteld door een moleculaire vingerafdruk (fingerprint) te maken van kolonies die op een vast voedingmedium groeien, waarbij de arbeidsintensiviteit het grootste nadeel is. In hoofdstuk 2 wordt de ontwikkeling en validatie beschreven van een methode, welke een combinatie is van een genus-specifieke PCR op het 16S ribosomaal RNA (rDNA) gen en denaturerende gradiënt gel electroforese (DGGE). De PCR-DGGE techniek is een kwalitatieve methode waarmee, met een enkele PCR reactie en de scheiding van de ontstane amplicons op basepaar-samenstelling door DGGE, de soort en/ of stamsamenstelling van een complexe gemeenschap vastgesteld kan worden. De methode werd gebruikt om de diversiteit binnen de groep van bifidobacteriën in humane feces vast te stellen. Deze benadering vereenvoudigt de bestudering van de bifidobacteriële diversiteit en eventuele verschuivingen, welke spontaan voorkomen dan wel geïnduceerd worden door de consumptie van probiotica (hoofdstuk 2, 5 en 6). Middels klonering en sequentie-analyse van DGGE fragmenten kunnen de ontstane DGGE patronen worden geïdentificeerd. Deze werkwijze wordt echter bemoeilijkt doordat binnen de groep van *Bifidobacterium* de sequentie-overeenkomsten in het 16S rDNA groot zijn.

De PCR-DGGE methode maakte duidelijk dat er intragenomische 16S rDNA sequentie-heterogeniteit bestaat in *B. adolescentis* E-981074T (hoofdstuk 2). Het bleek dat in deze stam 5 kopieën van het 16S rDNA op het genoom aanwezig zijn. Van twee kopieën werd de sequentie bepaald en daaruit volgde dat er op 8 posities verschillen bestonden, die verspreid zijn over het gen. De kopieën vertoonde meer gelijkenis (>99%) met elkaar dan met alle andere sequenties aanwezig in de publieke databanken, en er werd geconcludeerd dat de variatie zijn oorsprong moest hebben gevonden in mutaties binnen één kopie van het gen. De intragenomische 16S rDNA heterogeniteit lijkt uitzonderlijk te zijn, maar zal zeker zijn weerslag hebben op de fylogenie van micro-organismen en de moleculaire studies die deze sequenties gebruiken voor een schatting van microbiële diversiteit. De genetische diversiteit van 16S rRNA sequenties in een organisme kan problemen veroorzaken op het vlak van de identificatie, zeker wanneer sequenties grote verschillen vertonen door bijvoorbeeld horizontale genoverdracht. Daarom moet worden gestreefd naar een polyfasische taxonomy,

waarin fenotypische, genotypische en fylogenetische informatie wordt geïntegreerd.

Het *Lactobacillus* cluster van *L. casei* (*L. casei*, *L. paracasei*, *L. rhamnosus en L. zeae*) vormen een belangrijke groep van darmlactobacillen en wordt gebruikt in probiotische preparaten. De identificatie van de *L. casei*-groep met de traditionele fenotypische methoden is niet betrouwbaar. Dit initieerde de ontwikkeling van genotypische methoden voor de identificatie en karakterisering van deze groep lactobacillen (hoofdstuk 3). De ontwikkeling en validatie van een *L. rhamnosus* soort-specifieke PCR is beschreven in dit proefschrift. Specifieke PCR primers zijn nu beschikbaar voor alle leden van de *L. casei*-groep, en zijn door de snelle en praktische werkwijze eenvoudig toe te passen voor de identificatie van soorten binnen dit cluster van lactobacillen (hoofdstuk 3). De soortspecifieke PCR's en drie genetische fingerprint methoden, pulsed field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD) en ribotypering, werden gebruikt voor de identificatie en karakterisering van 24 stammen van de *L. casei*-groep lactobacillen. De verkregen resultaten onderbouwen de voorgestelde nieuwe classificatie van de *L. casei*-groep, namelijk de herklassificatie van *L. casei* stam (ATCC 393) als *L. zeae* en de verwerping van *L. paracasei* en het behoren tot *L. casei*. Verder werd het onderscheidende vermogen van de drie fingerprint methoden vergeleken. Gezamenlijk zijn de technieken zeer onderscheidend en in staat tot het differentieren op stamniveau. Op volgorde van onderscheidend vermogen werd tot de volgende conclusie gekomen: PFGE>ribotypering>RAPD, wat overeenkomt met een aantal eerdere studies waarin twee of meer moleculaire methoden werden vergeleken bij de typering van lactobacillen. Door het hoge discriminatoire vermogen op bacterieel stamniveau, zijn de beschreven moleculaire typeringstechnieken accurate methoden voor de bepaling van de genetische diversiteit in een populatie, het volgen van probiotische stammen en identificatie.

Nieuw ontwikkelde moleculaire methoden werden gebruikt om het lot van ingenomen probiotische stammen *L. rhamnosus* GG (LGG) en *B. lactis* Bb12 in de darm te volgen (hoofdstuk 4 en 5). Beide probiotische stammen lieten een tijdelijke kolonisatie van de darm zien, maar waren in de meeste proefpersonen 1 week nadat het toedienen was gestopt, niet meer te detecteren. De persistentie

van een probiotische stam in de darm werd niet bevorderd door het simultaan toedienen van galactooligosacchariden (GOS) en Bb12 (synbiotische benadering). In de proef met *L. rhamnosus* GG werden biopten bestudeerd naast de genomen fecale monsters. Dit bevestigt de *in vivo* aanhechting van *L. rhamnosus* GG aan mucosaecellen van de humane dikke darm en correleert met de hechting welke deze stam aan darmcellen vertoont onder laboratorium omstandigheden (*in vitro*). Daarnaast was het opmerkelijk dat *L. rhamnosus* GG 1 à 2 weken en zelfs langer na het stoppen van inname persisteerde in mucosae, terwijl de stam op de genoemde tijdstippen niet meer gedetecteerd werd in feces. Dit plaatst het onderzoek van alleen feces in een ander daglicht en kan leiden tot een onderschatting van de werkelijke persistentie van probiotische stammen. Het overleven van het maagdarmkanaal is een vooropgestelde eigenschap voor de functionaliteit van een probiotische stam. Adhesie aan het darmepithelium wordt als zeer belangrijk beschouwd voor de persistentie en bepaalde probiotische eigenschappen als immuunstimulatie. De vergelijking van *in vivo* en *in vitro* eigenschappen van een probiotische stam is relevant om de huidige *in vitro* onderzoekmethoden te evalueren en te verbeteren om tot een meer rationele selectie te komen van nieuwe probiotische stammen. De succesvolle introductie van exogene bifidobacteriën en lactobacillen in het maagdarmkanaal en hun adhesie aan het darmepithelium, opent nieuwe wegen voor het gebruik ervan als transportmiddel voor therapeutisch actieve stoffen, zoals antigene epitopen (vaccines) of enzymen naar specifieke locaties in het maagdarmkanaal.

In hoofdstuk 5 worden kwalitatieve veranderingen bestudeerd met PCR-DGGE. De feces van volwassenen werd onderzocht op *Bifidobacterium* populaties in reactie op het toedienen van *B. lactis* Bb12 en /of GOS toediening. In de meeste proefpersonen had het twee weken toedienen van Bb12 en /of GOS (respectievelijk, 3 x 10^{10} cfu en 8 g per dag) geen effect op de compositie van de endogene bifidobacteriële populatie, terwijl Bb12 de darm wel tijdelijk kon koloniseren. Eerder werd aangetoond dat GOS toediening (8 tot 14 g per dag voor tenminste twee weken), geen effect had op de fecale plaattellingen van bifidobacteriën. Hypothetiserend kan worden gesteld, dat de samenstelling van de darmmicrobiota in gezonde volwassen proefpersonen over het algemeen meer beïnvloed wordt door van de gastheer afkomstige nutriënten (mucine, dode epithelium cellen) en andere gastheerfactoren (zoals immuunrespons), dan door

dieet. Verschillende observaties onderbouwen deze hypothese. Ten eerste werden eerder geen grote verschillen gevonden in aantallen van de dominante microbiële populaties van bevolkingsgroepen met een verschillend dieet. Ten tweede blijkt de dominante fecale microbiota in gezonde volwassenen zeer stabiel over een periode van 6 tot 7 maanden, wanneer een normaal niet-restrictief dieet wordt gevolgd. Verder is aangetoond dat het genotype van de gastheer en /of de kolonisatie geschiedenis meer van invloed op de bacteriële gemeenschap is dan dieet of omgeving. Daarom is het in gezonde volwassenen met een volledig ontwikkelde en diverse microbiota zeer onwaarschijnlijk dat grote fluctuaties zullen optreden. Over het algemeen zal, de mogelijkheid dat pro- en prebiotica grote veranderingen in de darmmicrobiota veroorzaken, zeer marginaal zijn. Het blijft speculeren of zelfs kleine veranderingen een significant effect zouden hebben op de gezondheid van de gastheer en of probiotica een rol zouden kunnen spelen in het voorkomen van verschillende ziektes. Veel succesvolle proeven zijn uitgevoerd met probiotica welke symptomen verlichten van verscheidene aandoeningen. Er moeten echter meer klinische proeven worden opgezet welke de uitwerking moeten bevestigen. Voor de toekomst is een belangrijke taak voor het probioticumonderzoek weggelegd in het ophelderen van mechanismen waarmee bifidobacteriën en lactobacillen de humane gastheer beïnvloeden.

Hoofdstuk 6 beschrijft een kwalitatieve moleculaire analyse van de dominante bacteriële, en specifieke bifidobacteriële en lactobacillen populaties in feces van borst- en flesgevoede baby's. Als meetpunten werden ongeveer de eerste en zevende maand na geboorte gekozen, waarin de zevende maand de periode vormt waarin wordt overgegaan op vast voedsel. De genus/ groep-specifieke PCR gecombineerd met DGGE and het vervolgens sequentie-analyse van de 16S rDNA amplicons, liet geen verschillen tussen beide groepen baby's zien in de soortsamenstelling van *Bifidobacterium* en *Lactobacillus*. Over het algemeen lieten DGGE patronen van het 16S rDNA eenzelfde complexiteit van bacteriële gemeenschappen zien in borstgevoede en flesgevoede baby's. In de overgang van vloeibaar naar vast voedsel was eenzelfde verandering te zien. Humane moedermelk bevat bifidogene factoren en bifidobacteriën worden als de meest karakteristieke microbiële groep gezien in baby's die borstvoeding krijgen. Onze resultaten laten echter zien dat in twee van de zeven borstgevoede

baby's geen *Bifidobacterium* werd aangetoond, en dat het krijgen van moedermelk de ontwikkeling van een bifidobacteriële gemeenschap daarom niet kan verzekeren. Aan de andere kant kan flesvoeding (aangevuld met bifidogene factoren) de groei van bifidobacteriën ondersteunen. Deze en een aantal andere studies benadrukken de betekenis van andere factoren, dan het type voeding, in de ontwikkeling van de microbiële gemeenschap in babies. Als voorbeeld geldt de al te hygiënische leefstijl met maar weinig blootstelling aan microben of de geboorte via een keizersnede, welke een veranderde kolonisatie van de darm tot gevolg kan hebben. Er wordt gedacht dat de darmmicrobiota van invloed is op de ontwikkeling van de gastheer, met als voorbeeld de ontwikkeling van het immuunsysteem. Het is niet duidelijk hoe in het bijzonder bifidobacteriën en lactobacillen de postnatale ontwikkeling beïnvloeden, en of gezonde babies die geen bifidobacteriën en lactobacillen herbergen een voordeel zouden hebben wanneer ze worden geïnocculeerd met probiotische preparaten.

Concluderend kunnen we stellen dat de moleculaire en conventionele technieken die vandaag de dag beschikbaar zijn, een krachtig methodisch gereedschap vormen in de vergelijkende analyse van verschillende microbiële groepen in de darm van uiteenlopende bevolkingsgroepen. Momenteel wordt daar optimaal gebruik van gemaakt door een combinatie van verschillende methoden die elkaar goed aanvullen. Verdere verbeteringen in de moleculaire analyse van de microbiota, zullen tot stand komen wanneer nieuwe *high-troughput* methoden zoals de DNA-microarray techniek aangepast worden voor de bestudering van de microbiële diversiteit in de darm. Hierdoor zal men uiteindelijk tot een beter begrip komen van de darmecologie van *Bifidobacterium* en *Lactobacillus* en de mogelijkheid de darmmicrobiota te veranderen.

Vertaald in het Nederlands door Hans Heilig.

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Omnia vincit Amor: et nos cedamus Amori, Tawfiq.

Helsinki, 11 November 2001

Curriculum vitae

The author of this thesis was born 1st January 1970 in Raisio, Finland. She graduated from the high school of Raisio in 1989, after which she moved to Moscow, where she first studied Russian for one year and then biology for three years in the Moscow State University. In 1993 she returned back to Finland to continue her biology studies in Helsinki University and graduated as MSc in microbiology in 1996. After the graduation she received a position as a researcher in VTT Biotechnology, Finland, where she worked on the development of molecular detection methods for beer spoilage bacteria and probiotic lactobacilli. She compiled her research work with further studying and obtained the degree of Licentiate of Philosophy from Helsinki University in 1999 with the thesis 'Nucleic acid based methods for the detection and characterisation of beer spoilage bacteria'. From June 1999 to July 2001 she worked in the Laboratory of Microbiology of Wageningen University studying human intestinal bifidobacteria and lactobacilli by using molecular techniques. The results of her research on intestinal bifidobacteria and lactobacilli carried out both in Wageningen University and VTT Biotechnology are compiled in this thesis.

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List of publications

Satokari, R. M., Vaughan, E. E., Doré, J., and de Vos, W. M. Diversity of *Bifidobacterium* and *Lactobacillus* spp. in breast-fed and formula-fed infants as assessed by 16S rDNA sequence differences. Submitted for publication.

Satokari, R. M., Vaughan, E. E., Akkermans, A. D. L., Saarela, M., and de Vos, W. M. 2001. Polymerase chain reaction and denaturing gradient gel electrophoresis monitoring of fecal *Bifidobacterium* populations in a prebiotic and probiotic feeding trial. System. Appl. Microbiol. 24:227–231.

Satokari, R. M., Vaughan, E. E., Akkermans, A. D. L., Saarela, M., and de Vos, W. M. 2001. Bifidobacterial diversity in human feces detected by genus-specific PCR and denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. 67:504–513.

Satokari, R., Mattila-Sandholm, T., and Suihko, M.-L. 2000. Identification of pediococci by ribotyping. J. Appl. Microbiol. 88:260–265.

Tynkkynen, S., Satokari, R., Saarela, M., Mattila-Sandholm, T., and Saxelin, M. 1999. Comparison of ribotyping, randomly amplified polymorphic DNA analysis, and pulsed-field gel electrophoresis in typing of *Lactobacillus rhamnosus* and *L. casei* strains. Appl. Environ. Microbiol. 65:3908–3914.

Alander, M., Satokari, R., Korpela, R., Saxelin, M., Vilpponen-Salmela, T., Mattila-Sandholm, T., and von Wright, A. Persistence of colonization of human colonic mucosa by a probiotic strain *Lactobacillus rhamnosus* GG after oral consumption. 1999. Appl. Environ. Microbiol. 65:351–354.

Vaughan, E. E., Heilig, H. G. H. J., Zoetendal, E. G., Satokari, R., Collins, J. K., Akkermans, A. D. L., de Vos, W. M. Molecular approaches to study probiotic bacteria. 1999. Trends Food Sci. Technol.10:400–404

Juvonen, R., Satokari, R., Mallison, K., and Haikara, A. 1999. Detection of spoilage bacteria in beer by PCR. J. Am. Soc. Brew. Chem 57:99–103.

Satokari, R., Juvonen, R., Mallison, K., von Wright, A., and Haikara, A. 1998. Detection of beer spoilage bacteria *Megasphaera* and *Pectinatus* by PCR and colorimetric microplate hybridization. Int. J. Food Microbiol. 45:119–127.

Satokari, R., Juvonen, R., von Wright, A., and Haikara, A. 1997. Detection of *Pectinatus* beer spoilage bacteria by using the polymerase chain reaction. J. Food Protection 60:1571–1573.

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