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Quantitative Real-Time PCR Analysis of Fecal *Lactobacillus* Species in Infants Receiving a Prebiotic Infant Formula

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The developing intestinal microbiota of breast-fed infants is considered to play an important role in the priming of the infants' mucosal and systemic immunity. Generally, *Bifidobacterium* and *Lactobacillus* predominate the microbiota of breast-fed infants. In intervention trials it has been shown that lactobacilli can exert beneficial effects on, for example, diarrhea and atopy. However, the *Lactobacillus* species distribution in breast-fed or formula-fed infants has not yet been determined in great detail. For accurate enumeration of different lactobacilli, duplex 5′ nuclease assays, targeted on rRNA intergenic spacer regions, were developed for *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus delbrueckii*, *Lactobacillus fermentum*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, and *Lactobacillus rhamnosus*. The designed and validated assays were used to determine the amounts of different *Lactobacillus* species in fecal samples of infants receiving a standard formula (SF) or a standard formula supplemented with galacto- and fructo-oligosaccharides in a 9:1 ratio (OSF). A breast-fed group (BF) was studied in parallel as a reference. During the 6-week intervention period a significant increase was shown in total percentage of fecal lactobacilli in the BF group (0.8% ± 0.3% versus 4.1% ± 1.5%) and the OSF group (0.8% ± 0.3% versus 4.4% ± 1.4%). The *Lactobacillus* species distribution in the OSF group was comparable to breast-fed infants, with relatively high levels of *L. acidophilus*, *L. paracasei*, and *L. casei*. The SF-fed infants, on the other hand, contained more *L. delbrueckii* and less *L. paracasei* compared to breast-fed infants and OSF-fed infants. An infant milk formula containing a specific mixture of prebiotics is able to induce a microbiota that closely resembles the microbiota of BF infants.

The intestinal microbiota composition is regarded as an important factor for infant health and well-being (15, 32). A lower incidence of gastrointestinal and other infections has been found in breast-fed infants (43), which partly may be related to their microbiota composition. The intestinal microbiota of breast-fed infants is generally dominated by the genera *Bifidobacterium* and *Lactobacillus* (35), which are able to inhibit the growth of pathogens by lowering the pH, due to the production of lactic and acetic acid (1), or by competing for nutrients and epithelial adhesion sites (2). In contrast to breast-fed infants, formula-fed infants possess a more diverse microbiota which is mainly composed of *Bacteroides*, *Bifidobacterium*, *Staphylococcus*, *Escherichia coli*, and *Clostridium* spp. (19).

Several concepts are being used to modify the intestinal microbiota, such as nutritional changes or the consumption of pro- and/or prebiotics (10). Prebiotics are defined as nondigestible food ingredients that selectively stimulate the growth and/or activity of one or more bacteria in the colon and thereby beneficially affect the host (14). For infant formulas, a specific prebiotic mixture of galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) has been described that can stimulate the growth of bifidobacteria and lactobacilli similar to milk oligosaccharides in human breast milk (6, 8, 42). Several reports showed that the supplementation of infant formulas with this specific mixture of GOS and FOS increases the numbers of *Bifidobacterium* (7, 21, 36) and the total numbers of *Lactobacillus* (28), reduces the numbers of pathogens (20), and induces a short-chain fatty acid profile similar to that found in breast-fed infants (4, 21). Addition of the specific prebiotic mixture of GOS and FOS also results in a distribution of the different *Bifidobacterium* species similar to that found in breast-fed infants (16).

Although the supplementation of specific *Lactobacillus* strains, such as *Lactobacillus rhamnosus*, *Lactobacillus reuteri*, *Lactobacillus acidophilus*, and *Lactobacillus fermentum*, to infant formulas has been reported (2), the distribution of the different *Lactobacillus* species in breast-fed or formula-fed infants has not been studied in detail. To determine the composition of the different *Lactobacillus* species in breast-fed and formula-fed infants and to study the effects of nutritional interventions, it is relevant to quantitatively determine lactobacilli at the species level. For this purpose, species-specific duplex 5′ nuclease assays (quantitative real-time PCR) were developed for *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus delbrueckii*, *Lactobacillus fermentum*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, and *Lactobacillus rhamnosus*. With these assays the different *Lactobacillus* species were quantified in breast-fed infants (BF) and infants receiving a standard formula (SF) or a standard formula supplemented with the specific prebiotic GOS-FOS mixture (OSF).

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MATERIALS AND METHODS

Study design and sample collection. Fecal samples were collected from an intervention trial with exclusively formula-fed infants, aged 28 to 90 days, receiving a standard formula (SF group; age, 60.3 ± 6.9 days [mean ± the standard

TABLE 1. Bacterial strains used in this study

Lactobacillus strains	Origin[a]	Other strains	Origin[a]
Lactobacillus acidophilus	ATCC 4356	*Bacillus cereus*	ATCC 11778
	ATCC 521	*Bacteroides fragilis*	LMG 10263^T
	DSM 20079^T	*Brevibacterium casei*	ATCC 35513^T
Lactobacillus amylovorus	DSM 20552	*Clostridium difficile*	ATCC 9689^T
Lactobacillus bavaricus	JCM 1128	*Enterococcus faecalis*	DSM 20478^T
Lactobacillus brevis	LMG 18022	*Escherichia coli*	ATCC 35218
Lactobacillus bulgaricus	ATCC 11842^T	*Listeria monocytogenes*	ATCC 7644
Lactobacillus casei	ATCC 393^T	*Pediococcus acidilactici*	DSM 20284^T
	DSM 20011^T	*Proplonibacterium avidum*	DSM 4901
Lactobacillus crispatus	ATCC 33820^T	*Pseudomonas aeruginosa*	DSM 1117
Lactobacillus curvatus	ATCC 51436	*Saccharomyces cerevisiae*	DSM 2548
Lactobacillus delbrueckii	JCM 1106	*Salmonella enterica* serovar Typhimurium	ATCC 14028
	JCM 1248^T	*Staphylococcus aureus*	ATCC 29213
Lactobacillus fermentum	DSM 20052^T	*Bifidobacterium adolescentis*	ATCC 15703^T
Lactobacillus gasseri	LMG 11496^T	*Bifidobacterium angulatum*	DSM 20098^T
Lactobacillus helveticus	CNRZ 3	*Bifidobacterium bifidum*	DSM 20456^T
Lactobacillus johnsonii	ATCC 33200^T	*Bifidobacterium animalis*	ATCC 25527^T
Lactobacillus kefir	LMG 11496	*Bifidobacterium gallicum*	DSM 20093^T
Lactobacillus kefirgranum	DSM 10550^T	*Bifidobacterium dentium*	ATCC 27534^T
Lactobacillus paracasei	ATCC 11582	*Bifidobacterium breve*	ATCC 15700^T
	ATCC 27216	*Bifidobacterium catenulatum*	ATCC 27539^T
Lactobacillus pentosus	JCM 8334	*Bifidobacterium infantis*	LMG 8811^T
	JCM8338	*Bifidobacterium longum*	ATCC 15707^T
Lactobacillus plantarum	DSM 20174^T		
	NCIMB 8826		
Lactobacillus reuteri	LMG 9213^T		
Lactobacillus rhamnosus	ATCC 53103		
	ATCC 7469^T		
Lactobacillus sake	DSM 6333		
Lactobacillus salivarius	ATCC 11741^T		

[a] ATCC, American Type Culture Collection, United States; CNRZ, Centre National de Recherches Zootechniques, France; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany; JCM, Japan Collection of Microorganisms, Japan; LMG, Laboratory for Microbiology, University of Gent, Belgium; NCIMB, National Collections of Industrial and Marine Bacteria, United Kingdom.

error of the mean], ranging from 29.0 to 85.0 days) or a prebiotic formula containing 0.8 g of GOS-FOS/100 ml in a 9 to 1 ratio (OSF group; age, 51.9 ± 7.2 days, ranging from 30.0 to 86.0 days). A group of exclusively breast-fed infants was studied in parallel and used as a reference (BF group; age: 56.7 ± 7.4 days, ranging from 27.0 to 88.0 days). For study details, see also references 16 and 21.

Bacterial strains and culture conditions. All bacterial strains used in the present study are listed in Table 1. All *Bifidobacterium*, *Lactobacillus*, *Propionibacterium*, *Saccharomyces*, *Enterococcus*, and *Pediococcus* strains were cultured in Mann Rogosa Sharp broth (Oxoid, Basingstoke, United Kingdom) at 37°C under anaerobic conditions.

Gut commensals and pathogens, such as *Bacteroides fragilis* and *Pseudomonas aeruginosa*, were cultured in brain heart infusion broth (Oxoid, Basingstoke, United Kingdom) at 37°C, and *Bacillus cereus*, *Brevibacterium casei*, and *Listeria monocytogenes* were cultured at 30°C. Overnight cultures were stored at −20°C until further processing.

Qualitative PCR analysis. For the species-specific qualitative PCR, DNA was isolated as described previously (16) earlier. PCRs were carried out as described previously (37, 40, 41) by using a PTC-200 Peltier Thermal Cycler (Biozym, Landgraaf, The Netherlands). Amplification products were checked by agarose gel electrophoresis and ethidium bromide staining.

Species-specific quantitative real-time PCR. For the selection of primer and probe sequences, the 16S-23S intergenic spacer regions of the different *Lactobacillus* species were retrieved from the GenBank, EMBL, and DDBJ databases as follows: *L. acidophilus* (AB102855 [25], AF182726 [37], and U32971 [39]), *L. alimentarius* (AF500493 [33] and AF500492 [33]), *L. amylovorus* (AF182732 [37]), *L. animalis* (AY526616 and AY526614), *L. brevis* (AB102858 [25] and AF405353 [11]), *L. bulgaricus* (Z75475), *L. casei* (AB102854 [25], AF405352 [11], AF182729 [37], and AF121200 [38]), *L. collinoides* (AB117957 and AB117955), *L. crispatus* (AF182719 [37] and AF074857 [38]), *L. curvatus* (AF074858 [38], U97135 [5], and U97129 [5]), *L. delbrueckii* (]AB102856 [25], AB035485 [37], AB035484 [37], U32969 [39], U32968 [39], and U32967 [39]), *L. farciminis* (AF500491 [33] and AF500490 [33]), *L. fermentum* (AF182720 [37]), *L. frumenti* (AJ616011), *L. gasseri* (AB102860 [25], AF182721 [37], and AF074859 [38]), *L. graminis* (U97136 [5] and U97130 [5]), *L. hamsteri* (AF113601), *L. helveticus* (AF182728 [37]), *L. jensenii* (AB035486 [37] and U32970 [39]), *L. johnsonii* (AF074860 [38]), *L. mindensis* (AJ616016), *L. panis* (AJ616012), *L. paracasei* (AB035487 [37], AF182724 [37], and U32964 [39]), *L. paralimentarius* (AJ616014), *L. paraplantarum* (U97138 [5] and U97132 [5]), *L. pentosus* (U97141 [5], U97140 [5], and U97134 [5]), *L. plantarum* (AB102857 [25], AF405354 [11], AF182722 [37], U97139 [5], and U97133 [5]), *L. sakei* (U97137 [5] and U97131 [5]), *L. salivarius* (AB102859 [25], AB03488 [37], and AF182725 [37]), *L. sharpeae* (AF074861 [38]), *L. reuteri* (AF182723 [37]), *L. rhamnosus* (AF182730 [37], AF121201 [38], and U32966 [39]), *L. ruminis* (AF080103), *L. vaginalis* (AF182731), and *L. zeae* (AF074862). Sequences were aligned and the conserved regions were determined by using DNASIS for Windows V2.5 (Hitachi Software Engineering Co., Ltd., Wembley, United Kingdom). Using Primer Express 1.5a (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands), specific sequences were identified to design primers and probes for, respectively, all lactobacilli and the species: *L. acidophilus*, *L. casei*, *L. delbrueckii*, *L. fermentum*, *L. paracasei*, *L. plantarum*, *L. rhamnosus*, and *L. reuteri*. All primers and probes were tested for specificity using the basic local alignment search tool (BLAST) (3) and fulfilled the criteria described previously (16).

The probe for the detection of the genus *Lactobacillus* is labeled with the 5′ reporter dye VIC and the 3′ quencher NFQ-MGB (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The different lactobacilli species probes are labeled with the 5′ reporter dye 6-carboxy-fluorescein (FAM) and the 3′ quencher NFQ-MGB (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). To even further increase specificity and sensitivity, TaqMan minor groove binding probes were used (22).

For determination of the total bacterial load, an already-described probe and primer set was used (30). This universal oligonucleotide probe is labeled with the 5′ reporter dye FAM and the 3′ quencher dye 6-carboxy-tetramethyl-rhodamine (TAMRA).

The 5′ nuclease assays were performed as described earlier (16). Sequences of primers and probes are listed in Table 2. The optimized concentrations of the primers and probes are presented in Table 3.

TABLE 2. Primers and probes used in the duplex 5′ nuclease assays

Target	Primers and probes	Sequence (5′→3′)	T_m (°C)	% GC	BLAST ID number	Amplicon length (bp)
L. acidophilus	F_acid_IS	GAA AGA GCC CAA ACC AAG TGA TT	59	43	1089017502-26171-202965955840	85
	R_acid_IS	CTT CCC AGA TAA TTC AAC TAT CGC TTA	59	37	1089017571-27139-52545094772	
	P_acid_IS	TAC CAC TTT GCA GTC CTA CA	70	45	1089017717-29310-154296055415	
L. casei	F_case_IS	CTA TAA GTA AGC TTT GAT CCG GAG ATT T	59	36	1037022798-023495-2136	132
	R_case_IS	CTT CCT GCG GGT ACT GAG ATG T	59	55	1037022917-024843-29627	
	P_case_IS	ACA AGC TAT GAA TTC ACT TGC	70	38	1037022752-023005-20772	
L. delbrueckii	F_delb_IS	CAC TTG TAC GTT GAA AAC TGA ATA TCT TAA[a]	58	30	1089018504-4206-64529811906	94
	R_delb_IS	CGA ACT CTC TCG GTC GCT TT	58	55	1089018475-6841-166657768151	
	P_delb_IS	CCG AGA ATC ATT GAG ATC	68	44	1089018437-6309-163988227498	
L. fermentum	F_ferm_IS	AAC CGA GAA CAC CGC GTT AT	58	50	1036676682-09669-23287	88
	R_ferm_IS	ACT TAA CCT TAC TGA TCG TAG ATC AGT CA	58	38	1036676709-010209-2351	
	P_ferm_IS	TAA TCG CAT ACT CAA CTA A	68	32	1036676736-010547-20717	
L. paracasei	F_paca_IS	ACA TCA GTG TAT TGC TTG TCA GTG AAT AC	60	38	1038306417-016220-23561	80
	R_paca_IS	CCT GCG GGT ACT GAG ATG TTT C	60	55	1038306445-016796-3050	
	P_paca_IS	TGC CGC CGG CCA G	70	85	1038306524-018375-2626	
L. plantarum	F_plan_IS	TGG ATC ACC TCC TTT CTA AGG AAT	58	42	1038305707-03107-18756	144
	R_plan_IS	TGT TCT CGG TTT CAT TAT GAA AAA ATA[a]	58	26	1038305742-04177-12861	
	P_plan_IS	ACA TTC TTC GAA ACT TTG T	68	32	1038305778-04682-12880	
L. reuteri	F_reut_IS	ACC GAG AAC ACC GCG TTA TTT	59	48	1089025339-29395-129280047216	93
	R_reut_IS	CAT AAC TTA ACC TAA ACA ATC AAA GAT TGT CT	59	28	1089025385-30347-37558232754	
	P_reut_IS	ATC GCT AAC TCA ATT AAT	69	28	1089025413-30287-26112845854	
L. rhamnosus	F_rham_IS	CGG CTG GAT CAC CTC CTT T	59	58	1023708254-09591-2284	97
	R_rham_IS	GCT TGA GGG TAA TCC CCT CAA	59	52	1023708352-010389-16127	
	P_rham_IS	CCT GCA CAC ACG AAA	69	55	1023708453-011313-6655	
Lactobacillus spp.	F_alllact_IS	TGG ATG CCT TGG CAC TAG GA	58	55	1024485925-024664-30598	92
	R_alllact_IS	AAA TCT CCG GAT CAA AGC TTA CTT AT	58	35	1024478788-024701-16287	
	P_alllact_IS	TAT TAG TTC CGT CCT TCA TC	68	40	1024478009-017753-28422	
All bacteria	F_eub	TCC TAC GGG AGG CAG CAG T	59		–[b]	466[a]
	R_eub	GGA CTA CCA GGG TAT CTA ATC CTG TT	58			
	P_eub	CGT ATT ACC GCG GCT GCT GGC AC	70			

[a] Concessions to the probe and primer design had to be made in these cases (more than three consecutive nucleotides are the same or an amplicon length greater than 150 bp).
[b] Nadkami et al.

The relative amounts of the different *Lactobacillus* species in fecal samples were calculated after correction for differences in the amplification efficiencies of the duplex PCR as described previously (16, 24). The total counts of bacteria (cells per gram of feces) were determined by automated counting of microscopic images of fluorescently labeled cells. These counts, in combination with the percentages as determined with the duplex 5′ nuclease assays, were subsequently used to determine the numbers of lactobacilli per gram (wet weight) of feces (16).

The sensitivity of these duplex 5′ nuclease assays was compared to "conventional" PCR by testing dilution series of specific monocultures with both techniques. To determine the detection limit of the assay in CFU per milliliter, monocultures were also plated on Mann Rogosa Sharp agar and incubated under anaerobic conditions for 24 h at 37°C. The specificity of the assays was tested with the bacterial strains listed in Table 1.

The coefficients of variation (CV) within each duplex 5′ nuclease assay were determined by testing DNA isolated from feces spiked with a monoculture. This was performed 10 times for determination of the reproducibility and three times in quadruplicate for repeatability.

Data analyses. For statistical analysis, the software package SPSS for Windows (version 12.0.1; SPSS, Inc., Chicago, Ill.) was used. All values were checked for normality by visual inspection of the normal probability plots. Differences were tested with paired sample *t* tests, and if P was <0.05 the difference was considered statistically significant. Although the breast-fed group is compared to the formula groups, it has to be kept in mind that no complete randomization was obtained because it is not possible to double blindly assign infants to a breast-fed group.

TABLE 3. Optimized primer and probe concentrations for the duplex 5′ nuclease assays

Target	5′ Nuclease assay	Concn (nM)		
		Forward primer	Reverse primer	Probe
L. acidophilus	*L. acidophilus*	900	900	200
	All lactobacilli	900	900	200
L. casei	*L. casei*	900	900	200
	All lactobacilli	300	300	50
L. delbrueckii	*L. delbrueckii*	300	300	100
	All lactobacilli	900	900	200
L. fermentum	*L. fermentum*	300	300	100
	All lactobacilli	300	300	100
L. paracasei	*L. paracasei*	300	300	100
	All lactobacilli	300	300	100
L. plantarum	*L. plantarum*	300	300	100
	All lactobacilli	300	300	100
L. reuteri	*L. reuteri*	300	300	100
	All lactobacilli	900	900	200
L. rhamnosus	*L. rhamnosus*	900	450	200
	All lactobacilli	150	100	100
Genus *Lactobacillus*	All lactobacilli	600	600	100
	All bacteria	300	300	100

TABLE 4. Detection limits and CV for reproducibility and repeatability of the duplex 5′ nuclease assays

Target organism	Detection limit (CFU/ml)	CV	
		Reproducibility	Repeatability
L. acidophilus	0.75	0.11	0.13
L. casei	1.00	0.058	0.063
L. delbrueckii	1.15	0.059	0.035
L. fermentum	1.25	0.067	0.048
L. paracasei	1.25	0.052	0.11
L. plantarum	1.10	0.13	0.14
L. reuteri	1.00	0.062	0.11
L. rhamnosus	0.75	0.047	0.053

RESULTS

Species-specific quantitative real-time PCR. The 5′ nuclease assay for detection of the genus *Lactobacillus* detected all *Lactobacillus* species tested, but no other closely related genera such as *Enterococcus* or *Propionibacterium*. The duplex 5′ nuclease assays for the detection of the different *Lactobacillus* species were specific as tested with the other (lactobacilli) strains.

Overall, the 5′nuclease assays were more sensitive than the conventional PCR assays (1,000- to 10,000-fold) and, by comparing conventional plating techniques with the duplex 5′ nuclease assays, the detection limits of the nuclease assays were found to range from 0.75 to 1.25 CFU/ml (Table 4). RNase-free and RNase-treated samples showed identical results demonstrating that contaminating RNA does not disturb the assays.

L. acidophilus as a percentage of the total bacterial load was determined directly, but also by combining the data for *L. acidophilus* as a percentage of the lactobacilli with the *Lactobacillus* data indicated as a percentage of the total bacterial load. There were no statistically significant differences between results obtained with the two methods (Fig. 1).

The CV values for reproducibility and repeatability of the different assays ranged between 0.04 and 0.14 (Table 4).

Lactobacilli in fecal samples from the intervention study. The levels of the different *Lactobacillus* species in fecal samples of breast-fed infants and infants receiving a standard formula or a standard formula supplemented with GOS/FOS were determined with the duplex 5′ nuclease assays. The number of lactobacilli as a percentage of the total bacteria is shown in Fig. 2. At the start of the study the percentages of lactobacilli in the OSF and SF group were not statistically different (0.8% ± 0.3% and 0.5% ± 0.3%, respectively). After 6 weeks of intervention, at the end of the study period, the percentage of lactobacilli in the OSF group (4.4% ± 1.4%) was significantly higher ($P = 0.019$) than in the SF group (0.4% ± 0.2%). Furthermore, there was a statistically significant increase in the percentages lactobacilli during the study period in the OSF group (0.8% ± 0.3% at the start versus 4.4% ± 1.4% at the end [$P = 0.026$]) and the BF group (0.8% ± 0.3% at start versus 4.1% ± 1.5% at the end [$P = 0.034$]).

At the end of the study, breast-fed infants showed $3.0 \pm 1.2 \times 10^8$ lactobacilli per g (wet weight) of feces, OSF-fed infants showed $3.3 \pm 1.0 \times 10^8$ lactobacilli per g (wet weight) of feces, and SF-fed infants showed $5.4 \pm 3.1 \times 10^7$ lactobacilli per g (wet weight) of feces.

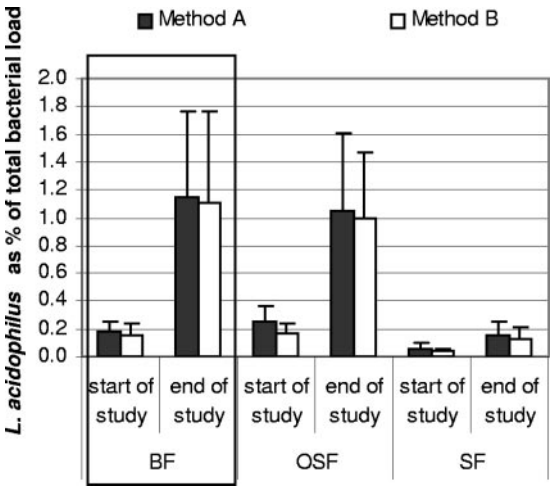

FIG. 1. Comparison of two methods to determine *L. acidophilus* as percentage of total bacterial load in breast-fed infants (BF) and infants receiving a standard formula supplemented with GOS-FOS (OSF) or a standard formula (SF). Bars represent the standard error. Method A shows a combination of the data of *L. acidophilus* as a percentage of the total lactobacilli and the genus *Lactobacillus* as a percentage of the total bacterial load. Method B shows *L. acidophilus* as a percentage of the total bacterial load.

The different *Lactobacillus* species expressed as a percentage of all lactobacilli are given in Table 5. In breast-fed infants *L. acidophilus*, *L. paracasei*, and *L. casei* were the most dominant species throughout the study period. The breast-fed infants also showed a significant increase during the study period of *L. acidophilus* (13.6% ± 3.4% versus 23.5% ± 4.5% [$P = 0.017$]), *L. paracasei* (7.2% ± 3.3% versus 22.1% ± 6.1% [$P = 0.027$]), and *L. casei* (4.0% ± 1.3% versus 6.0% ± 1.8% [$P = 0.028$]). At inclusion, the infants receiving OSF or SF showed a *Lactobacillus* distribution with relatively high proportions of *L.*

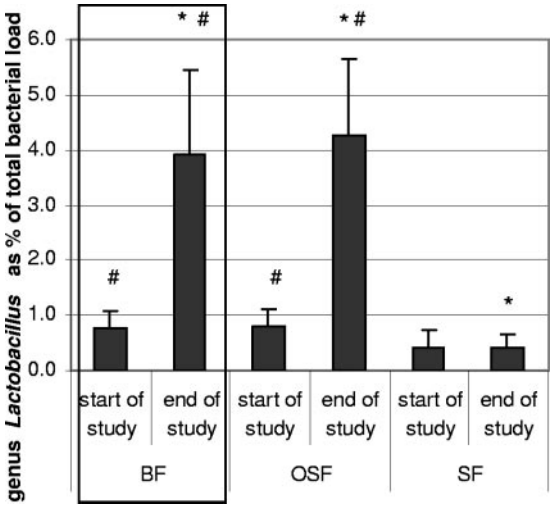

FIG. 2. Lactobacilli as a percentage of the total bacterial load in fecal samples of breast-fed infants (BF) and infants who received a standard formula supplemented with GOS-FOS (OSF) or a standard formula (SF). Bars represent SE. ✻, significant difference ($P < 0.05$) between the BF and SF group or the OSF and SF group at the end of the study; #, significant increase ($P < 0.05$) during the study period.

TABLE 5. *Lactobacillus* species as a percentage of the total *Lactobacillus* population in fecal samples of infants receiving breast milk, a standard formula supplemented with GOS and FOS, or a standard formula[a]

Lactobacillus sp.	% *Lactobacillus* spp. (SE) at:					
	Start of the study (n = 10)			End of the study (n = 10)		
	BF	OSF	SF	BF	OSF	SF
L. acidophilus	13.6 (3.4)^{A}	16.6 (3.3)^{A}	16.8 (4.1)	23.5 (4.5)^{A}	24.5 (3.9)^{A}	19.2 (4.1)
L. casei	4.0 (1.3)^{A}	5.6 (2.4)^{A}	5.5 (1.5)^{A}	6.0 (1.8)^{A}	10.7 (2.5)^{A}	8.3 (2.0)^{A}
L. delbrueckii	1.1 (0.8)	2.5 (1.1)^{B}	1.8 (0.7)^{A}	<0.001 (0.00)^{C}	0.01 (0.01)^{B,D}	6.9 (2.8)^{A,C,D}
L. fermentum	<0.001 (0.00)	0.2 (0.2)	0.3 (0.3)	<0.001 (0.00)	<0.001 (0.00)	0.05 (0.03)
L. paracasei	7.2 (3.3)^{A}	0.8 (0.6)^{A}	0.9 (0.5)	22.1 (6.1)^{A}	16.8 (4.2)^{A}	5.6 (3.3)
L. plantarum	<0.001 (0.00)	<0.001 (0.00)	<0.001 (0.00)	<0.001 (0.00)	<0.001 (0.00)	<0.001 (0.00)
L. reuteri	2.2 (1.5)	2.1 (0.8)	1.9 (1.5)	1.4 (0.6)	1.3 (0.4)	6.4 (3.2)
L. rhamnosus	<0.001 (0.00)	0.2 (0.2)	0.2 (0.2)	<0.001 (0.00)	<0.001 (0.00)	<0.001 (0.00)
Others	71.9 (10.3)^{B}	72.1 (8.6)^{B}	72.5 (8.7)^{B}	47.0 (13.0)^{B}	46.8 (12.5)^{B}	53.5 (15.3)^{B}

[a] BF, breast milk; OSF, standard formula supplemented with GOS and FOS; SF, standard formula. Superscripts: A, a significant increase ($P < 0.05$) during the study period; B, a significant decrease ($P < 0.05$) during the study period; C, a significant difference ($P < 0.05$) between the BF and SF groups; D, a significant difference ($P < 0.05$) between the OSF and SF groups.

acidophilus, *L. casei*, *L. delbrueckii*, and *L. reuteri*. During the intervention period a significant increase was shown for *L. acidophilus* (16.6% ± 3.3% versus 24.5% ± 3.9% [$P = 0.001$]), *L. paracasei* (0.8% ± 0.6% versus 16.8% ± 4.2% [$P = 0.011$]), and *L. casei* 5.6% ± 2.4% versus 10.7% ± 2.5% ($P = 0.005$)) as well as a significant decrease for *L. delbrueckii* (2.5% ± 1.1% versus 0.01% ± 0.01% [$P = 0.045$]) in infants receiving OSF. Consequently, the *Lactobacillus* distribution of the OSF group, at the end of the intervention study, mimics the distribution of breast-fed infants with *L. acidophilus*, *L. paracasei*, and *L. casei* as the predominant strains. In infants receiving SF a significant increase was seen in *L. casei* from 5.5% ± 1.5% to 8.3% ± 2.0% ($P = 0.017$) and in *L. delbrueckii* from 1.8% ± 0.7% to 6.9% ± 2.8% ($P = 0.049$). Also, a significant difference was found between the percentages of *L. delbrueckii* in infants receiving OSF and SF (0.01% ± 0.01% and 6.9% ± 2.8% [$P = 0.033$], respectively). At the end of the intervention period, the composition of the *Lactobacillus* microbiota in the SF-group represented more *L. delbrueckii* and *L. reuteri* and less *L. acidophilus* and *L. paracasei* compared to the BF and OSF groups.

L. fermentum, *L. plantarum*, and *L. rhamnosus* strains were present in very low percentages at the start of the intervention period, and these strains seemed to disappear completely during the intervention in all feeding groups.

DISCUSSION

Duplex 5′ nuclease assays were designed, optimized, validated, and used to study the distribution of *Lactobacillus* species in fecal samples of infants obtained from a nutritional intervention study. With these accurate assays, it was demonstrated that after an intervention with a mixture of galacto- and fructo-oligosaccharides the *Lactobacillus* species distribution in the feces of formula-fed infants closely resembles the distribution in breast-fed infants. Infants receiving SF showed a somewhat different pattern with relatively high levels of *L. delbrueckii* and lower levels of *L. paracasei*.

Species-specific quantitative real-time PCR. Currently, traditional plating methods, conventional PCR, or fluorescent in situ hybridization (FISH) are used for the enumeration of lactobacilli. Traditional plating methods have some major disadvantages compared to modern molecular techniques, such as insufficient selectivity and the presence of "nonculturable" bacteria in fecal samples (31). The FISH technique is currently used to quantify the genus *Lactobacillus* in feces. However, with the commonly used FISH probe (S-G-Lab-0158-a-A20) for quantification of the genus *Lactobacillus*, genera such as *Enterococcus*, *Pediococcus*, *Weissella*, *Vagococcus*, *Leuconostoc*, and *Oenococcus* are also detected (17). In addition, the detection limit of FISH is rather high, which disables the quantification of very low bacterial numbers present in fecal samples of, for example, the different lactobacilli species. The conventional PCR is sufficiently sensitive for the detection of the genus *Lactobacillus* (40) and the different *Lactobacillus* species (37, 41). However, the conventional PCR can only be used for semiquantitative assessment, due to endpoint analyses limitations such as the plateau phase (29) and diminishing effects of differences in PCR product abundance (26). Contemporary quantitative real-time PCR allows the monitoring of the complete amplification and, as a consequence, overcomes the limitations correlated with endpoint analyses of the PCR process. To follow the PCR process, the use of specific fluorescently labeled probes or a minor-groove binding dye, like SYBR Green, can be utilized (9). A major disadvantage of the minor groove binding dyes is that these bind nonspecifically to all double-stranded DNA and may therefore reduce the specificity of a PCR.

For enumeration of the relatively small amounts of the different *Lactobacillus* species in fecal samples duplex 5′ nuclease assays were developed. These assays use a specific fluorescently labeled (TaqMan) probe during the amplification to ensure a high specificity and sensitivity.

The 16S-23S intergenic spacer rRNA gene sequences were used for the design of specific primers and probes for the duplex 5′ nuclease assays instead of the 16S rRNA gene, which is commonly used for the phylogenic analyses and specific detection of bacteria. Due to high similarities of the 16S rRNA gene sequences of the different *Lactobacillus* species, it is not feasible to develop highly specific primer and probe sets (23) for this gene. The intergenic spacer of 16S-23S rRNA gene can

be used for a more detailed analysis of *Lactobacillus* species because sequences are less conserved than the 16S rRNA gene sequence (31).

The CV values (0.04 to 0.14) for the different species-specific duplex 5′ nuclease assays are acceptable and comparable to the CV values (0.09 to 0.28) reported earlier for determination of bacteria in fecal samples with the FISH technique (12, 18).

Lactobacilli in fecal samples from the intervention study. In fecal samples of breast-fed infants, as well as in infants receiving a standard formula containing GOS-FOS, a significant increase in the percentage of lactobacilli was demonstrated during the study period. In contrast, the numbers in infants receiving a standard formula remained constant. The data presented here, obtained using quantitative molecular methods, support an earlier study in which traditional plating methods were used to show that GOS-FOS stimulates fecal lactobacilli (28). The sum of bifidobacteria and lactobacilli at the end of the study reaches ~80% for the BF and OSF groups, whereas this percentage is ~50% for the SF group. This is in correspondence with earlier findings, which state that the intestinal microbiota of breast-fed infants is generally dominated by the genera *Bifidobacterium* and *Lactobacillus*. Infants fed a standard formula are reported to have a more diverse microbiota with higher numbers of *Bacteroides* and *Clostridium* spp. (19, 35).

At the start of the study a higher percentage of lactobacilli was expected in the breast-fed group compared to the OSF and SF group since earlier reports state that breast-fed infants have relatively high levels of lactobacilli (19, 35). The level of the genus *Lactobacillus* was, however, not elevated in breast-fed infants compared to infants receiving OSF or SF at the start of the present study, although they were exclusively breast-fed for 4 weeks before the start of the study. On the other hand, the *Lactobacillus* species distribution of breast-fed infants already differed from that of OSF- and SF-fed infants at study start and was mainly composed of *L. acidophilus*, *L. casei*, and *L. paracasei*.

A major finding of the present study is that GOS-FOS supplemented in a standard formula results in a *Lactobacillus* distribution with relatively high levels of *L. acidophilus*, *L. casei*, and *L. paracasei*, which is rather similar to that of breast-fed infants. Infants receiving a standard formula showed more *L. delbrueckii* and *L. reuteri* and less *L. paracasei* and *L. acidophilus* at the study end. In literature, it has only been described that *L. acidophilus* is one of the most common *Lactobacillus* species in infants (35) and also that *L. reuteri*, *L. gasseri*, *L. paracasei*, *L. rhamnosus*, and *L. fermentum* are commonly present (34, 44). In the present study, relatively high levels of *L. acidophilus* were also found in all of the infants. Conversely, no or very low levels of *L. rhamnosus* or *L. fermentum* were found in the feces of these infants. A large group of lactobacilli in the fecal samples of these infants (~70% at the study start and ~50% at the study end) is still unknown. This percentage of lactobacilli could consist partly of *L. gasseri* or other known human lactobacilli strains, such as *L. crispatus*, *L. salivarius*, *L. johnsonii*, *L. ruminus*, *L. vitulinis*, and *L. brevis* (13, 27, 34). The distribution of the unknown *Lactobacillus* species might still differ between the BF, OSF, and SF groups.

As previously shown for the *Bifidobacterium* population (16), an infant milk formula containing a specific mixture of prebiotics is also able to induce a *Lactobacillus* species distribution that mimics the distribution of breast-fed infants.

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