Lactoferrin (LF) is a beneficial multifunctional protein in milk. The objective of this study was to determine whether bovine transgenic milk containing recombinant human lactoferrin (rhLF) can modulate intestinal flora in the neonatal pig as an animal model for the human infant. We fed 7-day-old piglets (i) ordinary whole milk (OM), (ii) a 1:1 mixture of OM and rhLF milk (MM), or (iii) rhLF milk (LFM). LFM provided better average daily mass gain than OM (P = 0.007). PCR-denaturing gradient gel electrophoresis and 16S rDNA sequencing analysis revealed that the LFM piglets exhibited more diversity of the intestinal flora than the OM group. Except for the colon in the LFM group, an increasing trend in microbial diversity occurred from the duodenum to the colon. Fecal flora was not different across different ages or different treatment groups, but a cluster analysis showed that the fecal flora of OM- and MM-fed piglets had a higher degree of similarity than that of LFM-fed piglets. Based on culture-based bacterial counts of intestinal content samples, concentrations of Salmonella spp. in the colon and of Escherichia coli throughout the intestine were reduced with LFM (P < 0.01). Concentrations of Bifidobacterium sp. in the ileum and of Lactobacillus spp. throughout the intestine were also increased with LFM (P ≤ 0.01). We suggest that rhLF can modulate the intestinal flora in piglets.

Key words: recombinant human lactoferrin (rhLF), piglets, intestinal flora.

Résumé : La lactoferrine (Lf) est une protéine du lait multifonctionnelle bénéfique. L’objectif de cette étude était de déterminer si un lait transgénique de vache contenant de la lactoferrine humaine (rhLF) pouvait moduler la flore intestinale du porcelet nouveau-né, utilisé comme modèle animal du bébé humain. Nous avons nourri des porcelets de 7 jours avec : (i) du lait entier normal (OM); (ii) un mélange 1 : 1 de lait entier normal et de lait contenant de la rhLF (MM); ou (iii) du lait contenant de la rhLF (LFM). Le LFM était plus avantageux sur le plan du gain de poids quotidien comparativement au OM (P = 0.007). Une analyse par PCR et par électrophorèse sur gel en gradient dénaturent, ainsi que le séquençage de l’ADNr 16S ont révélé que les porcelets nourris au LFM montraient une flore intestinale plus diversifiée comparativement au groupe OM. À l’exception du côlon du groupe LFM, la diversité microbienne tendait à s’accroître du duodénum au côlon. La flore fécale n’était pas différente selon l’âge ou entre les différents groupes de traitement, mais une analyse de grappes géniques a montré que la flore fécale des porcelets nourris au OM et au MM montrait un degré de similitude plus élevé que celle des porcelets nourris au LFM. Selon la numération bactérienne basée sur la culture du contenu des échantillons intestinaux, les concentrations de Salmonella sp. du côlon et d’Escherichia coli tout au long de l’intestin étaient réduites par le LFM (P < 0.01). Les concentrations de Bifidobacterium sp. de l’iléon et de Lactobacillus sp. au long de l’intestin étaient aussi augmentées par le LFM (P ≤ 0,01). Nous suggérons que la rhLF peut moduler la flore intestinale des porcelets.

Mots-clés : lactoferrine recombinante humaine (rhLF), porcelets, flore intestinale.

Introduction

Lactoferrin (LF) is a natural iron-binding glycoprotein of the transferrin family. LF is ~80 kDa, contains 1 to 4 glycans, and is widely present in human milk and the milk of most other mammals, including the swine, bovine, goat, rabbit, and mouse (Steijns and van Hooijdonk 2000), but not in the milk of the dog or rat (Levay and Viljoen 1995). LF is also found in saliva, tears, neutrophils, bile, and pancreatic juice (Levay and Viljoen 1995; Steijns and van Hooijdonk 2000). LF is a beneficial multifunctional protein that regulates iron homeostasis and also has bacteriostatic, bactericidal, antioxidant, anticancer, immune-modulating, and antiinflammatory activities (Iyer and Lönnerdal 1993; Ward et al. 2005).

LF is a prominent component of the secondary granules of neutrophils (PMNs), which release LF after migrating to in-
fected tissues or in blood as part of the inflammation process (Legrand et al. 2005). In milk and fluids of the digestive tract, LF is expressed and secreted abundantly and participates in the first line of host defense (Legrand et al. 2005). In the intestine, LF directly interacts with luminal microbes and has multiple effects on enteropathogens (Appelmelk et al. 1994; Brandenburg et al. 2001; Ochoa et al. 2004; Ochoa and Cleary 2009). Of particular note, LF can inhibit the growth of pathogens such as Escherichia coli, Salmonella typhimurium, and Shigella flexneri (Tigyi et al. 1992; Gomez et al. 2002; Bessler et al. 2006; Ochoa and Cleary 2009) and promotes the growth of beneficial bacteria such as Bifidobacteria (Ochoa and Cleary 2009). The effect of LF should be ascribed to an inhibitory effect on pathogenic flora rather than to a direct stimulus to the development of Bifidobacteria (Coppa et al. 2006).

Most research regarding the interaction between LF and enteric pathogens has been performed in vitro or in mice. Thus, it remains unknown whether the same interactions occur in the human intestinal tract. Some (Roberts et al. 1992; Zavaleta et al. 2007) but not all (Balmer et al. 1989) published studies have shown beneficial effects of LF on intestinal flora in human infants. These inconsistent results may be explained by factors such as LF quality, origin, study design (group size, infant age, and length of study), and the detection methods (Tomita et al. 2009). More accurate assessments of the effect of LF on human intestinal microflora and in appropriate animal models, along with modern molecular biological methods, are needed to draw definite conclusions.

Human lactoferrin (hLF) is one of the most abundant whey proteins in milk. Its concentration is >7.0 g/L in colostrum and approximately 1–2 g/L in mature milk (Steijns and van Hooijdonk 2000). The concentration of bovine lactoferrin (bLF) is >1.5 g/L in cow’s colostrum and is reduced to about 20–200 mg/L in milk at mid-lactation (Steijns and van Hooijdonk 2000). We have produced transgenic cows that secrete recombinant human lactoferrin (rhLF) at high levels (>200 mg/L of irradiation, the milk was stored at 4 °C. We used ordinary whole-milk powder, which was supplied by Inner Mongolia Yili Industrial Group Co., Ltd., as the control. The concentration of bovine lactoferrin (bLF) was 2 mg/g in the transgenic milk powder and the control ordinary whole-milk powder.

**Materials and methods**

**Milk powder processing**

We collected mature milk from the rhLF transgenic cow named Wanwa at mid-lactation for 3 months continuously and converted the rhLF-containing milk to solid powder with vacuum freeze-drying technology, then homogeneously mixed the milk powder. After sterilization with a 2 kGy dose of irradiation, the milk was stored at 4 °C. We used ordinary whole-milk powder, which was supplied by Inner Mongolia Yili Industrial Group Co., Ltd., as the control. The concentration of rhLF in the transgenic milk powder was 20 mg/g, thus bLF was 2 mg/g in the transgenic milk powder and the control ordinary whole-milk powder.

**Experimental animals**

Eighteen male Landrace 7-day-old piglets from 3 litters with nearly identical genetics and similar body masses were divided into the following 3 feeding groups (Table 1; n = 6 each): (i) ordinary-milk (OM); (ii) a 1:1 mix of ordinary milk and recombinant human lactoferrin (rhLF) milk; and (iii) rhLF milk (LFM). All piglets were raised in the same room with 50%–70% humidity. Room temperature was 33 °C at 7 to 14 days of age and then decreased 1 °C every week. Each piglet had an independent sty, nipple-type waterer, and milk container. All piglets were housed under conditions similar to a specific pathogen-free environment. In the current study, animal experiments have been approved by the Animal Care and Use Committee of China Agricultural University.

**Diet**

All piglets were nursed by sows from day 0 to day 6 after birth and then transferred into the piglet room for weaning under 24 h care. From postnatal day 7 to 28, the animals in the 3 groups were given reconstituted milk that was a mixture of milk powder and water and placed in the containers. From 29 to 37 days of age, animals were directly fed dry milk powder instead of reconstituted milk. We fed the piglets every 3 to 4 h (6 or 7 times a day), providing 24 h of uninterrupted feeding, which mimics the natural milk-drinking process of piglets. Reconstituted milk and dry milk powder were fed for a total of 30 days, and each piglet in the 3 groups was fed the same amount of milk throughout the experiment, ~130 g milk powder per day.

Table 1. Experimental groups.

<table>
<thead>
<tr>
<th>Group name</th>
<th>Piglet No.</th>
<th>Sow No.</th>
<th>Birth mass (kg)</th>
<th>Masst at 7 days (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OM</td>
<td>1-1</td>
<td>54</td>
<td>1.50</td>
<td>2.62</td>
</tr>
<tr>
<td></td>
<td>1-2</td>
<td>54</td>
<td>1.40</td>
<td>2.44</td>
</tr>
<tr>
<td></td>
<td>1-3</td>
<td>57</td>
<td>1.46</td>
<td>2.32</td>
</tr>
<tr>
<td></td>
<td>1-4</td>
<td>56</td>
<td>1.12</td>
<td>2.12</td>
</tr>
<tr>
<td></td>
<td>1-5</td>
<td>56</td>
<td>1.10</td>
<td>1.96</td>
</tr>
<tr>
<td></td>
<td>1-6</td>
<td>57</td>
<td>1.14</td>
<td>1.90</td>
</tr>
<tr>
<td>Average mass</td>
<td></td>
<td></td>
<td>1.287</td>
<td>2.227</td>
</tr>
<tr>
<td>MM</td>
<td>2-7</td>
<td>57</td>
<td>1.54</td>
<td>2.06</td>
</tr>
<tr>
<td></td>
<td>2-8</td>
<td>54</td>
<td>1.52</td>
<td>2.54</td>
</tr>
<tr>
<td></td>
<td>2-9</td>
<td>56</td>
<td>1.28</td>
<td>2.22</td>
</tr>
<tr>
<td></td>
<td>2-10</td>
<td>57</td>
<td>1.40</td>
<td>2.10</td>
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<td></td>
<td>2-11</td>
<td>54</td>
<td>1.44</td>
<td>2.58</td>
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<tr>
<td></td>
<td>2-12</td>
<td>56</td>
<td>1.02</td>
<td>1.88</td>
</tr>
<tr>
<td>Average mass</td>
<td></td>
<td></td>
<td>1.367</td>
<td>2.23</td>
</tr>
<tr>
<td>LFM</td>
<td>3-13</td>
<td>54</td>
<td>1.48</td>
<td>2.56</td>
</tr>
<tr>
<td></td>
<td>3-14</td>
<td>54</td>
<td>1.56</td>
<td>2.48</td>
</tr>
<tr>
<td></td>
<td>3-15</td>
<td>54</td>
<td>1.42</td>
<td>2.28</td>
</tr>
<tr>
<td></td>
<td>3-16</td>
<td>57</td>
<td>1.10</td>
<td>2.08</td>
</tr>
<tr>
<td></td>
<td>3-17</td>
<td>56</td>
<td>1.14</td>
<td>2.06</td>
</tr>
<tr>
<td></td>
<td>3-18</td>
<td>56</td>
<td>1.09</td>
<td>1.92</td>
</tr>
<tr>
<td>Average mass</td>
<td></td>
<td></td>
<td>1.298</td>
<td>2.23</td>
</tr>
</tbody>
</table>

Note: OM, ordinary-milk feeding group; MM, feeding group given a 1:1 mix of ordinary milk and recombinant human lactoferrin (rhLF) milk; and LFM, feeding group given rhLF milk. The masses for each group did not differ significantly at birth (P = 0.754) or at 7 days of age (P = 1.000).
Mass and sample collection
Piglets were weighed every 3 days, and SPSS 11.5 (SPSS Inc.) was used for statistical analysis. Fresh feces (5 mL) were immediately collected after defecation from piglets in the morning once a week. The fecal samples were pooled within the same group in 50 mL sterile centrifuge tubes and stored at −70 °C for subsequent DNA extraction and PCR – denaturing gradient gel electrophoresis (PCR–DGGE) analysis.

Necropsy
At the end of the feeding period (38 days of age), animals were euthanized, and the contents of the middle sections of the duodenum, jejunum, ileum, and colon were obtained. The samples used for PCR–DGGE analysis were stored at −70 °C. The samples used for culture-based bacterial count analysis were stored for less than 12 h at 4 °C and then used for the experiment.

PCR–DGGE analysis
Microbial DNAs were extracted from intestinal contents and fecal samples using a QIAamp DNA stool mini kit (Qiagen, Germany) and E.Z.N.A.™ Soil DNA kit (Omega Bio-Tek), respectively, in accordance with the manufacturer’s instructions. Extracted DNA was quantified by a Tecan infinite 200 microware plate reader (Tecan, Switzerland) at 260 and 280 nm (Xu et al. 2011). The variable V3 region of bacterial 16S rDNA was amplified with PCR using the primer pair 338F 5′-ACTCTTACGGGAGGCAGCAG-3′ and 518R 5′-ATTACCGCGGTGTGCTGG-3′. A GC clamp (GCCCGGCAGGCGCGGCGGCGGCGGCGGCGGCGGCGG) was added to the 5′ terminus. PCR amplification was carried out in 50 μL reaction mixtures containing 1 μL template DNA (2.5 ng/μL), 0.25 μL Taq polymerase (5 U/μL; Takara, Dalian, China), 1 μL of each primer (20 μM/μL), 4 μL dNTP mix (2.5 mmol/L; Takara), and 5 μL of 10× PCR buffer (Takara). The PCR cycle was 94 °C for 10 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min 30 s, and then a final extension at 72 °C for 7 min. PCR products were examined with electrophoresis (2% (w/v) agarose) and then used for DGGE analysis with the Dcode Universal Mutation Detection System (Bio-Rad, Hercules, Calif.). The PCR products were loaded onto 8% (w/v) polyacrylamide gels in 1× Tris–EDTA buffer. The gels were run at 150 V for 420 min and then silver stained (Xu et al. 2011).

Cloning and sequencing
Prominent bands from the PCR amplification were excised from the DGGE gel, purified with a Watson Gel Recovery Purification kit (Watson Biotechnologies, China), cloned into the pMD18-T vector (Takara), and transformed into E. coli DH5α competent cells. Ten clones from each band were randomly selected, and plasmids were isolated with the E.Z.N.A.™ DNA Isolation System (Omega Bio-Tek). An ABI 3730XL DNA sequencer (Applied Biosystems) was used for sequencing using the universal primer pair for the PMD18-T vector, M13F (−40) 5′-GTTTTCCAGTACGAC-3′ and M13R (−26) 5′-CAGGAAACAGCTATGAC-3’ (Xu et al. 2011).

Diversity index
A diversity index was used to describe the diversity of a microbial community. Here, we used the Shannon’s diversity index to evaluate the diversity of the microflora (Shannon 1997). Quantity One analysis software (Bio-Rad) was used for quantitative analysis of DGGE bands for each sample and to calculate the preliminary data of the diversity index, which was then calculated with the Shannon formula.

Cluster analysis
Cluster analysis was used to study the similarity of microflora among samples. The method we used is derived from Dice’s algorithm and uses the unweighted pair group method with arithmetic averages to construct a similarity matrix (Yang et al. 2001; Fromin et al. 2002; Boon et al. 2002; Smalla et al. 2007; Kim et al. 2008; Xu et al. 2011). The software programs BioEdit 7.0, Phylip 4.0, and MEGA 4.0 were used for cluster analysis.

Phylogenetic analysis and phyla distribution
16S rDNA gene sequences were submitted to the National Center for Biotechnology Information (NCBI) to obtain closely related sequences by BLAST, and all sequences with >97% similarity were chosen for construction of phylogenetic trees (Xing et al. 2010; Xu et al. 2011). Neighboring phylogenetic trees were constructed using MEGA 4.0 (Tamura et al. 2007; Xu et al. 2011) and assessed using a bootstrap analysis with 1000 replications (Xu et al. 2011). The percent of each genus or phylum was derived from phylogenetic analysis (Xu et al. 2011). Phyla distribution was used to show the composition of microflora as compared with the percent of each genus or phylum in different samples.

Nucleotide sequence acc. Nos.
The 16S rDNA gene sequences used for phylogenetic analysis have been submitted to the GenBank nucleotide sequence database. The accession numbers are JN624931–JN625028.

Culture-based bacterial count analysis
We selected 6 types of bacteria for culture-based analysis, each of which grew in an appropriate medium (Table 2). One gram of each intestinal sample was rapidly mixed with 9.0 mL sterile diluent in an aseptic homogenate cup, homogenized at 8000 r/min (9100g) for 1–2 min, and a 10−1 homogeneous sample diluent was prepared. Then, each diluent was used to make a series of liquid samples with different dilution ratios, such as 10−2, 10−3, 10−4, etc. All procedures were completed within 15 min. Using the appropriate medium (Table 2), we prepared 2 or 3 consecutive appropriate dilutions of each sample and plated 0.1 mL of homogeneous sample diluent per plate, making 2 replicates and 1 blank control. Culture time, temperature, and condition are presented in Table 2. The morphology of the flora and individual cells, Gram staining, and oxygen consumption were used to identify the type of bacteria. The logarithm of the number of bacteria per gram of intestinal contents (logCFU/g) was used to show the results. SPSS 11.5 (SPSS Inc.) was used for statistical analysis.

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Table 2. Cultured bacteria and their different media.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Medium</th>
<th>Culture condition</th>
<th>Culture time (h)</th>
<th>Culture temperature (°C)</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total aerobic bacteria</td>
<td>Brain heart infusion agar medium</td>
<td>Aerobic culture</td>
<td>48±2</td>
<td>36±1</td>
<td>Qingdao Hope Bio, China</td>
</tr>
<tr>
<td>Total anaerobic bacteria</td>
<td>Wilkins–Chalgren anaerobe agar medium</td>
<td>Anaerobic culture</td>
<td>48±2</td>
<td>36±1</td>
<td>Qingdao Hope Bio, China</td>
</tr>
<tr>
<td>Salmonella spp.</td>
<td>Salmonella spp. chromogenic medium</td>
<td>Aerobic culture</td>
<td>22–24</td>
<td>36±1</td>
<td>Qingdao Hope Bio, China</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>E. coli chromogenic medium</td>
<td>Aerobic culture</td>
<td>18–24</td>
<td>36±1</td>
<td>Qingdao Hope Bio, China</td>
</tr>
<tr>
<td>Bifidobacterium spp.</td>
<td>Bifidobacterium spp. selective medium</td>
<td>Anaerobic culture</td>
<td>48±2</td>
<td>36±1</td>
<td>Qingdao Hope Bio, China</td>
</tr>
<tr>
<td>Lactobacillus spp.</td>
<td>Lactobacillus spp. selective medium</td>
<td>Anaerobic culture</td>
<td>48±2</td>
<td>36±1</td>
<td>Qingdao Hope Bio, China</td>
</tr>
</tbody>
</table>

Results

Mass
We weighed each piglet every 3 days, determined the mass change over the study period (Fig. 1A), and calculated the average daily gain (Fig. 1B) for each of the 3 groups. The masses of the LFM and OM group differed beginning at 29 days of age (P = 0.045). The LFM piglets grew faster than the OM group. The average daily gain of the LFM group was greater (P = 0.007) than that of the OM group (Fig. 1B).

PCR–DGGE analysis and 16S rDNA sequences of intestinal content samples
After necropsy, the contents of the duodenum, jejunum, ileum, and colon from each piglet were individually pooled with the replicates for each different group, resulting in a total of 12 pooled samples (samples are labeled as in Fig. 2A).

The 16S rDNA PCR products of the 12 intestinal content samples were electrophoresed on the same DGGE gel plate for comparison. Based on the PCR–DGGE analysis, different intestinal compartments and groups showed different DGGE band patterns (Fig. S1).2 We performed further analysis using 16S rDNA sequencing and the following statistical methods.

Cluster analysis of the 16S rDNA sequences showed that the similarity was ~54%–94% among the different samples (Fig. 2A). The similarity between DF1 and DF2 was close to 94%, and comparison of DF3 with DF1/DF2 revealed a similarity of 77%. The similarity among the 3 jejunum fecal samples (JF1, JF2, and JF3) was ~84%–85%. The similarity among IF1, IF2, and IF3 was 54%–70%, and the similarity among CF1, CF2, and CF3 was 61%–65% (Fig. 2A). Only the similarity between DF1 and DF2 was >90%.

Phyla distribution was derived from phylogenetic analysis (Fig. S3A).2 When the 16S rDNA clone libraries were examined at the phylum level, the prominent groups of phyla were Firmicutes and Gammaproteobacteria, which appeared in all the intestinal compartments of the 3 feeding groups and had the highest percents (Fig. 3A). Some sequences could not be classified and were designated as unknown.

When we examined the clone libraries at the genus level (Fig. 3B), the genus distribution result seemed more specific. We classified all 16S rDNA clones into the following 18 genera: Pseudomonas, Lactobacillus, Robinsoniella, Escherichia, Clostridium, Ruminococcus, Enterococcus, Streptomyces, Acinetobacter, Streptococcus, Erwinia, Aeromonas, Lactococcus, Ewingella, Dorea, Rothia, Salmonella, and Mega- sphera. Some sequences could not be classified and were designated as unknown. The prominent groups at the genus level were Lactobacillus and Pseudomonas in all the intestinal content samples.

Regarding the genus complexity of the different intestinal compartments, the duodenum was the simplest, and we observed a trend toward increasing complexity from the duodenum to the colon (Fig. 3B). Regarding the genus complexity of the different feeding groups, in the duodenum and ileum, the LFM group showed the most complex genera, especially in the ileum, where the percent of unknown genera observed in the LFM group was higher (34.48%) than in the OM (14.29%) and MM (16.67%) groups, revealing more complexity. Unknown microbes in the other intestinal content samples comprised only 0%–25%.

Different diversity indices among the different intestinal compartments of the 3 feeding groups indicated an increasing trend from the duodenum to the colon, except for the colon of the LFM group. However, the diversity index of the LFM group in the duodenum, jejunum, and ileum was higher than that in the OM or MM groups (Fig. 4A).

To summarize, PCR–DGGE analysis and 16S rDNA sequencing of intestinal content samples revealed low similarity among the intestinal content samples. Specifically, the LFM piglets exhibited more complexity and diversity than the control group did in the duodenum and ileum and a trend toward an increase in complexity and diversity from the duodenum to the colon.

PCR–DGGE analysis and 16S rDNA sequencing of fecal samples
Fresh fecal samples of piglets were collected at 13, 20, 28, and 35 days of age, and samples were pooled into the same group and collection time. PCR products of the 12 pooled samples (samples are labeled as in Fig. 2B) were

2Supplementary data are available with the article through the journal Web site (www.nrcresearchpress.com/doi/suppl/10.1139/o2012-003).
From the DGGE profile, there appeared to be few major differences among the different groups of fecal samples or samples collected at different times (Fig. S2).² The diversity index values were not different among the 3 groups of piglets (Fig. 4B).
Fig. 2. Cluster analysis of 16S rDNA clone libraries. Numbers along the bottom indicate the similarity coefficient. (A) Cluster analysis of 16S rDNA clone libraries derived from intestinal content samples. DF1, duodenum content of the ordinary-milk feeding (OM) group; DF2, duodenum content of the 1:1 mix of ordinary milk and recombinant human lactoferrin (rhLF) milk feeding (LFM) group; JF1, jejunum content of the OM group; JF2, jejunum content of the MM group; JF3, jejunum content of the LFM group; IF1, ileum content of the OM group; IF2, ileum content of the MM group; IF3, ileum content of the LFM group; CF1, colon content of the OM group; CF2, colon content of the MM group; and CF3, colon content of the LFM group. (B) Cluster analysis of 16S rDNA clone libraries derived from fresh fecal samples. F1-1, fresh fecal sample mixture of OM at 13 days of age; F1-2, fresh fecal sample mixture of MM at 13 days of age; F1-3, fresh fecal sample mixture of LFM at 13 days of age; F2-2, fresh fecal sample mixture of MM at 20 days of age; F2-3, fresh fecal sample mixture of LFM at 20 days of age; F3-1, fresh fecal sample mixture of OM at 28 days of age; F3-2, fresh fecal sample mixture of MM at 28 days of age; F3-3, fresh fecal sample mixture of LFM at 28 days of age; F4-1, fresh fecal sample mixture of OM at 35 days of age; F4-2, fresh fecal sample mixture of MM at 35 days of age; and F4-3, fresh fecal sample mixture of LFM at 35 days of age.

Fecal flora phyla distribution was derived from fecal flora phylogenetic analysis (Fig. S3B).2 Regarding the phyla distribution at the phylum level (Fig. 5A), the dominant groups were Firmicutes and Gammaproteobacteria, which appeared in all fecal samples and showed the highest percents (Fig. 5A). Similar results were obtained for the phyla distribution in the intestinal content samples (Fig. 3A).

Regarding the phyla distribution at the genus level (Fig. 5B), we classified all 16S rDNA clones into the following 10 genera: Lactobacillus, Robinsoniella, Escherichia, Clostridium, Ruminococcus, Klebsiella, Gemmiger, Faecalibacterium, Erysipelotrichaceae, and Phascolarctobacterium. The prominent groups at the genus level were Lactobacillus, Robinsoniella, and Escherichia in all fecal samples.

Regarding genus complexity, there were no major differences among the different periods or groups (Fig. 5B). The fecal flora was not richer than the intestinal content in the classified microbes, whereas unknown microbes were present at higher percents in all the fecal samples (41.67%–61.11%).

Based on cluster analysis (Fig. 2B), the similarity among all 12 pooled fecal samples ranged from 60% to 92%. F1-1 and F1-2 were clustered together, as were F2-1 and F2-2 and F3-1 and F3-2. Compared with the LFM group, the fecal flora of the OM and MM groups had a higher degree of similarity at 13, 20, and 28 days of age. At 35 days of age, the similarity between F4-1 and F4-2 was also higher than their similarity with F4-3. Thus, the OM and MM groups exhibited a higher degree of similarity in fecal flora than did the LFM group.

To summarize, PCR–DGGE analysis and 16S rDNA sequencing revealed no apparent differences in the complexity and diversity of fecal samples, but the OM and MM groups had greater similarity in fecal flora than the LFM group.

Culture-based bacterial count analysis of intestinal content samples

Intestinal content samples were analyzed with culture-based bacterial count analysis. Six types of bacteria were selected for culture as follows: total aerobes, total anaerobes, Salmonella spp., Escherichia coli, Bifidobacterium spp., and Lactobacillus spp.

For total aerobes (Table 3), we observed no differences in the duodenum or jejunum, but we observed a difference in the ileum and colon among the 3 feeding groups. The total number of aerobes in the ileum of the OM group was the highest (P < 0.001). For total anaerobes (Table 3), we observed no differences in the duodenum, jejunum, or colon. Only in the ileum the total anaerobe number in the MM group was lower than those in the OM and LFM groups (P = 0.003), but the OM and LFM groups were not different. For Salmonella spp., we observed no differences in the duodenum, jejunum, or ileum, but the number of Salmonella spp. in the OM group in the colon was higher than that in the LFM group (P = 0.005). For E. coli, the OM group had a higher number than the LFM group in the duodenum (P < 0.001), jejunum (P < 0.001), ileum (P < 0.001), and colon (P = 0.006). For Bifidobacterium spp. (Table 3), we also observed no differences in the duodenum, jejunum, or colon. The number of Bifidobacterium spp. in the ileum of the OM group was the lowest and different from the number in the MM and LFM groups (P = 0.011). For Lactobacillus spp., the number in the LFM group was higher than that in the OM duodenum (P = 0.001), jejunum (P = 0.002), ileum (P < 0.001), and colon (P = 0.002). The LFM group had greatly increased levels of Lactobacillus spp. in the intestine, which was also the dominant genus in the intestine of piglets weaned at a very young age.

Discussion

When human infants are born, the gastrointestinal tract is sterile and is then exposed to an external environment that is rich in various bacteria. These microbes continuously populate the infant gastrointestinal tract. After growth and reproduction, the microbes gradually form a complex microbial community (Coppa et al. 2006). The type of feeding is the main factor that contributes to the development of the intestinal ecosystem of newborns. Bifidobacteria and Lactobacillus represent up to 90% of the intestinal flora of breast-fed infants, whereas in formula-fed infants, these 2 types of bacteria represent only 40%–60% of the flora (Coppa et al. 2006). This bifidogenic effect has been reported in several studies (Roberts et al. 1992; Rubaltelli et al. 1998; Harmsen et al. 2000). In breast-fed infants, the minor components in fecal samples were mainly streptococci, but fecal samples from formula-fed infants are often contaminated with phylloccoci, E. coli, and clostridia (Harmsen et al. 2000), which include harmful microorganisms, such as Staphylococcus aureus, enterotoxigenic E. coli (ETEC), enteropathogenic E. coli (EPEC), enterohemorrhagic E. coli (EHEC), shiga toxin-producing E. coli (STEC), Clostridium tetani, Clostridium perfringens, and Clostridium botulinum. Bifidobacteria and Lactobacillus have many beneficial effects on infants, including modulation of mucosal physiology and barrier function, inhibition of the growth of pathogenic bacteria, and participation in systemic...
immunologic and inflammatory responses (Coppa et al. 2006).

Breast milk contains a complex mixture of unique components and nutrients, and the bifidogenic effect is related to a complex of interacting factors, such as lactose, oligosaccharides, and a low concentration of proteins, especially LF (Coppa et al. 2006). When LF is added to formula as a supplement, infants can also establish a bifidus flora that is
somewhat similar to the flora established in breast-fed infants (Roberts et al. 1992). This effect is, however, still controversial (Balmer et al. 1989).

We chose piglets that were weaned early as an animal model to evaluate the effect of oral rhLF-containing transgenic milk in modulating intestinal flora. A critical stage in pig development is weaning, which alters the gastrointestinal tract architecture and function, including the immune response and adaptations to enteric microbiota. Weaning stress will result in impaired absorption of nutrients, diarrhea, and

Fig. 3. (A) Phyla and (B) genus distribution of 16S rDNA clone libraries derived from intestinal content samples. Samples are labeled as in Fig. 2A.
decreased growth performance (Liu et al. 2008). In our study, 7-day-weaned piglets in the rhLF milk feeding group showed better average daily mass gain than control piglets, suggesting that rhLF may have a beneficial effect on the growth of piglets.

To understand the mechanisms responsible for the effect of rhLF on the intestinal microflora ecosystem, we used the molecular-biology-based approach (PCR–DGGE) followed by 16S rDNA sequence analysis. This method permits qualitative analysis of the abundance of bacterial species and how their presence interacts with diet. Simpson et al. (1999) studied changes in pig intestinal flora from birth to weaning using PCR–DGGE and found that pig intestinal flora varied with age, individuals, and the compartments of the intestine. Zhu et al. (2003) used PCR–DGGE to analyze the fecal flora variations of 12 weaning piglets and found that on the weaning day, the pattern of the DGGE bands was simple. After weaning, the DGGE bands gradually increased over time, became complex and diverse, and individual differences increased. When examining the effect of fermentable carbohydrates on piglet fecal bacterial communities with DGGE analysis of 16S rDNA, Konstantinov et al. (2003) found that compared with a regular diet, supplementing fermentable carbohydrates to piglets produces more diverse and more stable intestinal flora.

In intestinal samples, we observed a low degree of similarity among intestinal content samples. Cluster analysis identified the similarity of most samples as below 90%. From the diversity index and phyla distribution statistical analysis, the higher diversity and complexity in the LFM group than in the control may mean that the LFM piglets developed intestinal flora faster than the OM and MM groups did after early weaning and that the different doses of rhLF may be the reason for the differential microflora ecosystems.

Fig. 4. Diversity index analysis. (A) Diversity index analysis of the intestinal content samples. OM, ordinary-milk feeding group; MM, feeding group given a 1:1 mix of ordinary milk and recombinant human lactoferrin (rhLF) milk; LFM, feeding group given rhLF milk; DF, duodenum; JF, jejunum; IF, ileum; and CF, colon. (B) Diversity index analysis of fecal samples.
In the DGGE profile of the fecal samples, there were no differences in the complexity or diversity of flora. But with cluster analysis, the OM and MM groups had greater similarity in their fecal flora than they did with the LFM group. Thus, rhLF may have a dosage compensation effect on the composition of the developing intestinal flora. When the rhLF dose reaches the level in the LFM group, this protein may have a greater influence on the composition of the flora.

Large amounts of *Lactobacillus* and anaerobic bacteria usually live in the digestive tracts of healthy animals, and the number of *Lactobacillus* controls the intestinal microbial balance (Morotomi et al. 1975; Conway et al. 1990). *Lactobacillus* and some other anaerobic bacteria are also the main microbes in the gastrointestinal tract of nursing pigs. *Escherichia coli* are part of the normal flora in the pig gastrointestinal tract, but under normal circumstances, aerobic bacteria such as *E. coli* account for only ∼1% of the total bacteria. Why do pathogenic bacteria such as *E. coli* not easily proliferate in nursing pigs? An important reason is the supply of breast milk and the effective mucosal barrier. When close to weaning, *Lactobacillus*, *Enterobacteriaceae*, and *Bifidobacterium* become the advantageous bacteria. Early weaned pigs often experience ablactation hyperirritability, which greatly influences microbial numbers in the intestine. After weaning,
the *Lactobacillus* population declines over a short period of time, and the ratio of total bacteria to *Enterobacteriaceae* (especially *E. coli*) rises (Su and Zhu 2006). Changes in piglet intestinal microbial composition and number are the underlying causes of disease when weaning.

From the results of the quantitative culture-based bacterial count analysis, the LFM piglets showed reduced levels of *E. coli* and *Salmonella* and increased numbers of *Lactobacillus* and *Bifidobacterium*, which creates an advantageous microflora in the gut. rhLF-containing transgenic milk appears to beneficially modulate the intestinal flora both in the number and concentrations of bacteria.

In summary, the results of this study indicate that feeding of the rhLF-containing transgenic cow’s milk resulted in higher average daily mass gain than the ordinary whole-milk. Additionally, the PCR–DGGE and 16S rRNA sequence analysis revealed that the intestinal microbiota in the LFM piglets had higher diversity and complexity. The different doses of rhLF in milk may be the major factor contributing to the differential microbial ecosystems in the gut. Our findings have important implications for the use of bovine-derived rhLF in the infant formula industry.

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