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Comparison of Bacterial Composition in Blood and Placentas Using Conventional and Molecular Methods

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Abstract

Fetus was considered to be sterile and little work is done to explore the ignored microbial diversity in it. To investigate the bacterial diversity in the feces, blood and placentas of pregnant mice and to assess the translocation capability of the administered strains, conventional culture and PCR denaturing gradient gel electrophoresis (PCR-DGGE) were used in the present study. The culture method detected microbes (*Lactobacilli*, *Enterobacter* and *Enterococcus*) in the blood and in the placentas at positive rates of 27.8%, 55.6% and 11.1% and 22.2%, 66.7% and 16.7%, respectively. The PCR-DGGE results showed that the *E. faecalis*, *L. lactis* and an uncultured bacterium were the dominant bacteria found in the feces, blood and placentas, and the transport of *E. faecalis* FD3 from the intestinal tract to the blood and to the placenta was also observed, revealing a potential risk to the health of both mother and fetus. In conclusion, the combination of classical cultivation and PCR-DGGE methods provides a superior strategy for the fast and accurate monitoring of the microbial composition in the blood and in the placentas of pregnant mice.

Keywords: PCR-DGGE; Bacteria; Blood; Viable cell count; Placenta

Introduction

Fetuses have been considered to be sterile since Tissier's era [1], and it is generally believed that the initial bacterial colonization in the newborn's intestinal tract is from the cross-contamination with the vaginal and fecal bacteria of the maternal microflora during the transit through the birth canal [2-5]. In this context, the meconium, amniotic fluid, chorioamnion and placenta tissue have been considered sterile under normal conditions unless there are symptoms of infection or circumstances that may facilitate infection (e.g., premature rupture of membranes or cervical dilation) [4]. However, recent studies have indicated that bacteria could be isolated and/or detected by PCR in the umbilical cord blood, amniotic fluid, meconium and fetal membranes without any clinical or histological evidence of infection or inflammation in the mother-infant pair [4,6-8].

The placenta is an organ that connects the developing fetus to the uterine wall to allow nutrient uptake, waste elimination, and gas exchange via the mother's blood supply. The presence of any microorganism in the placenta is a major concern because of the production of inflammatory mediators, which may lead to the increased production of prostaglandins that are possible mediators of preterm birth, the leading cause of infant morbidity and mortality in the United States [9]. Presently, the knowledge of the bacterial diversity in the placenta is very limited and is almost exclusively based on the use of culture media or the PCR method with specific primers. This limitation implies that the presence of additional bacterial species that are not cultivable and are unsuitable for the specific PCR may have been overlooked.

Bacteria have been isolated/detected from breast milk, umbilical cord blood, amniotic fluid and chorioamnion, which had been considered sterile environments under normal conditions [3,4,10-13]. Therefore, the identification of any microorganism in these sources is a potential concern for the risk of preterm birth and for the impact on the neonatal immune system [9,14]. Although universal and specific bacterial primers have been used in PCR to detect the presence of the bacteria, the low-resolution of agarose gel electrophoresis makes it impossible to distinguish the different PCR products (of the same/similar lengths) that are clustered in the same band. DGGE is a powerful tool that can distinguish different PCR products, even those with a 1 bp difference [15]. As DGGE cannot detect the minor species that represent <1% of the total bacterial population, the combination of culture-dependent and culture-independent methods is a good solution to study microbial diversity [16,17].

In the present study, we evaluated the bacterial diversity in the feces, blood and placentas of pregnant mice, which is helpful for the health of mothers and infants.

Materials and Methods

Oral administration of bacteria to pregnant mice

Ten-week-old female BALB/c mice were used in this study. The mice were kept in a pathogen-free facility, and all protocols that were used in this study were described previously [18]. Pregnant females were purchased from the experimental animal center of nanchang university, housed in a specific pathogen-free facility and randomly divided into 6 groups (3 mice/group). Mice in groups 1-5 were orally administered 0.5 ml of water containing 10^8 colony-forming units

(CFUs) of *L. plantarum* WCFS1, *B. longum* NCC2705, *E. faecalis* FD3, *E. coli* O157:H7, or the DNA mixture (of equal amounts of DNA of *L. plantarum* WCFS1, *B. longum* NCC2705, *E. faecalis* FD3 and *E. coli* O157:H7) for 1 week, respectively, and the mice in group 6 were administered water alone. The oral administration of bacteria was continued daily at the same dosage until labor. One day before the predicted date of labor, the mice were euthanized with CO_2, and their blood and placentas were immediately collected using aseptic procedures by Caesarean section [19]. The samples and the feces collected before and after bacterial administration were used in the evaluation of bacterial biomass and species. The study was approved by the ethics committee of nanchang university (China).

Cultivation of bacteria

All samples were processed within 3 h after collection. For the placental samples, 0.1 g of tissue was minced with a sterile, sharp blade, suspended in 0.9 ml of sterile 0.9% sodium chloride solution and then serially diluted 10-fold. Fifty microliters of each dilution was plated separately in duplicate on de man-rogosa-sharpe (MRS) agar plates for *Lactobacilli*, slanetz-bartley medium (SBM) for *Enterococci*, and MacConkeyagar (Mac) for *Escherichia coli*. The plates were incubated aerobically or anaerobically at 37°C for 24 h-36 h. The blood and fecal samples were diluted and cultured using the same procedure as for the placental samples.

DNA extraction and PCR amplification

DNA was extracted and purified according to a bead-beating method [20]. After phenol-chloroform extraction, DNA was precipitated with ethanol and resuspended in 50 μl of TE buffer. Primers 357f (5′-TACGGGAGGCAGCAG-3′) and 519r (5′-ATTACCGCGGCTGCTGG-3′) were used to amplify the V3 regions of bacterial 16S rDNA [21]; a GC clamp in each primer was used to produce PCR products that were suitable for separation by DGGE [22]. PCR was performed with the Taq DNA polymerase kit from Life Technologies (Takara, Shanghai, China) according to the manufacturer's instructions. The PCR reaction mixture (25 μl) consisted of 0.125 μl of Taq DNA polymerase (1.25 U), 0.5 μl of each primer (concentration), 1 μl of ten-fold diluted DNA template (approximately 1 ng), 2.5 μl of 10 × PCR buffer, 1.5 μl of $MgCl_2$ (50 mM), and 18.875 μl of UV-sterilized water. DNA was amplified in a biosci thermal cycler (with 30 cycles of 94°C for 30 s, 56°C for 30 s, and a final extension at 72°C for 5 min. Five microliters of the PCR mixtures was analyzed by electrophoresis on an agarose gel (1%), followed by staining with ethidium bromide and visualization under UV light to determine the sizes and amounts of the amplicons.

PCR-DGGE and DNA sequencing

The DGGE analysis of PCR amplicons was performed according to the method that was described by Nicolaisen and Ramsing [23]. Briefly, after the PCR reaction, the PCR products (3.5 μl) were separated on a 35%-65% denaturing polyacrylamide gradient gel by electrophoresis. The gel was then stained with silver. The DGGE fingerprints obtained were subsequently normalized and analyzed using the bio numeric software version 2.0 (applied math, London, England). During the analysis, the different lanes were defined, and the background was subtracted. The differences in the intensity of the DGGE lanes were compensated for during normalization, and the correlation matrix was calculated. Clustering was performed using the pearson correlation and the UPGMA methods. The bands of interest were then excised from the gel using a sterile blade and incubated overnight at 4°C in TE buffer (pH 8.0) to allow DNA diffusion out of the polyacrylamide matrix. The solution was used directly for further amplifications. For sequencing, the eluted DNA was amplified using the same primer pairs but without the GC clamps. The PCR products for sequencing were purified using the QIAquick PCR purification kit. The PCR products were subcloned using the pMD18-T vector system I (Takara) according to the manufacturer's instructions. Escherichia coli DH5α cells were electrotransformed with recombinant plasmids using a standard method [24]. Selection of transformants was performed on LB agar containing 100 mg of Ampicillin/ml. Transformants were randomly picked and sequenced by invitrogen (Shanghai, China). The sequences were checked using the BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The bacterial species that contained the highest sequence similarities to the partial 16S rDNA sequences were determined by using BLASTN 2.2.1 (http://www.ncbi.nlm.nih.gov/blast/).

Statistical analysis

The data are expressed as the mean ± SE. The groups were compared using Student's t-test; statistical significance was defined as $P<0.05$, and high statistical significance was defined as $P<0.01$.

Results

Bacterial number identified in blood and placenta

Blood and placental samples were collected from treated mice and were used in conventional agar plate cultivation. In general, the positive identification rates of *Lactobacilli*, *Enterobacter* and *Enterococcus* present in the blood and in the placenta were 27.8%, 55.6% and 11.1% and 27.8%, 66.7% and 16.7%, respectively (Table 1). The microbial density in most samples was less than 10^3 CFU/ml, and the unexpected abundance in Ctrl-2 might be caused for the individual difference. A total of 54 clones were selected according to their colonies, cell morphologies and culture conditions (aerobic/anaerobic); DNA from these colonies was extracted and used in the sequencing of the 16S ribosomal rDNA for species identification. Nine distinct isolates were confirmed as *Enterococcus faecium*, *Staphylococcus epidermidis*, *Weissella confusa*, *Lactobacillus johnsonii*, *Lactobacillus reuteri*, *Lactobacillus murinus*, *Lactococcus lactis*, *Streptococcus thoraltensis*, and *Staphylococcus* species (Table 2).

Group	Blood (CFU/ml)				Placenta (CFU/ml)		
	MRS	Mac	SBM		MRS	Mac	SBM
Ctrl-1	-	440	-	Ctrl-1	-	-	-
Ctrl-2	720	400	580	Ctrl-2	12360	8680	8440
Ctrl-3	-	540	-	Ctrl-3	-	780	-
LaP-1	-	-	-	LaP-1	-	-	-
LaP-2	-	-	-	LaP-2	240	160	-
LaP-3	-	-	-	LaP-3	-	260	-
BaL-1	-	-	-	BaL-1	-	-	-
BaL-2	300	100	-	BaL-2	40	20	

BaL-3	-	220	-	BaL-3		100	100
EnF-1	-	540	-	EnF-1	-	100	-
EnF-2	-	-	-	EnF-2	-	-	-
EnF-3	-	-	-	EnF-3	-	100	-
EnC-1	-	340	-	EnC-1	-	240	-
EnC-2	260	40	-	EnC-2	300	60	-
EnC-3	-	-	-	EnC-3	-	-	200
DNA-1	340	-	-	DNA-1	-	-	-
DNA-2	-	340	-	DNA-2	420	100	-
DNA-3	120	200	220	DNA-3	-	240	-
Positive NO.	5	10	2		5	12	3

Table 1: Bacterial detection from blood and placental samples obtained from each pregnant mouse using the conventional agar cultivation method.

Strain No.	Closest relatives	Similarity (%)	GeneBank No.	Length (bp)
1	*Enterococcus faecalis*	99%	JF728295.1	1465
2	*Staphylococcus epidermidis*	99%	AF270147.1	1465
3	*Weissella confusa*	98%	DQ321751.1	1465
4	*Lactobacillus johnsonii*	99%	EU381128.1	1465
5	*Lactobacillus reuteri*	98%	CP000705.1	1465
6	*Lactobacillus murinus*	99%	AB326349.1	1465
7	*Lactococcus lactis*	99%	CP002094.1	1465
8	*Streptococcus thoraltensis*	99%	NR-026368.1	1465
9	*Staphylococcus* species	99%	DQ337534.1	1465

Table 2: Identification of isolates from Table 1 using 16S ribosomal DNA fragment sequencing.

Bacterial diversity in feces before and after the oral administration of bacteria

PCR-DGGE, a culture-independent method, has been proven to be more sensitive and faster than the conventional culture-dependent cultivation method [25]. To further investigate the bacterial diversity in pregnant mice, we also included feces, along with the blood and placental samples, in the PCR-DGGE analysis. DNA was extracted from only feces before the oral administration of bacteria and from feces, blood, and placenta after the oral administration of bacteria, and DGGE was performed to compare the bacterial composition in each sample.

Our viable cell count results indicated that there were significant differences in the number of *Lactobacilli* before and after the oral administration of bacteria; however, there was a slight drop in the cell number mainly due to the physical damage from the gavages. The increase in the number of *Enterobacter* was significant or highly significant after the oral administration of water, *E. faecalis* FD3, *E. coli* O157:H7, or the DNA mixture. Similar to *Lactobacilli*, the number of *Enterococcus* changed slightly except for the group that was administered *E. faecalis* FD3, whose significant increase relied mainly on the administration of viable *E. faecalis* (Figure 1). Interestingly, the numbers of bands in the DGGE fingerprint of the treated group were significantly decreased compared with the control group (Figure 2).

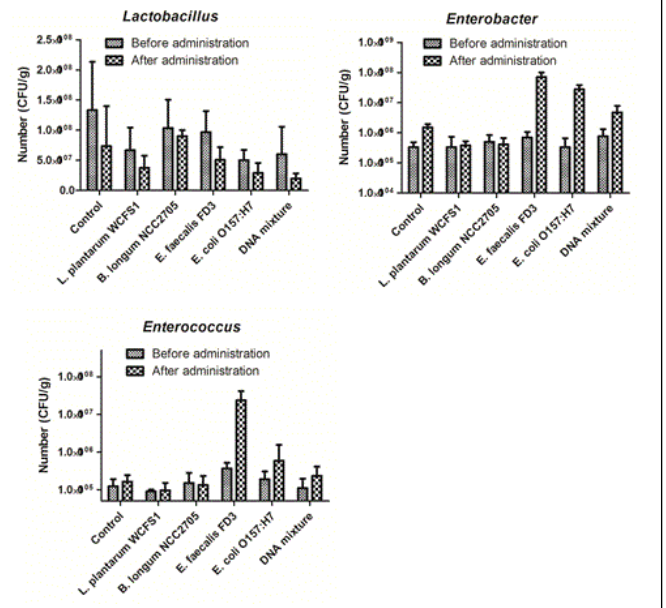

Figure 1: The bacterial number of *Lactobacillus* **(A)**, *Enterobacter* **(B)** and *Enterococcus* **(C)** before and after the administration of *L. plantarum* WCFS1, *B. longum* NCC2705, *E. coli* O157:H7 and the DNA mixture ($^{*}p<0.05$; $^{**}p<0.01$).

Bacterial translocation from the intestinal tract to the blood and placenta

To determine the capability of bacterial translocation from the intestinal tract to the blood and from the blood to the placenta, we compared the dominant bacterial species in the blood and placentas of the pregnant mice using DGGE and sequencing. Our results demonstrated that only *E. faecalis*, whose DNA showed as a distinctive DGGE band (d1) in the fingerprint (Figure 2D), was detected in the blood and in the placenta, indicating its translocation from the intestinal tract to the blood and to the placenta.

Compared with the feces, the bacterial compositions in the blood and placentas of the pregnant mice were much less diverse. The dominant bacteria found in the blood and placental samples were strikingly similar and were composed mainly of *E. faecalis*, *L. lactis*, and an uncultured bacterium that was detected in most samples (Figure 2 and Table 3).

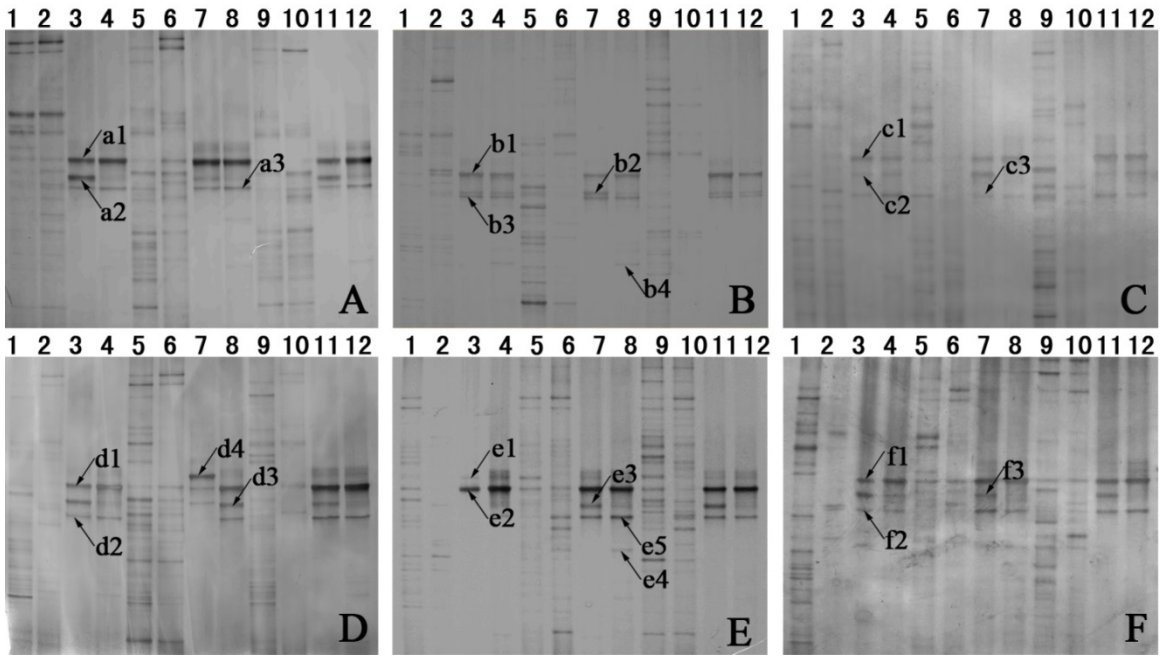

Figure 2: The DGGE fingerprint of the bacterial 16S ribosomal DNA gene fragments that were amplified from the feces, blood, and placental samples obtained from three BalB/c mice that were orally administered sterile water **(A)**, *L. plantarum* WCFS1 **(B)**, *B. longum* NCC2705 **(C)**, *E. faecalis* FD3 **(D)**, *E. coli* O157:H7 **(E)** and the DNA mixture **(F)** (DNA from *L. plantarum* WCFS1, *B. longum* NCC2705, *E. faecalis* FD3 and *E. coli* O157:H7). Samples that were loaded in Lanes 1~4, 5~8 and 9~12, were from three different mice, respectively. Lanes 1, 5, and 9 represent the fecal samples before administration, and lanes 2, 6, and 10 represent those after administration. Lanes 3, 7, and 11 represent the blood samples after administration; lanes 4, 8, 12 represent the placental samples after administration. The typical bands in the DGGE patterns, which are marked with arrows, were selected, sequenced and categorized as the different species listed in Table 3.

Strain No.	Closest relatives	Similarity (%)	GeneBank No.	Length (bp)
Control				199
a1	*Enterococcus faecalis*	99	JF728295.1	199
a2		100	FJ471462.1	199
a3	*Lactococcus lactis*	99	JF703669.1	199
L. plantarum WCFS1				
b1	*Enterococcus faecalis*	99	JF728295.1	199
b2		100	FJ471462.1	199
b3	*Lactococcus lactis*	99	JF703669.1	199
b4	*Lactococcus lactis*	99	HQ721275.1	199
B. longum NCC2705				
c1	*Enterococcus faecalis*	99	JF728295.1	199
c2		100	FJ471462.1	199
c3	*Lactococcus lactis*	99	JF703669.1	199
E. faecalis FD3				
d1	*Enterococcus faecalis*	99	HQ603180.1	199

d2	*Enterococcus faecalis*	99	JF728295.1	199
d3		100	FJ471462.1	199
d4	*Enterococcus faecalis*	99	JF728295.1	199
E. coli O157:H7				
e1	*Enterococcus faecalis*	99	JF728295.1	199
e2	*Enterococcus faecalis*	99	JF728295.1	199
e3		100	FJ471462.1	199
e4	*Erwinia psidii*	99	EU490596.1	199
e5	*Lactococcus lactis*	99	JF703669.1	199
DNA mixture				
f1	*Enterococcus faecalis*	99	JF728295.1	199
f2	*Lactococcus lactis*	100	JF703669.1	199
f3		100	FJ471462.1	199

Table 3: Identification of bacterial species based on the 16S ribosomal DNA fragment sequencing results of the selected bands in the DGGE fingerprint shown in Figure 1.

Discussion

In the present study, four strains (*L. plantarum* WCFS1, *B. longum* NCC2705, *E. faecalis* FD3, and *E. coli* O157:H7) that are commonly isolated from food were used to evaluate their effects on the bacterial diversity in the feces, blood and placentas of pregnant mice. In addition, *E. faecalis* FD3 (which was reported to possess the translocation capability) and a DNA mixture of all four strains (which can exert immunostimulatory effects and improve the survival of both pregnant and fetal mice) were also evaluated [3,4,26,27].

In previous studies, scientists had isolated *E. fecalis*, *S. epidermidis* and *E. coli* from the meconium and noted that the *Lactobacilli* or *Bifidobacteria* should be present in these "sterile sites" in higher numbers; our identification of *W. confusa*, *L. johnsonii*, *L. reuteri*, *L. murinus* and *L. lactis* proved the presence of *Lactobacilli* [3,4,9]. The mouse intestines were predominantly occupied by *Lactobacilli*. Therefore, it is not surprising that *Lactobacilli*, rather than *Bifidobacteria*, were detected in the placentas of the pregnant mice; hence, *Bifidobacteria* may found in the human placenta based on their dominance in the human intestine.

The bacterial groups play important biological roles in the bacterial diversity in the neonatal gut and in the neonatal immunity [3,10]. In previous studies, *S. epidermidis* was reported to be the predominant bacterial species in breast milk of healthy women, and its presence was associated with a decrease in infection rates; *W. confusa*, *L. johnsonii*, *L. reuteri*, *L. murinus* and *L. lactis* are commonly seen in human/animal intestines and in fermented foods, which are claimed to ease health problems, ranging from constipation to diarrhea to colds (both preventing and fighting them), and are associated with a slightly higher birth weight and delivery date [3]. Moreover, *L. lactis*, a Gram-positive bacterium that is used extensively in the production of buttermilk and cheese but has recently also become noted as the first genetically modified organism to be used live in the treatment of human disease [6,28], was first isolated and identified from the blood and placentas of pregnant mice. However, most of the bacterial species isolated in this study are known to be opportunistic pathogens in the placenta of a pregnant mouse suffering from other underlying conditions; this negative role most likely reflects the easy access of such bacteria to predisposed infants and may be a part of their endogenous microbiota, even at the fetal stage. *E. fecalis* was traditionally used as food starter cultures and as probiotic or bioprotective cultures [29] but is currently banned for use in food and drugs because of its vancomycin resistance [30]. Our study [31], along with the research performed by Jiménez et al [4], have confirmed the translocation capability of *E. fecalis* from the mouse intestines to the placenta (Figure 2); therefore, its effects (beneficial or harmful) on the health of the mother and the fetus during pregnancy require a more detailed study.

In addition, we also evaluated the bacterial changes in mouse feces before and after the oral administration of bacteria. The slight changes in the numbers of *Lactobacilli* and *Enterococcus* (Figure 1) indicated the minor effects of foreign bacteria on the total amounts of *Lactobacilli* and *Enterococcus*, but there was a significant/highly significant increase in *Enterobacter* after the oral administration of water, *E. faecalis* FD3, *E. coli* O157:H7, and the DNA mixture, indicating that the physical damage and administration of additional bacteria enhanced the number of pathogens. However, the low number of *Enterobacter* in the *L. plantarum* WCFS1 and *B. longum* NCC2705 groups suggested that the administration of probiotics could inhibit the growth of the pathogens and reduce the harm to the host. Interestingly, the number of DGGE bands in the treated groups (not only for the pathogens group but also for the probiotics groups) significantly decreased compared with the control group (Figure 2), indicating a reduction in bacterial diversity in these groups. Generally, a higher abundance and larger species diversity of the human gut microbiota were viewed as beneficial for host protection [32-34]; the reduction of bacterial diversity after probiotics administration (*L. plantarum* WCFS1 and *B. longum* NCC2705) suggested that the

administration of probiotic products may pose a negative effect on the health of both mother and fetus (a detailed research study is needed to describe the microbial discrepancy between human and mice intestines).

The adult human intestine is colonized by 10 trillion to 100 trillion microbes of more than 1000 species, among which the probiotic microorganisms are predominant [35,36]. The gnotobiotic models have revealed that the microbiota facilitates the breakdown of otherwise indigestible polysaccharide components in our diet, regulates the storage of calories that are extracted from the diet in the adipocytes, metabolizes xenobiotics (including carcinogens), modulates intestinal epithelial cell turnover, and educates the immune system [36]. Compared with the complexity of intestinal bacteria, the microbiota composition in the blood and in the placenta is simpler and is mainly composed of *E. faecalis*, *L. lactis*, and an uncultured bacterium present in almost all of the samples (dependent mainly on the intestinal barrier and other other defense mechanism in the animal) (Figure 2). This study is the first to reveal the bacterial diversity in the blood and in the placentas of pregnant mice, and the bacterial dominance of *E. faecalis* and *L. lactis* in mouse feces, blood and placenta indicated a bacterial relationship at these sites. Moreover, understanding the nature of host-microbial mutualism in the placenta will be vital in improving our prediction in disease development, in disease treatment and in elucidating its effects on the health of mother and fetus.

Conclusion

In this study, we demonstrated the bacterial diversity in mouse feces, blood and placenta and that *E. faecalis* FD3 is capable of translocating from the intestinal tract to the blood and from the blood to the placenta, which suggests gut colonization may initiate before birth.

Conflicts of Interest

All contributing authors declare no conflict of interest.

Acknowledgments

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