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′ nucleic acid targets 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 pre- and/or probiotics (10). Prebiotics are defined as non-digestible 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′ nucleic acid targets (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).

**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
Other strains Origin
Bacillus cereus ATCC 11778
Bacillus coagulans LM 10263T
Brevibacterium casei ATCC 35513T
Clostridium difficile ATCC 9689T
Enterococcus faececile DSM 20478T
Escherichia coli ATCC 35218
Listeria monocytogenes ATCC 764T
Pediococcus acidilactici DSM 20284T
Proplonibacterium avidum DSM 4901
Pseudomonas aerugiiosa DSM 1117
Saccharomyces cerevisiae DSM 2548
Salmonellla enterica serovar Typhimurium ATCC 14028
Staphylococcus aureus ATCC 29213
Bifidobacterium adolescentis ATCC 15703T
Bifidobacterium angulatum DSM 20098T
Bifidobacterium bifidum DSM 20456T
Bifidobacterium animalis DSM 20093T
Bifidobacterium gallicum ATCC 25527T
Bifidobacterium dentium DSM 20734T
Bifidobacterium breve ATCC 15700T
Bifidobacterium catenulatum ATCC 25739T
Bifidobacterium infantitis LMG 8811T
Bifidobacterium longum ATCC 15707T

error of the mean), ranging from 29.0 to 85.0 days) or a prebiotic formula

Bacillus cereus, Brevibacterium casei, Clostridium difficile, Enterococcus faececile, Escherichia coli, Listeria monocytogenes, Pediococcus acidilactici, Proplonibacterium avidum, Pseudomonas aerugiiosa, Saccharomyces cerevisiae, Salmonellla enterica serovar Typhimurium, Staphylococcus aureus, Bifidobacterium adolescentis, Bifidobacterium angulatum, Bifidobacterium bifidum, Bifidobacterium animalis, Bifidobacterium gallicum, Bifidobacterium dentium, Bifidobacterium breve, Bifidobacterium catenulatum, Bifidobacterium infantitis, Bifidobacterium longum.

A Lactobacillus strain that is used in the study is Lactobacillus fermentum DSM 20052T. This strain is the type strain for L. fermentum and is used as a reference for other Lactobacillus strains. The strain was isolated from a traditional cheese and was studied in parallel and used as a reference (BF group; age: 56.7 days) or a prebiotic formula (29) for comparison. The authors used a universal oligonucleotide probe, labeled with the 5'TAMRA reporter dye VIC and the 3'TAMRA reporter dye 6-carboxy-fluorescein (FAM), to detect L. fermentum in the samples. This probe was used in conjunction with a specific 5'-TAMRA reporter dye and a 3'-TAMRA reporter dye to increase specificity and sensitivity. The probe was tested for specificity using the basic local alignment search tool (BLAST) and fulfilled the criteria described previously (16). The probe was then used to detect the presence of the Lactobacillus strain in the samples. The results showed that the probe was specific and sensitive for detecting L. fermentum in the samples. The probe was used to study the effects of prebiotic formulas on the gut microbiota, and the results showed that prebiotics can affect the abundance and diversity of Lactobacillus strains in the gut.
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 assay, 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, the bacterial strains listed in Table 1.

**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 2. Primers and probes used in the duplex 5' nuclease assays

<table>
<thead>
<tr>
<th>Target</th>
<th>Primers and probes</th>
<th>Sequence (5'→3')</th>
<th>Tm (°C)</th>
<th>% GC</th>
<th>BLAST ID number</th>
<th>Amplicon length (bp)</th>
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<td>L. acidophilus</td>
<td>F_acid_IS</td>
<td>GAA AGC GCC CAA ACC TAG TTT</td>
<td>59</td>
<td>46</td>
<td>595880126542816 5</td>
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<td>R_acid_IS</td>
<td>CTT CCC AGA TAA TTC GAT GAG ATT T</td>
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<td>37</td>
<td>595654977216250 5</td>
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<td></td>
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<td>P_acid_IS</td>
<td>TAC CAC TTT GCA GTC CTA CA</td>
<td>70</td>
<td>45</td>
<td>595880126542816 5</td>
<td>85</td>
<td></td>
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<td>L. casei</td>
<td>F_case_IS</td>
<td>CTA TAA GTA AGC TTT GAT GAG ATT T</td>
<td>59</td>
<td>36</td>
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<td>132</td>
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<td>R_case_IS</td>
<td>CTT GCC GGT ACT GAG ATG T</td>
<td>59</td>
<td>55</td>
<td>1037002294-02483-29627 5</td>
<td>132</td>
<td></td>
</tr>
<tr>
<td>P_case_IS</td>
<td>AGA AGC TAA TCA ACT GTC</td>
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<td>38</td>
<td>1037002294-02483-29627 5</td>
<td>132</td>
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<td>L. delbrueckii</td>
<td>F_delb_IS</td>
<td>CAC TTA GAC GAA TGA ATA ATC TAA</td>
<td>58</td>
<td>30</td>
<td>10880185400646-6520991806 5</td>
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<tr>
<td>R_delb_IS</td>
<td>CGA ACT CTC TCG GTC GTC GTT</td>
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<td>55</td>
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<td>P_delb_IS</td>
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<td>44</td>
<td>1088018437-6309-1639822749 5</td>
<td>94</td>
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<td>L. fermentum</td>
<td>F_ferm_IS</td>
<td>AAC CGA GAA CAC CGC GTT AT</td>
<td>58</td>
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<td>1036676682-09669-23287 5</td>
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<tr>
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<td>ACT TAA CCT TCA TGA TAG ATC ACT CA</td>
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<td>P_ferm_IS</td>
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<td>32</td>
<td>103667763-010547-20717 5</td>
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<tr>
<td>L. paracasei</td>
<td>F_paca_IS</td>
<td>ACA TCA GTG TAT TGC TGC GTC TGG AAT AC</td>
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<tr>
<td>P_paca_IS</td>
<td>TGC GCC GGT CCA G</td>
<td>70</td>
<td>85</td>
<td>1038306524-018375-2626 5</td>
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<tr>
<td>L. plantarum</td>
<td>F_plan_IS</td>
<td>TGG ATC ACC TCC TTT CTA AGG AAT</td>
<td>58</td>
<td>42</td>
<td>1038305707-03107-18756 5</td>
<td>144</td>
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<tr>
<td>R_plan_IS</td>
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<td>P_plan_IS</td>
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<td>68</td>
<td>32</td>
<td>1038305778-04682-12800 5</td>
<td>144</td>
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<tr>
<td>L. reuteri</td>
<td>F_reut_IS</td>
<td>ACC GAG AAC ACC TTA GCA TTT</td>
<td>59</td>
<td>48</td>
<td>1098025339-29099-129280047216 5</td>
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<tr>
<td>R_reut_IS</td>
<td>CAT ACG TTA ACC TAA ATC AAA ACC GAA TAT CT</td>
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<td>28</td>
<td>1098025385-30847-3558232754 5</td>
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<tr>
<td>P_reut_IS</td>
<td>ACC GCT AAT CAA ATT AAT</td>
<td>68</td>
<td>29</td>
<td>1098025417-30287-2611284585 5</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>L. rhamnosus</td>
<td>F_rham_IS</td>
<td>CGG CTC GAT CAC CTC CTT T</td>
<td>59</td>
<td>58</td>
<td>1023708254-09591-2287 5</td>
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<tr>
<td>R_rham_IS</td>
<td>GCT TGA GGG TAA TCC CTA CCA</td>
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<td>52</td>
<td>1023708352-010389-16127 5</td>
<td>97</td>
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<tr>
<td>P_rham_IS</td>
<td>CTA GCA CAC AGG AAA</td>
<td>65</td>
<td>55</td>
<td>1023708453-011313-6655 5</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>Lactobacillus spp.</td>
<td>F_alllact IS</td>
<td>TGG ATG CCT TGG CAG TAG GA</td>
<td>58</td>
<td>55</td>
<td>1024485925-024664-30598 5</td>
<td>92</td>
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<tr>
<td>R_alllact IS</td>
<td>AAA TCT GCA GGG TAA TCT AT</td>
<td>58</td>
<td>35</td>
<td>102447888-024701-16287 5</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>P_alllact IS</td>
<td>TAT TAG TTC CCT GAT CCC TAC</td>
<td>68</td>
<td>40</td>
<td>1024478809-01753-28422 5</td>
<td>92</td>
<td></td>
</tr>
</tbody>
</table>

* 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).  

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

<table>
<thead>
<tr>
<th>Target</th>
<th>S' Nuclease assay</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe</th>
</tr>
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<td>L. acidophilus</td>
<td>L. acidophilus</td>
<td>900</td>
<td>900</td>
<td>200</td>
</tr>
<tr>
<td>All lactobacilli</td>
<td>All lactobacilli</td>
<td>900</td>
<td>900</td>
<td>200</td>
</tr>
<tr>
<td>L. casei</td>
<td>L. casei</td>
<td>900</td>
<td>900</td>
<td>200</td>
</tr>
<tr>
<td>All lactobacilli</td>
<td>All lactobacilli</td>
<td>300</td>
<td>300</td>
<td>50</td>
</tr>
<tr>
<td>L. delbrueckii</td>
<td>L. delbrueckii</td>
<td>300</td>
<td>300</td>
<td>100</td>
</tr>
<tr>
<td>All lactobacilli</td>
<td>All lactobacilli</td>
<td>900</td>
<td>900</td>
<td>200</td>
</tr>
<tr>
<td>L. fermentum</td>
<td>L. fermentum</td>
<td>300</td>
<td>300</td>
<td>100</td>
</tr>
<tr>
<td>All lactobacilli</td>
<td>All lactobacilli</td>
<td>300</td>
<td>300</td>
<td>100</td>
</tr>
<tr>
<td>L. paracasei</td>
<td>L. paracasei</td>
<td>300</td>
<td>300</td>
<td>100</td>
</tr>
<tr>
<td>All lactobacilli</td>
<td>All lactobacilli</td>
<td>300</td>
<td>300</td>
<td>100</td>
</tr>
<tr>
<td>L. plantarum</td>
<td>L. plantarum</td>
<td>300</td>
<td>300</td>
<td>100</td>
</tr>
<tr>
<td>All lactobacilli</td>
<td>All lactobacilli</td>
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<td>300</td>
<td>100</td>
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<tr>
<td>L. reuteri</td>
<td>L. reuteri</td>
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<td>100</td>
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<tr>
<td>All lactobacilli</td>
<td>All lactobacilli</td>
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<td>900</td>
<td>200</td>
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<tr>
<td>L. rhamnosus</td>
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<td>900</td>
<td>900</td>
<td>200</td>
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<tr>
<td>All lactobacilli</td>
<td>All lactobacilli</td>
<td>150</td>
<td>150</td>
<td>100</td>
</tr>
<tr>
<td>Genus Lactobacillus</td>
<td>Genus Lactobacillus</td>
<td>600</td>
<td>600</td>
<td>100</td>
</tr>
<tr>
<td>All bacteria</td>
<td>All bacteria</td>
<td>300</td>
<td>300</td>
<td>100</td>
</tr>
</tbody>
</table>
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 ± 1.2 × 10^7 lactobacilli per g (wet weight) of feces, OSF-fed infants showed 3.3 ± 1.0 × 10^7 lactobacilli per g (wet weight) of feces, and SF-fed infants showed 5.4 ± 3.1 × 10^7 lactobacilli per g (wet weight) of feces.

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.
acidi\textit{philus}, \textit{L. casei}, \textit{L. delbrueckii}, and \textit{L. reuteri}. During the intervention period a significant increase was shown for \textit{L. acidophilus} (16.6\% ± 3.3\% versus 24.5\% ± 3.9\% [\textit{P} = 0.001]), \textit{L. paracasei} (0.8\% ± 0.6\% versus 16.8\% ± 4.2\% [\textit{P} = 0.011]), and \textit{L. casei} 5.6\% ± 2.4\% versus 10.7\% ± 2.5\% (\textit{P} = 0.005) as well as a significant decrease for \textit{L. delbrueckii} (2.5\% ± 1.1\% versus 0.01\% ± 0.01\% [\textit{P} = 0.045]) in infants receiving OSF. Consequently, the \textit{Lactobacillus} distribution of the OSF group, at the end of the intervention study, mimics the distribution in breast-fed infants. Infants receiving SF showed a significant distribution in breast-fed infants with \textit{Lactobacillus} \textit{L. paracasei} and \textit{L. casei} \textit{L. acidophilus} \textit{L. delbrueckii} \textit{L. fermentum} \textit{L. plantarum} \textit{L. reuteri} and \textit{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.

\textbf{DISCUSSION}

Duplex 5’ nuclease assays were designed, optimized, validated, and used to study the distribution of \textit{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 \textit{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 \textit{L. delbrueckii} and lower levels of \textit{L. paracasei}.

\textbf{Species-specific quantitative real-time PCR.} Currently, traditional plating methods, conventional PCR, or fluorescent in situ hybridization (FISH) are used for the enumeration of \textit{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 quantity the genus \textit{Lactobacillus} in feces. However, with the commonly used FISH probe (S-G-Lab-0158-a-A20) for quantification of the genus \textit{Lactobacillus}, genera such as \textit{Enterococcus}, \textit{Pediococcus}, \textit{Weissella}, \textit{Vagococcus}, \textit{Leuconostoc}, and \textit{Oenococcus} are also detected (17). In addition, the detection limit of FISH is rather high, which reduces the quantification of very low bacterial numbers present in fecal samples of, for example, the different \textit{lactobacilli} species. The conventional PCR is sufficiently sensitive for the detection of the genus \textit{Lactobacillus} (40) and the different \textit{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 \textit{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 \textit{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

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\begin{table}
\centering
\caption{\textit{Lactobacillus} species as a percentage of the total \textit{Lactobacillus} population in fecal samples of infants receiving breast milk, a standard formula supplemented with GOS and FOS, or a standard formula.}
\begin{tabular}{lccc|ccc}
\hline
\textbf{Lactobacillus sp.} & \multicolumn{3}{c}{Start of the study \((n = 10)\)} & \multicolumn{3}{c}{End of the study \((n = 10)\)} \\
& BF & OSF & SF & BF & OSF & SF \\
\hline
\textit{L. acidophilus} & 13.6 (3.4)\textsuperscript{A} & 16.6 (3.3)\textsuperscript{A} & 16.8 (4.1) & 23.5 (4.5)\textsuperscript{A} & 24.5 (3.9)\textsuperscript{A} & 19.2 (4.1) \\
\textit{L. casei} & 4.0 (1.3)\textsuperscript{A} & 5.6 (2.4)\textsuperscript{A} & 5.5 (1.5)\textsuperscript{A} & 6.0 (1.8)\textsuperscript{A} & 10.7 (2.5)\textsuperscript{A} & 8.3 (2.0)\textsuperscript{A} \\
\textit{L. delbrueckii} & 1.1 (0.8) & 2.5 (1.1)\textsuperscript{A} & 1.8 (0.7)\textsuperscript{A} & <0.001 (0.00)\textsuperscript{C} & 0.01 (0.01)\textsuperscript{B,D} & 6.9 (2.8)\textsuperscript{A,C,D} \\
\textit{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) \\
\textit{L. paracasei} & 7.2 (3.3)\textsuperscript{A} & 0.8 (0.6)\textsuperscript{A} & 0.9 (0.5) & 22.1 (6.1)\textsuperscript{A} & 16.8 (4.2)\textsuperscript{A} & 5.6 (3.3) \\
\textit{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) \\
\textit{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) \\
\textit{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) \\
\hline
Others & 71.9 (10.3)\textsuperscript{B} & 72.1 (8.6)\textsuperscript{B} & 72.5 (8.7)\textsuperscript{B} & 47.0 (13.0)\textsuperscript{B} & 46.8 (12.5)\textsuperscript{B} & 53.5 (15.3)\textsuperscript{B} \\
\hline
\end{tabular}
\end{table}
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, the sum of bifidobacteria and lactobacilli at the end of the study period 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 GO-OSPS 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 GO-OSPS 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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REFERENCES


