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Intestinal microbiota diversity and expression of pattern recognition receptors in newly weaned piglets

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ABSTRACT

This study evaluated the gastrointestinal microbial diversity and the expression of pattern recognition receptors (PRRs) of the small intestine during the first week post-weaning in newly weaned piglets. Sixteen piglets were sacrificed on days 0, 1, 4, and 7 post-weaning. Luminal contents from the stomach, ileum, and colon were collected to determine the microbiota diversity; intestinal mucosa from the ileum was collected to assess mRNA expression of PRRs, including toll-like receptors (TLRs) and NOD-like receptors (NLRs); sections of ileum were examined immunohistochemically to assess the immunoglobulin-secreting cells. The results showed that the number of denaturing gradient gel electrophoresis (DGGE) bands from the ileum and colon contents were significantly reduced in the d 4 post-weaning group. Biodiversity indexes (Shannon–Wiener index, richness index, and evenness index) were significantly decreased in the ileum of weaning groups. These indexes decreased in the colon of the d 4 post-weaning group. No significant differences were obtained in the stomach. With the exception of TLR5, the mRNA expressions of TLR2, TLR4, and TLR7 increased post-weaning. The mRNA expressions of NOD1 and NOD2 were significantly affected in the d 4 post-weaning group, and there were no significant differences in the d 1 or d 7 post-weaning groups. Analysis of the immunoglobulin-secreting (IgA, IgG, and IgM) cells showed that the ratio of each immunoglobulin was significantly higher on d 7 than d 0. The results revealed that microbial diversity was lower in the ileum and on d 4 post-weaning. Weaning significantly affected the expression of intestinal PRRs mainly on d 1 and d 4 post-weaning. The expression of specific PRRs was triggered by weaning to recognize distinctive microbiota and promote the development and maturation of the intestinal mucosal immunity.

1. Introduction

The weaning process is one of the most stressful events in the life of pigs that can contribute to gastrointestinal and immune system imbalances, particularly during the first week post-weaning [1]. With the introduction of solid foods and the deprivation of maternal milk, piglets must cope with a rapidly changing microbiota [2,3]. Recent studies have shown that the microbiota is involved in the maturation, differentiation, and proliferation of the intestinal mucosa at the cellular and molecular levels [4,5]. Furthermore, pattern recognition receptors (PRRs) have been shown to mediate the above process [6–8]. PRRs in the intestinal mucosa recognize and bind to distinctive microbial associated molecular patterns (MAMPs, also known as PAMP) such as lipopolysaccharide (LPS), peptidoglycan, zymosan, lipoproteins and lipoteichoic acid, etc [9]. By detecting MAMPs, PRRs activate a sequence of intracellular signaling pathways, such as nuclear factor κB(NF-κB) and mitogen-activated protein kinase (MAPK) pathways, which result in the induction of a range of cytokines and chemokines [10]. PRRs mainly include toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors, and C-type lectin receptors [11], in which TLRs and NLRs are especially important [12]. TLRs can differentiate among different types of MAMPs and induce the activation of the innate immune system [13]. TLRs are largely expressed within the digestive tract and are key components that mediate bacterial–host interactions and microbial recognition, specifically TLR subtypes 2, 4, 5, and 7 (TLR2, TLR4, TLR5, and TLR7) [14]. Similarly, NLRs are also important for the recognition of MAMPs [15]. NOD1 and NOD2 are specialized NLRs that participate in the recognition of a subset of pathogenic microorganisms. TLRs are localized either to the cell surface or within endosomes, whereas NODs are located within the cytoplasm [16]. Both TLRs and

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NLRs activate NF-κB and activated NF-κB stimulates the synthesis of proinflammatory cytokines, including TNF-α, IL-1β, and IL-6 [17]. Therefore, the objective of this study was to investigate changes in intestinal microbiota and mRNA expression of TLRs and NLRs during the first week post-weaning in newly weaned piglets.

2. Materials and methods

2.1. Animals

This study was conducted in accordance with the principles and procedures outlined by the Zhejiang Farm Animal Welfare Council of China and approved by the ethics committee of Zhejiang Academy of Agricultural Sciences. In this study, 16 Duroc × Yorkshire × Landrace piglets from four litters were used. The 7-day old piglets had ad libitum access to water and ground pre-starter feed. At 25 d of age, all animals with a pre-weaning body weight of 7.06 ± 0.23 kg were removed from their sows. Four of the pre-weaning piglets were sacrificed and assigned to the control group (d 0 group or suckling piglets). The remaining 12 piglets were weaned and had ad libitum access to water and ground starter feed. Four piglets were sacrificed at days 1, 4, and 7 post-weaning (d 1, d 4, and d 7 post-weaning groups, respectively). To correct for genetic differences, a piglet from each litter was randomly allocated to each of the four groups.

2.2. Sample preparation

The piglets were sedated with xylazine and ketamine and sacrificed with an overdose of intravenous pentobarbital via a catheterized ear vein. The abdominal cavity of the piglets was opened and the gastrointestinal tissue was removed. The luminal contents of the stomach from three different sites (cardia, fundus, and pylorus), and digesta from the midsection of the ileum and ascending colon were collected, transferred to an ice bucket, and subsequently stored −20 °C. For total RNA extraction, mucosal samples from the ileal sections were collected by scraping with a glass slide. Mucosal samples were immediately frozen in liquid nitrogen and subsequently stored at −80 °C. A block of ileum (about 1 cm in length) were removed and fixed in buffered formalin for immunohistochemical studies.

2.3. PCR/denaturing gradient gel electrophoresis

Total genomic DNA of the luminal contents was extracted using the QIAamp DNA stool kit (QIAGEN, CA, USA). DNA concentrations were measured in a NanoDrop1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) and standardized to 20 μg/ml prior to PCR. The variable V6–V8 region of bacterial 16S rRNA was amplified by PCR using the following primer sets with a GC clamp at the 5′ end, U968: 5′ -CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGG GAACGCGAA GAACCTTAC-3′ and L1401: 5′-CGGTGTGTACAAGACCC-3′ [18]. Amplicons of the V6–V8 region were used for sequence-specific separation by denaturing gradient gel electrophoresis (DGGE).

DGGE was performed in a DCode™ Universal Mutation Detection System (Bio-Rad, CA, USA). PCR fragments were separated in an 8% polyacrylamide gel in 1.0 × TAE buffer (20 mM trisacetate, pH 7.4, 10 mmol/L sodium acetate, and 0.5 mmol/L Na_2EDTA) with 35–47% linear gradients of denaturant [19]. The polyacrylamide gel was diluted from a non-deionized 40% acrylamide/bis stock solution (37.5:1). Electrophoresis was performed at 60 °C for 4.5 h at 200 V. Following electrophoresis, DGGE gels were silver-stained and scanned in an Image Scanner III (GE Healthcare, Waukesha, USA).

DGGE images were analyzed by Quantity One v.4.62 software (Bio-Rad, CA, USA). Three biodiversity indexes were calculated: 1) the richness index (S), which represents the number of bands in each lane; 2) the Shannon–Wiener index (H'), which was calculated by the equation, $H' = -\sum P_i \times \ln P_i$, where $P_i = n_i/N$ (n_i is the peak height of a band and N is the sum of all peak heights in the densitometric curve); and 3) the evenness index (E), which was calculated by the equation, $E = H'/\ln S$.

2.4. Real-time quantitative PCR

The mucosal samples were lyzed in a PRO 200 Post-Mounted Laboratory Homogenizer (PRO Scientific, Oxford, USA). Total RNA was extracted using an E.Z.N.A. HP Total RNA kit (Omega Bio-Tek, Norcross, USA); total RNA concentrations were determined in the NanoDrop1000 spectrophotometer. RNA quality was assessed by quantifying the amount of 28S and 18S rRNA following electrophoresis on a formaldehyde-containing 1% agarose gel and staining with ethidium bromide. Approximately 1 μg of the total RNA was reverse-transcribed in 20 μl of reaction mixture using the ReverTra Ace qPCR RT kit (TOYOBO, Osaka, Japan). All cDNA samples were diluted (1:10) with H_2O. The 18S rRNA gene was used as the endogenous reference gene [20]. The primers used in this study are shown in Table 1. Real-time quantitative PCR was performed using a standard SYBR Green Real-time PCR Master Mix (TOYOBO, Osaka, Japan) in an ABI StepOne Plus real-time PCR system (Applied Biosystems, Foster City, USA). Three replicates were run for every sample. Relative quantification (RQ) was performed by the $2^{-\Delta\Delta Ct}$ method.

2.5. Immunohistochemical examination

The assays were used to determine the ratio of ileac cells that secreted immunoglobulins(IgA, IgG and IgM). Sections of 2–3 μm were used to detect immunoglobulin-secreting cells by independent incubation with diluted rabbit primary antibodies (DAKO, Glostrup, Denmark) against IgA, IgG and IgM, respectively; HRP-conjugated goat anti-rabbit polyclonal antibodies were used as the secondary antibody, and counterstaining was performed with hematoxylin and eosin staining. The absolute number of positive cells for IgA, IgG and IgM were counted from the total number of immune cells, and the positive indices were expressed per 100 total immune cells for each immunoglobulin.

2.6. Statistical analyses

All statistical tests were performed using SPSS statistical software (SPSS, Chicago, USA). Comparisons between groups were performed using ANOVA or Kruskal–Wallis tests according to the Gaussian or non-Gaussian distribution of the data. Changes within each group were analyzed using one-way ANOVA or non-parametric tests. Data were expressed as the mean ± SEM. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Changes in gastrointestinal microbial diversity

The DGGE bands from the luminal contents of the stomach, ileum, and colon during the first week post-weaning are shown in Fig. 1. DGGE bands were successfully generated from the control (d 0) and weaned piglets (d 1, d 4, and d 7 post-weaning groups). Most of the predominant bands were present in all luminal contents of the control and weaned piglets. The results revealed that the DGGE