Short communication

Characterization of ileal bacterial microbiota in newly-weaned pigs in response to feeding lincomycin, organic acids or herbal extract

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Abstract

The changes of ileal bacterial microbiota in pigs during the first 2 weeks post-weaning in response to feeding lincomycin, organic acids or herbal extract were characterized using 16S rRNA gene-based PCR-denaturing gradient gel electrophoresis (DGGE) profiling, DNA sequencing, and real-time PCR (QPCR) techniques. Both time post-weaning and the dietary treatments resulted in a shift of the microbiota composition. While time post-weaning mostly influenced \textit{Clostridium} group, the feed additives increased the population of \textit{Lactobacillus} and related lactic acid bacteria by $\geq 3$-fold.

Keywords: Pigs; Ileal microbiota; Lincomycin; Organic acids; Herbal extract; 16S rRNA

1. Introduction

Piglets are under stress at weaning. The stressors include changes in diet composition, environment and bacterial challenges and contribute to digestive upsets and depressed growth rates (Pluske et al., 2003). Dietary antibiotics have been used to largely overcome weaning-associated disorders, such as diarrhea caused by \textit{Escherichia coli} K88 (Bosi et al., 2004), which significantly affect animal productivity. However, this practice has been forbidden today in Europe due to public concerns over the potential link of the use of antibiotics in feed to the wide spread of bacterial antibiotic-resistance that threatens human health. To develop viable alternatives to dietary antibiotics, various feed additives such as organic acids, copper sulphate, zinc oxide, probiotics, prebiotics, and herbs have been tested on newly-weaned piglets (NRC, 1998). Partanen and Mroz (1999) and Piva et al. (2002) reported that the inclusion of organic acids in the diet can enhance growth performance and modulate swine intestinal fermentation and microbial proteolysis. Lactic acid in particular has been reported to reduce proliferation of an enterotoxigenic \textit{E. coli} (Thomlinson and Lawrence, 1981) and to be more effective than other organic acids in improving pig growth performance (Tsiloyiannis et al., 2001). Recently we also observed that supplementing diets with lincomycin and a blend of acids, containing a...
large amount of lactic acid, showed a similar degree of growth promotion in pigs during week 2 post-weaning. This observation coincided with changes in the ileal bacterial microbiota that were not investigated in detail (Namkung et al., 2004). The objective of the present study was to characterize the changes of ileal microbiota in response to the dietary treatments, including analyses of the diversity of the microbiota and identification of affected bacteria.

2. Materials and methods

2.1. Animals, diets, and sample collection

Ileal digesta samples were obtained from pigs that were used in a large-scale pig performance study. General experimental procedures, including a detailed description of dietary treatments, and growth performance data have been reported previously (Namkung et al., 2004). Briefly, pigs were not fed creep feed before weaning, weaned at 16 to 19 days of age with an average body weight (BW) of 4.90±0.67 kg. Pigs were exposed to one of five dietary treatments, with two pens of six pigs per treatment in each of three equal blocks: 1) basal diet (Pig Starter); 2) basal diet with lincomycin (110 mg/kg); 3) basal diet with a herbal extract (0.75%); 4) basal diet with acid blend 1 (1.1%; Acids-1); and 5) basal diet with acid blend 2 (2.1%; Acids-2). Treatments 1 and 2 served as a negative and a positive control, respectively. At day 14 post-weaning, one pig from each of two pens per treatment and with growth rate closest to the pen average was sacrificed for sampling of ileal digesta. Digesta samples from two pigs of the same treatment were pooled. In addition, ileal digesta was obtained and pooled from five pigs just before weaning. Collection and preparation of the digesta samples from the pig ileum for bacterial DNA extraction were conducted as described previously (Li et al., 2003).

2.2. Cell lysis, DNA extraction, and PCR–DGGE analysis

Cell lysis, chromosomal and plasmid DNA extraction, PCR amplification, DGGE electrophoresis of PCR amplicons, and visualization of DNA bands were conducted as described previously (Li et al., 2003). PCR primers were HD1-GC (5'-CGC CCG GGG CGC GCC CCG GGG GGG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG T-3'; the GC clamp is in boldface) and HD2 (5'-GTA TTA CCG CGG CTG CTG GCA C-3') (Walter et al. (2000) against the V3 region of the 16S rRNA genes (position 339 to 539 in the E. coli gene) of bacteria.

The similarities of PCR–DGGE profiles were analyzed with BioNumerics software version 3.0 (Applied Maths, Sint-Martens-Latem, Belgium) using the Dice function. DNA bands were manually assigned in the software and compared using a positional tolerance of 0.5% with manual correction where required. A distance matrix was calculated by the Dice and dendograms were constructed from this matrix using the unweighted pair group mean average (UPGMA). The degree of similarity was represented by a similarity coefficient. Both Shannon Index and Simpson’s Diversity Index were determined based on the presence and density of DNA bands using the R vegan Package (Version 1.8-8) (Oksanen et al., 2007).

2.3. Isolation and sequence analysis of DNA bands from DGGE gels

To determine the bacteria represented by DNA bands in DGGE gels that were either major representative bands or
Table 1
Putative identity of ileal bacteria affected by lincomycin and feed additives

<table>
<thead>
<tr>
<th>Band #</th>
<th>Closest sequence relative</th>
<th>Accession #</th>
<th>Similarity (%)</th>
<th>Closest cultivated relative</th>
<th>Accession #</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Uncultured bacterium clone p-527</td>
<td>AF371939</td>
<td>99</td>
<td>Veillonella dispar</td>
<td>AF439639</td>
<td>96</td>
</tr>
<tr>
<td>2</td>
<td>Uncultured bacterium clone p-271</td>
<td>AF371473</td>
<td>100</td>
<td>Lactobacillus delbrueckii</td>
<td>AY050171</td>
<td>97</td>
</tr>
<tr>
<td>3</td>
<td><em>Escherichia coli</em> MP2 1</td>
<td>AY186041</td>
<td>98</td>
<td><em>E. coli</em></td>
<td>AY186041</td>
<td>98</td>
</tr>
<tr>
<td>4</td>
<td>Swine manure bacterium 37-4</td>
<td>AY167966</td>
<td>99</td>
<td>Clostridium sp.</td>
<td>AF390549</td>
<td>99</td>
</tr>
<tr>
<td>5</td>
<td>Swine manure bacterium RT-1A</td>
<td>AY167932</td>
<td>99</td>
<td>Clostridium biferecmentans</td>
<td>AF320283</td>
<td>99</td>
</tr>
<tr>
<td>6</td>
<td>Uncultured bacterium clone p-458</td>
<td>AF371839</td>
<td>100</td>
<td>Clostridium disporicum</td>
<td>Y18176</td>
<td>99</td>
</tr>
<tr>
<td>7</td>
<td>Clostridium neonatale</td>
<td>AF275949</td>
<td>99</td>
<td>C. neonatale</td>
<td>AF275949</td>
<td>99</td>
</tr>
<tr>
<td>8</td>
<td><em>Lactobacillus crispatus</em> TSK V40-4</td>
<td>AY190625</td>
<td>98</td>
<td><em>L. crispatus</em></td>
<td>AY190625</td>
<td>98</td>
</tr>
<tr>
<td>9</td>
<td><em>Shigella boydii</em></td>
<td>X96965</td>
<td>100</td>
<td><em>S. boydii</em></td>
<td>X96965</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>Uncultured bacterium clone p-308</td>
<td>AF371487</td>
<td>99</td>
<td><em>Lactobacillus pontis</em></td>
<td>AJ422033</td>
<td>99</td>
</tr>
<tr>
<td>11</td>
<td><em>Lactobacillus oris</em> MAB23</td>
<td>AF375889</td>
<td>99</td>
<td><em>L. oris</em></td>
<td>AF375889</td>
<td>99</td>
</tr>
<tr>
<td>12</td>
<td><em>Lactobacillus mucosae</em></td>
<td>AY190614</td>
<td>99</td>
<td><em>L. mucosae</em></td>
<td>AY190614</td>
<td>99</td>
</tr>
<tr>
<td>13</td>
<td><em>S. boydii</em></td>
<td>X96965</td>
<td>98</td>
<td><em>S. boydii</em></td>
<td>X96965</td>
<td>98</td>
</tr>
</tbody>
</table>

* By sequence comparison through BLASTn analysis.
* Band numbers correspond to those in Fig. 1A.
* Bacterial names were assigned to rDNA sequences with closest BLASTn matches to named organisms located in GenBank.

...demonstrated a differential response to the experimental treatments, DNA fragments were isolated and sequenced using the procedure essentially as described by Nielsen et al. (1999). Cloning and sequence analysis of the PCR amplicons generated from the DNA fragments were conducted as described previously (Li et al., 2003).

2.4. QPCR analysis

QPCR was performed as described (Gong et al., 2007). Primers HDA1 and HDA2 were used for the measurement of the total population of eubacteria. The population of lactobacilli and related lactic acid bacteria including *Leuconostoc* and *Pediococcus* was estimated using primers Lab-0159 (5'-GGAAACAGRTGCTAATACCG-3') and Univ-0515 (5'-ATCGTATTACCAGCGGTGCTGGCA-3') (Heilig et al., 2002; Collier et al., 2003).

Serial dilutions with a test range (10⁻¹ to 10⁻⁴) of *Lactobacillus acidophilus* DNA had equivalent QPCR reaction efficiencies. The QPCR data were analyzed using the 2⁻ΔΔCt method of Livak and Schmittgen (2001) to determine the relative abundance (fold change) of the target gene representing treatment effects. The cycle threshold, Ct, is the point at which fluorescence above the background is statistically significant. Ct values were determined with the MX4000 software based on a threshold line that was manually defined above the non-informative fluorescent data. ΔCt represents the difference between the Ct value with the primers to lactobacilli and the Ct value with the primers to eubacteria. ΔΔCt represents the difference between the ΔCt value of each dietary treatment and the ΔCt value of the control (Basal diets). The reference sample (Basal diets) has the 2⁻ΔΔCt value of 1.

3. Results

A primary analysis of the PCR–DGGE bacterial profiles from the same samples used in the present study was presented previously (Namkung et al., 2004). Fig. 1 shows further characterization of the ileal microbiota in response to the dietary treatments, including similarity analysis. Ileal bacterial microbiota of pigs pre-weaning was different from those of pigs post-weaning (Fig. 1B). *Clostridium* (represented by Bands 5–7) was mostly affected by time post-weaning (Fig. 1A and Table 1). Samples collected before weaning had similarity coefficients only between 46–62% when compared to those collected from post-weaning pigs on different diets. The inclusion of lincomycin, herbal extract, and organic acids in the diet resulted in a shift of the microbiota composition (Fig. 1A). While all the ileal samples from the pigs on the dietary treatments had similar PCR–DGGE profiles with 69–83% similarity coefficients, they showed a lower degree of similarity (56–69% similarity coefficients) to the microbiota from the pigs on the basal diet (negative control). All the samples had similar Shannon Index and Simpson’s Diversity Index (Table 2), indicating a similar degree of bacterial diversity.

The shift of microbiota composition was investigated. The groups of bacteria that were affected most by dietary treatment were related to *L. crispatus*, *L. oris*, *E. coli*, *Shigella boydii*, and uncultured bacteria (Table 1). The
Table 3
Fold changes in the population of Lactobacillus and related lactic acid bacteria determined by QPCR.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>LAB Ct</th>
<th>HDA Ct</th>
<th>ΔCt</th>
<th>ΔΔCt</th>
<th>2−ΔΔCt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal diet</td>
<td>17.4±0.24</td>
<td>14.9±0.17</td>
<td>2.6±0.38</td>
<td>0.0±0.38</td>
<td>1.0±0.77</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>18.7±0.18</td>
<td>17.8±0.09</td>
<td>1.0±0.11</td>
<td>−1.6±0.11</td>
<td>3.0±0.93</td>
</tr>
<tr>
<td>Herbal extract</td>
<td>17.2±0.26</td>
<td>16.3±0.03</td>
<td>0.9±0.28</td>
<td>−1.7±0.28</td>
<td>3.2±0.83</td>
</tr>
<tr>
<td>Acids-1</td>
<td>12.1±0.19</td>
<td>11.5±0.38</td>
<td>0.6±0.34</td>
<td>−1.9±0.34</td>
<td>3.8±0.79</td>
</tr>
<tr>
<td>Acids-2</td>
<td>14.6±0.36</td>
<td>13.7±0.08</td>
<td>0.9±0.32</td>
<td>−1.6±0.32</td>
<td>3.1±0.80</td>
</tr>
</tbody>
</table>

* Samples were run in triplicate. LAB: QPCR with primers specific to Lactobacillus and related lactic acid bacteria; HDA: QPCR with primers to eubacteria. ΔΔCt represents the difference between the Ct value of the control (a basal diet) and the Ct value with the primers to lactobacilli and the ΔCt value with the primers to eubacteria. 2−ΔΔCt: fold changes to represent relative abundance of the population (Reference samples = 1.0).

uncultured bacteria were those reported previously from pigs and related to L. delbrueckii, L. pontis, and Veillonella dispar. Lactobacillus and related lactic acid bacteria were examined by QPCR for their abundance in the ileal bacterial microbiota in response to the dietary treatments. All dietary treatments increased the abundance of Lactobacillus and related lactic acid bacteria by ≥ 3-fold (Table 3).

4. Discussion

The effects of dietary antibiotics on intestinal microbiota have been studied previously by both conventional microbiological and molecular biological techniques. The use of antibiotics can reduce the overall numbers and/or the numbers of species of intestinal bacteria (Jensen, 1998; Gaskins et al., 2002). Lactobacilli are common commensal bacteria in animal digestive tracts and have often been used as probiotics (Ljungh and Wadstrom, 2006). The intestine G+ flora (including lactobacilli) is normally depressed by growth promoting antibiotics (Van Assche et al., 1975; Vervaet et al., 1979; Engberg et al., 2000; Knaareborg et al., 2002), although increases are sometimes noted (Collier et al., 2003; Dumonceaux et al., 2006). In the present study, lincomycin was found to increase the abundance of lactobacilli in addition to causing the shift of microbiota composition. Similar observations were obtained with feeding blends of acids and herb extract. No conclusions can be drawn from the present study that the changes in ileal microbiota were responsible for growth promotion, given that only one acid blend (Acids-2) and lincomycin promoted the growth of 2-weeks post-weaning pigs (Namkung et al., 2004).

Individual animals can have considerable variations in their microbiota. We have observed such individual variations in both pigs (Richards et al., 2005) and broiler chickens (Gong et al., 2005) in our previous studies. As such, it is difficult to determine if changes in the composition of microbiota are a result of experimental effects, such as antibiotic or probiotic treatment, or are due to natural individual variations. We recently determined the optimal sample size of individual broiler chickens to be pooled for minimizing the natural individual variations and identifying experimental treatment effects (Zhou et al., in press). Five birds provided an optimal sample size for pooling (5 birds a sample) to examine changes in the composition of cecal microbiota influenced by experimental treatments with the PCR–DGGE profiling techniques. While pooling digesta samples precludes the possibility to examine individual animal’s response to dietary treatments, it offers the advantage to reduce the degree of the natural individual variations and increase the possibility to identify experimental treatment effects.

It was pointed out previously that PCR–DGGE profiling was more sensitive than conventional culturing techniques to identify diet effects on intestinal microbiota (Namkung et al., 2004). By using molecular techniques and additional analysis, the present study has further advanced our understanding on the effect of dietary treatments on the microbiota composition, particularly on the population of lactobacilli and related lactic acid bacteria.

Acknowledgements

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References


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