Short Communication

Assessing changes in composition of intestinal microbiota in neonatal BALB/c mice through cluster analysis of molecular markers

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The present study introduced a molecular biological approach to demonstrate changes in the composition of intestinal microbiota in neonatal mice. Female BALB/c mice were fed either a control diet or a diet supplemented with fructo-oligosaccharide (FOS) at 50 g/kg diet, and then mated to male mice. A cultivation-independent approach, denaturing gradient gel electrophoresis (DGGE) of the PCR-amplified 16S rRNA gene, was performed to characterise changes in intestinal microbial populations in pups at 0, 7, 14 and 21 d old and their dams. Comparisons of DGGE profiles were performed using the Dice similarity coefficient and the unweighted pair group method with arithmetic mean (UPGMA) cluster analysis based on numbers, positions and intensities of bands. DGGE profiles differed between dams fed control and FOS-supplemented diets. Although profiles in pups on the day of birth showed a high similarity with dams, profiles in 7-d-old pups differed from dams and showed high similarity to littermates. In 14- and 21-d-old pups, profiles again showed high similarity with dams. DGGE profiles in pups were divided into two large clusters of control and FOS-supplemented diet groups in the range of 0- to 21-d-old, suggesting modulation of intestinal microbiota in infants by manipulation of microbiota in dams. The present study shows a useful technique for demonstrating changes in intestinal microbiota and provides a mouse model for modulation of intestinal microbiota in neonatal life.

Fructo-oligosaccharides: Intestinal microbiota: Polymerase chain reaction–denaturing gradient gel electrophoresis: Cluster analysis: Neonatal mice

About 400 different microbial species, mostly bacteria, have been estimated to inhabit the intestinal tract of mammals, and bacterial density can exceed 10^{11} cells/g contents[1]. The intestinal tract is sterile *in utero*, and the colonisation process begins as the neonate is exposed to bacteria in the environment, birth canal, maternal faeces and other sources during and after birth[2–5]. Intestinal microbiota thus develop rapidly after birth and are initially strongly dependent on maternal faeces and vaginal microbiota. Modulation of the intestinal microbiota in infants could thus theoretically be achieved by manipulating the maternal microbiota.

Experimental and clinical studies have reported that modulation of intestinal microbiota may be effective in preventing and treating allergic diseases in infancy and childhood, but not in later life[6–9]. To further investigate this, we aimed to modulate the intestinal microbiota in neonatal mice. As some indigestible oligosaccharides such as fructo-oligosaccharides (FOS) have been known to change the composition of intestinal microbiota[10–12], the present study investigated the development of intestinal microbiota in neonatal mice whose dams were fed FOS during periods of

pregnancy and lactation. To observe changes in the composition of intestinal microbiota, we used a cultivation-independent molecular biological technique based on bacterial 16S rRNA gene sequences. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA fragments is reportedly applicable to monitor changes in the composition of intestinal microbiota[13–15]. The present study compared DGGE profiles through hierarchical cluster analysis based on numbers, positions and intensities of bands[16].

Materials and methods

Animals and diets

Male (8 weeks old) and female (9 weeks old) BALB/cCr Slc mice were purchased from Japan SLC (Hamamatsu, Japan) and housed in plastic cages in a temperature-controlled (23 ± 2°C) room with a dark period from 20.00 to 08.00 hours. Mice were allowed *ad libitum* access to food and water, and were fed either a synthetic diet prepared according to AIN-93G guidelines[17] or the same diet supplemented with

FOS (Meioligo P; donated by Meiji Foodmateria, Tokyo, Japan) for 3 weeks. These diets were referred to as FOS (−) and FOS (+), respectively. FOS (+) was prepared by adding FOS at 50 g/kg diet to FOS (−) at the expense of α-maize starch. Three females were then mated to one male in one cage, and pregnant mice were housed individually. Day of birth was referred to as day 0 of neonatal life. Pups and dams were housed in the same cage until killing.

All study protocols were approved by the Hokkaido University Research Faculty of Agriculture Animal Use Committee (approval no. 16), and animals were maintained in accordance with the guidelines for the care and use of laboratory animals of Hokkaido University.

Extraction of RNA

Pups and dams were anaesthetised by diethyl ether and killed by exsanguination from the carotid artery. After laparotomy, the entire intestinal tract and contents were excised from pups at 0, 7 and 14 d old, and samples were subjected to RNA extraction. For pups at 21 d old and dams, caecal contents were used. Samples were immediately frozen in liquid N_2 and stored at −80°C until use. For RNA extraction, samples were homogenised after adding 1 ml Trizol reagent (Invitrogen, Carlsbad, CA, USA) using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) at 2000 rpm for 10 s. RNA was then extracted in accordance with the instructions of the manufacturer for Trizol reagent and stored at −80°C until use.

Reverse transcription and polymerase chain reaction

Total RNA samples were treated with DNase to remove any genomic DNA. Approximately 15 ng total RNA was then annealed with 100 pmol of primer L1401-r (5′ GCG TGT GTA CAA GAC CC)[18] at 65°C for 5 min, and first-strand cDNA was subsequently synthesised using a Super Script First-Strand Synthesis System (Invitrogen) according to the instructions of the manufacturer. Samples were then treated using a QIAquick PCR purification kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions.

PCR was performed to amplify V6 to V8 regions of bacterial 16S rRNA using a *Taq* Hot Start Version kit (Takara, Otsu, Japan). A first-strand cDNA sample (10 ng) was thus added to 25 μl of PCR reaction mixture comprising 0·5 μM of primers L1401-r and U968-GC-f (5′ CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC)[18], 1 × PCR buffer, 0·2 mM-dNTP, and 1·25 U of *Taq*-HS polymerase (Takara, Otsu, Japan). Samples were amplified in a PCR thermal cycler (Takara) using the following program: initial denaturation at 95°C for 1 min; thirty-five thermal cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 50 s; and final extension at 72°C for 7 min. Amplification was checked under electrophoresis by running samples of amplicons on a 1·5 % agarose gel.

Denaturing gradient gel electrophoresis of polymerase chain reaction products

Amplicons from RT-PCR were analysed by DGGE using a DCode system (Bio-Rad Laboratories, Tokyo, Japan) on an 8 % polyacrylamide gel (dimension, 160 × 180 × 1 mm) containing a 40–60 % gradient of urea–formamide, where 100 % was defined as 7 M-urea and 40 % formamide. Electrophoresis was performed in 1 × TAE (40 mM-tri(hydroxymethyl)-aminomethane, 20 mM-acetic acid, 1 mM-EDTA) at 80 V, 60°C for 16 h. The gel was stained for 30 min in 1 × TAE containing SYBR green nucleic acid gel stain (Cambrex Bio Science Rockland, Inc., Rockland, ME, USA). The gel image was incorporated into a computer file using a 400EX gel documentation device (Aishin Seiki, Kariya, Japan).

Data analysis

One lactating mouse and four pups at each time point for each feeding treatment were used for analysis. Quantity One software (version 4.6.0; Bio-Rad Laboratories) was used for identification of bands and normalisation of band patterns from DGGE gels and for comparison of DGGE band profiles. The Dice similarity coefficient (Cs) was used for computing sample similarity based on band position and intensity. The formula for Cs values is as follows:

$$Cs = 200 \times \frac{\sum Min(s, t)}{\sum (s + t)},$$

where s and t are vectors representing two lanes in the same band set that are being compared. The unweighted pair group method with arithmetic mean (UPGMA) algorithm was used to construct a dendrogram of DGGE band profiles. The UPGMA algorithm is defined as follows:

$$d_{ki} = \left(\frac{n_p}{n}\right) \times d_{pi} + \left(\frac{n_q}{n}\right) \times d_{qi},$$

where p and q indicate two clusters that are to be joined into a single cluster, k is the index of the cluster formed by joining clusters p and q, i is the index of any remaining clusters other than clusters p, q or k, n_p is the number of samples in the pth cluster, n_q is the number of clusters in the qth cluster, n is the number of clusters in the kth cluster formed by joining the pth and qth clusters (n = n_p + n_q), and d is the distance matrix (Quantity One Software Manual; Bio-Rad Laboratories).

Results and discussion

A representative DGGE gel image is shown in Fig. 1 (A). Dams 0, 1, 2 and 3 in Fig. 1 (A) indicate the lactating mice having 0-, 7-, 14- and 21-d-old pups, respectively. Consumption of indigestible oligosaccharides reportedly influences the intestinal microbiota. This has been demonstrated not only by traditional bacteriological techniques[10–12] but also by recent cultivation-independent approaches[19,20]. The dendrogram in Fig. 1 (B) shows two large clusters of dams fed FOS (−) and FOS (+). The results suggest that dietary FOS influences the intestinal microbiota in mice and are consistent with previous reports[19,20].

The molecular biological approach in the present study clearly demonstrated the transmission of intestinal microbiota from dams to neonates. On the day of birth (i.e. day 0), DGGE band profiles are divided into two large clusters: one comprising neonates and dams fed FOS (−), and the other comprising neonates and dams fed FOS (+) (Fig. 1 (C)). Band profiles in neonates on day 0 thus show high similarity with dams. Transmission of microbes from mothers to neonates has
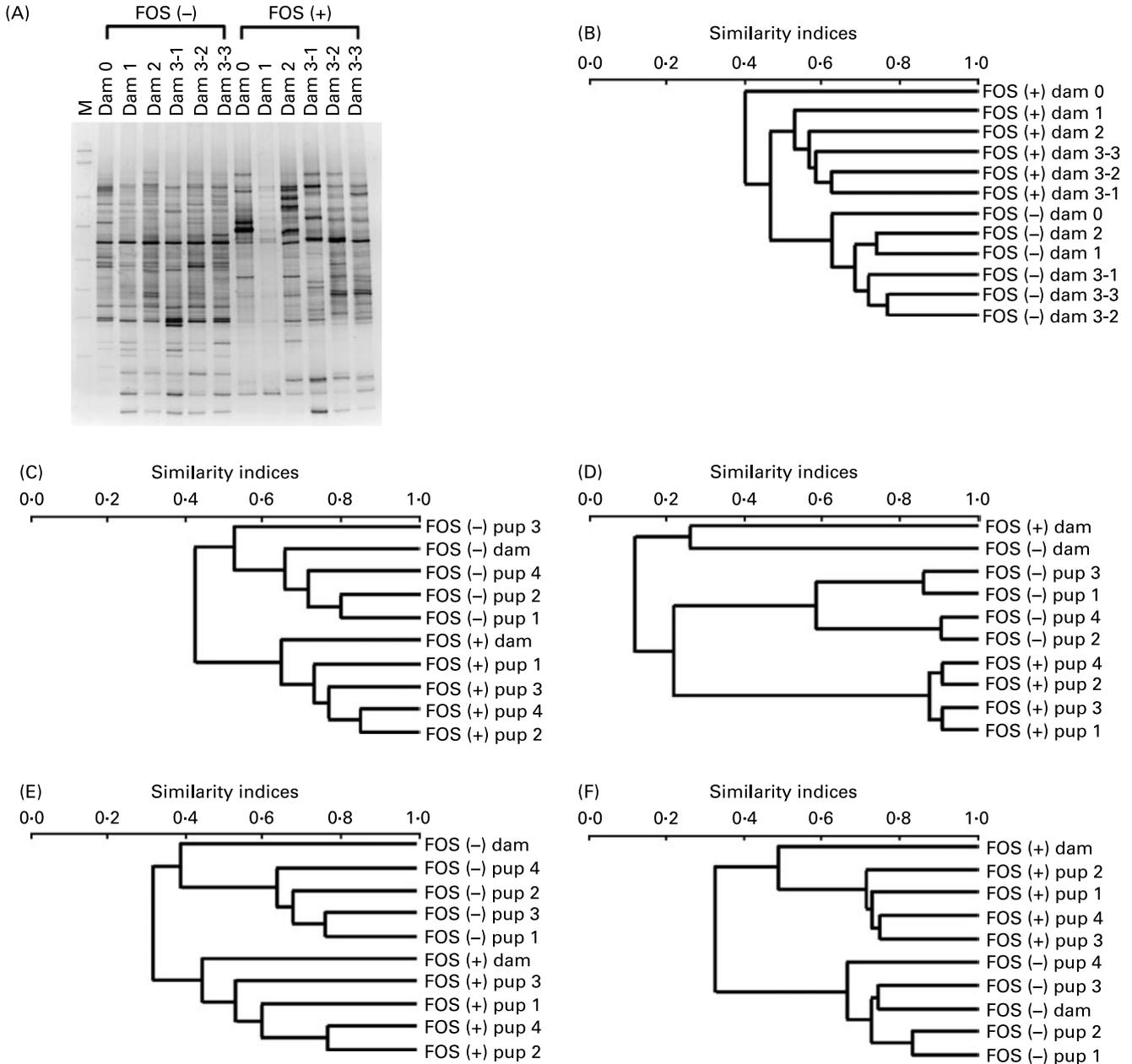
Fig. 1. PCR−denaturing gradient gel electrophoresis (DGGE) analysis of caecal microbiota based on 16S rRNA sequences in lactating mice (dams) fed a diet containing fructo-oligosaccharide (FOS (+)) or a diet containing no FOS ((FOS) (−)) and offspring (pups). (A) Representative DGGE gel image for lactating mice. Dams 0, 1, 2 and 3 represent the lactating mice with 0-, 7-, 14- and 21-d-old pups, respectively. Three lactating mice with 21-d-old pups are shown as dams 3-1, 3-2 and 3-3. (B−F) Dendrograms of DGGE band profiles constructed by the Dice similarity coefficient and the unweighted pair group method with arithmetic mean (UPGMA) cluster analysis in dams and 0-, 7-, 14- and 21-d-old pups with dams, respectively. Four pups in each group are shown. Distances are measured in arbitrary units. M, marker.

been shown in human subjects by traditional bacteriological techniques[21−23], suggesting that intestinal microbiota in neonates immediately after birth is strongly dependent on the mother, and the present data are in agreement with this.

In marked contrast to 0-d-old pups and dams, the dendrogram shows three large clusters of dams and littermates in FOS (−) and FOS (+) groups (Fig. 1 (D)), indicating that DGGE band profiles of 7-d-old pups differ substantially from dams in both FOS (−) and FOS (+) groups and that profiles of DGGE bands in littermates are similar in each group. As 7-d-old mouse pups are usually maintained entirely on breast milk, the complete difference in diets between 7-d-old pups and

their dams probably led to this difference in intestinal microbiota, which would be reflected in different profiles of DGGE bands. Nevertheless, band profiles in 7-d-old pups show two large clusters of FOS (−) and FOS (+) groups with high similarity between littermates. As pups and their dam were housed in the same cage, FOS in the dam's diet could influence intestinal microbiota in suckling mice through bacterial exposure from dams after birth. Alternatively, an attractive possibility is that FOS in the dam's diet may affect the composition of breast milk, and thus influence the intestinal microbiota in suckling mice. However, whether intestinal microbiota influences the composition of breast milk remains unclear.

In 14- and 21-d-old pups and their dams, the dendrogram shows two large clusters of FOS (−) and FOS (+) groups (Figs. 1 (E) and 1 (F), respectively). In each group, pups and their dams form the same cluster, while higher similarity is observed among littermates than between pups and dams. Similarities in intestinal microbiota between pups and dams were thus higher in 14- and 21-d-old pups than in 7-d-old pups. This may be due to the addition of solid food to the diet in addition to breast milk.

In conclusion, the present study clearly showed that PCR–DGGE coupled with cluster analysis is useful to study changes in the composition of intestinal microbiota. In addition, the present findings suggest that intestinal microbiota in infants could be modulated by manipulating microbiota in dams, and this could be achieved by controlling the maternal diet. The present study therefore provides a mouse model to modulate intestinal microbiota in neonatal life. This model could, for example, enable investigation of the mechanisms by which intestinal microbiota in neonates influences development of the immune system and onset of allergic diseases later in life. We are now examining whether administration of FOS to pregnant and lactating mice influences mucosal immune functions and severity of allergic symptoms in offspring after growth.

Acknowledgements

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