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Modification of Fecal Bacteria Counts and Blood Immune Cells in the Offspring of BALB/c and C57BL/6 Mice Obtained through Interstrain Mouse Embryo Transfer

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The reproducibility of results obtained with rodent models depends on the genetic purity of the strain and the stability of the environment. However, another potential factor is changes in the gut microbiota due to the transmission of mother's bacteria during embryo transfer. In this study, we demonstrate the transmission of the microbiota and immune cell blood phenotype to the offspring of 2 strains, C57BL/6JNskrc and BALB/cJNskrc, from surrogate dams of different genotypes. Interstrain embryo transfer resulted in a change in the number of *Enterococcus* spp. organisms, as shown by quantitative PCR analysis. The number of blood leukocytes was also affected, as estimated by flow cytometry. The number of blood leukocytes, including B cells and helper T cells, and the number of *Enterococcus* spp. organisms in male C57BL/6JNskrc offspring born to BALB/cJNskrc surrogate dams became similar to those of male BALB/cJNskrc mice born to BALB/cJNskrc dams. Likewise, the same parameters of male BALB/cJNskrc mice born to C57BL/6JNskrc dams became similar to those of male C57BL/6JNskrc offspring. Researchers should be aware of the possible transmission of the dam's microbiota and immune cell phenotypes to the experimental strains when planning embryo transfer experiments, because these factors could affect the experimental outcomes or the reproducibility of experimental results.

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The gut microbiota shapes the immune system and metabolism of the host.[51] Because bacterial colonization of the intestine occurs from the moment of birth or—according to recent studies—even from early prenatal stages,[22,52,58] the microbiota transferred from the mother can affect the neonatal development of the immune system.[10] Several bacterial taxa are known to affect the differentiation of naïve T cells and drive maturation of the host of immune system.[9] Furthermore, the proportion of immune cells in the blood in a strain is specified by genetics,[40] but can be altered by changes in microbiota caused by antibiotic treatment or diet.[6,26] Changes in microbiota composition can in turn affect the reproducibility of the phenotype of a model, and thereby, the results of animal experiments.[14] Differences in the gut microbiota of animals can occur even within the same facility; for example, as a result of the transmission of certain bacteria due to rederivation and embryo transfer.[17,19]

Immune parameters vary among mouse strains.[50] For example, Th1 responses predominate in C57BL/6 mice that are exposed to infection, whereas Th2 responses are predominant in BALB/c mice.[25] In addition, antigen-dependent activation of macrophages can be mediated by either by M1 or M2 macrophages.[32] BALB/c mice tend to develop M2 macrophage activation, whereas C57BL/6 show M1 type activation.[34] Furthermore, the ratio of T cells to B cells differs between these 2 strains.[21] The different responses of these 2 mouse strains bacterial antigens suggests that their gut microbiomes would differ, and indeed, their microbiome compositions do differ.[8,15]

Presently, C57BL/6 mice are often used to produce transgenic mice; the Balb/c strain is used less often.[35,36] Some evidence suggests that the genotype affects the development of the immune phenotype in knockout animals.[35,50] Rederivation is often used to eliminate of unwanted microbial pathogens.[43,46] Often transgenic embryos are stored in cryobanks, and embryos are transferred into surrogate dams.[29] Females of strain with good breeding characteristics—typically hybrids or outbred strains—are often used as surrogate dams.[2,47] The transfer into and carrying of embryos by a surrogate dam of a different genotype can affect the microflora, odor preference, and behavior of the offspring.[19,30,42]

This study was designed to study the transmission of microbiota and immune cell blood phenotypes to the C57BL/6JNskrc and BALB/cJNskrc offspring from surrogate dams of different genotypes. Mice of these 2 strains differed in composition of microbiota and blood lymphocyte counts. We used interstrain embryo transfer to determine the effects of a surrogate dam on the microflora and immune cell blood phenotype of the offspring.

Materials and Methods

Animals. C57BL/6JNskrc and BALB/cJNskrc mice were bred under SPF conditions at the Center for Genetic Resources of Laboratory Animals of the Institute of Cytology and Genetics,

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Siberian Branch, Russian Academy of Sciences, since December 2013. The animal colonies were negative for all the pathogens listed in the annual FELASA 2014 recommendations from the time of their transfer from the Jackson Laboratory (2013) to the completion of the experiment.[31] In accordance with the FELASA 2014 recommendations for surveillance, pathogens testing was conducted quarterly in sentinel mice that received both dirty bedding and water from colony mice for a 3-mo period. The pedigree nucleus of the substrains C57BL/6JNskrc and BALB/cJNskrc were bred from December 2013 until July 2017 and were maintained for 6 or 7 generations. Mice were housed as same-sex groups and male–female pairs in IVC systems (Optimice, Animal Care Systems, Centennial, CO) with autoclaved dust-free birch bedding, and they were provided sterile water and diet ad libitum (Mouse Maintenance autoclavable, V1534-300, Sniff Spezialdiäten, Soest, Germany). The room had a photoperiod of 14:10 light:dark cycle, a temperature of 22 ± 2 °C, and relative humidity of 40% to 50%.

All the procedures were conducted under Russian legislation according to Good Laboratory Practice (Directive of the Ministry of Health of the Russian Federation no. 267, 19.06.2003), European Directive 86/609/EEC,[12] and the European Convention for the protection of vertebrate animals used for scientific purposes. All the procedures were approved by the bioethical committee (no. 36a 23.03.2017).

Embryo transfer. Five BALB/cJNskrc and C57BL/6JNskrc female mice (age, 8 to 10 wk) were superovulated through intraperitoneal injection of 5 IU of pregnant mare serum gonadotropin (Folligon, MSD Animal Health. Millsboro, MD) during the light cycle, between 1700 and 1800 (local time). After 48 h, each female mouse received 5 IU of human chorionic gonadotropin (Chorulon, MSD Animal Health) intraperitoneally and was immediately mated with a male mouse (1:1) of the same strain and with proven fertility. For breeding, male mice (age, 12 to 14 wk) were maintained in individual cages.

The next morning, the female mice were checked for copulation plugs.[36] At 1.5 d after coitus, female mice with copulation plugs were euthanized by cervical dislocation, which was performed without anesthesia to avoid the undesirable effects of anesthesia on the embryo quality.[23] Oviducts were removed and placed into a plastic dish containing a drop of M2 medium (Sigma–Aldrich, St Louis, MO) at 37 °C. Next, 2-cell embryos were flushed from the oviducts by using M2 medium (Sigma–Aldrich) and a 32-gauge needle. Sixty embryos of each strain were obtained, transferred into a drop of KSOM medium (Cosmo Bio, Tokyo, Japan), covered with mineral oil (Sigma–Aldrich), and incubated at 37 °C under an atmosphere of 5% CO_2 until transplantation.[18]

For embryo transfer, 5 pseudopregnant female mice (age, 10 to 12 wk) were prepared as recipients by sterile mating with vasectomized males of the same strain (age, 14 to 16 wk). Vas occlusion was performed by thermal cautery at least 2 wk prior to mating. Surgical procedures were performed under general anesthesia (15 µg/100 g of body weight IP Domitor [Farnoss, Helsinki, Finland]; 3 mg/100 g of body weight IP Zoletil [Virbac Animal Health, Fort Worth, TX]). Embryos were transplanted on the day when the copulation plug was detected. The ovaries, oviducts, and part of the uterine horn of recipient female mice were exteriorized under isoflurane inhalation anesthesia (Aerrane, Baxter Healthcare, Marion, NC). Each recipient female mouse received twelve 2-cell embryos, which were transferred into the ampulla through the infundibulum by using a fine glass capillary. The ovaries, oviducts, and uterine horns were returned to the abdomen, and the wound was closed by using wound clips. Mice were kept on a warming plate at 37 °C until they recovered from the effects of the anesthesia.[18,36]

The number of successful surrogate pregnancies was 4 for the C57BL/6JNskrc dams and 5 for the BALB/cJNskrc dams. The female mice of the C57BL/6JNskrc and BALB/cJNskrc groups gave birth to 17 (8 male and 9 female) and 24 (11 male and 13 female) pups, respectively. Eight male and 5 female offspring of C57BL/6JNskrc dams and 5 male and 8 female offspring of BALB/cJNskrc dams survived until 12 to 14 wk of age (the end of the experimental period). Three female offspring of C57BL/6JNskrc dams and 6 male and 5 female offspring of BALB/cJNskrc dams died until 1-2 wk of age due to poor maternal behavior (hypothermia or starvation of pups).

Experimental groups. The experiment was performed on 12- to 14-wk-old male mice obtained by interstrain embryo transfer and 5 intrastrain breeding pairs of each substrain. We used only male offspring to avoid the hormonal and estrous-cycle effects on immune cell counts.[4,28] Four experimental groups were used: C57BL/6JNskrc mice born of C57BL/6JNskrc dam (B6 > B6) - 7 male mice were used for fecal analysis, and 11 were used for blood and spleen cell assays; C57BL/6JNskrc born of BALB/cJNskrc (B6 > C) - 5 mice were used for each assay; BALB/cJNskrc born of BALB/cJNskrc (C > C) - 7 mice were used for fecal analysis and 5 for cell assay; and BALB/cJNskrc born of C57BL/6JNskrc (C > B6) - 6 mice were used for fecal analysis and 8 for cell assays. Numbers in each group vary because all available male offspring were assigned to a group, with a minimum of 5 per group, in order to have more replicates and thus enhance statistical power. Based on power analysis, we had planned to analyze 31 samples for each assay. However, 6 mice (4 from the B6 > B6 group and 2 from the C > B6 groups) had too few fecal boli for testing and were therefore excluded from the fecal analysis. In addition, during the wash procedure, 2 blood samples from the B6 > C group were lost. Overall, a total of 29 samples were analyzed in the cell assay, and 25 were analyzed in the bacterial DNA assay. The tests for all the experimental groups were performed simultaneously for the same assay.

Assay for fecal bacteria. Immediately after blood sampling and CO_2-mediated euthanasia, 3 fecal boli from the distal to proximal colon were collected from each mouse. We used only male offspring, to keep the fecal collection the same for all mice. The fecal samples from each male mouse were collected in a sterile 1.5-mL plastic tube, immediately frozen in liquid nitrogen, and stored at –70 °C until the assays were performed.

Fecal DNA samples were extracted by using QIAamp Fast DNA Stool Mini Kit (catalog no. 51604, Qiagen, Hilden, Germany) according to the manufacturer's instructions. The 16S rRNA genes from *Bacteroides* spp., *Escherichia coli*, *Lactobacillus* spp., *Enterococcus* spp., and *Bifidobacteria* spp. were detected by using commercial real-time PCR kits for bacterial DNA detection (catalog nos. BACSPP96S, ECOLI96S, LSPP96S, ESPP96S, BIFSPP96S, Belki Biotechnologies, Novosibirisk, Russia) according to the manufacturer's instructions. PCR analysis was performed in duplicate in a total volume of 20 µL, which included 15 µL of master-mix (containing polymerase buffer, polymerase, dNTP, SYBR-Green I, and 0.5 µM of each of the 2 specific primers), and 5 µL of sample (approximately 50 ng of DNA). The positive control provided with the kit was used, and deionized ultrapure water (Milli-Q type I, Merck Millipore, Darmstadt, Germany) served as a negative control. According to manufacturer's instructions, primers were designed to detect specific regions of the 16S gene of each bacterial genus. The amplification reaction was run in a CFX96 real-time PCR instrument (Bio-Rad, Hercules, CA). DNA was denatured for 5

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min at 95 °C, and then amplified in 45 cycles of denaturation at 95 °C for 15 s and primer annealing and DNA synthesis at 62 °C for 50 s. Bacterial DNA was normalized to the 16S rRNA gene as follows: $\log_{10}[\delta Ct \times (\text{16S rRNA of each bacterial group / total 16S rRNA})] = \log_{10}[2^{(Ct \times (\text{total 16S rRNA}) - Ct \times (\text{16S rRNA of each bacterial group}))}]$. The threshold cycle (Ct) corresponded approximately to the middle of the exponential phase of PCR and was set at 400 fluorescent emission units for all the samples.

To identify and assay the additional bacteria present in the fecal samples, we used the primers for *Enterococcus faecalis*,[49] genus-specific *tuf* gene primers for *Enterococcus* spp. and primers for *Lactobacillus murinus* (Table 1). *E. faecalis* and *L. murinus* were detected by using real-time PCR analysis. PCR amplification was performed in duplicate according to the manufacturer's instructions by using Universal PCR Master Mix (HS-qPCR SYBR Blue, Biolabmix, Novosibirisk, Russia). The amplification reaction and normalization of bacterial DNA was same as previously described. Strains of *L. murinus* and *E. faecalis* (kindly provided by Galina Kalmikova [Siberian Federal Scientific Centre of Agro-BioTechnologies of the Russian Academy of Sciences]) were used as positive controls. The strains were previously isolated from the cecal content of laboratory mice by using a selective medium under aerobic conditions and subsequently identified as *L. murinus* and *E. faecalis* by sequence assay of the variable region of the 16s rRNA gene (data not shown). *Lactobacillus acidophilus* and *Enterococcus faecium*, pharmaceutical probiotics (Linex, Sandoz, Ljubljana, Slovenia), and deionized ultrapure water (Milli-Q type I, Merck Millipore) served as negative controls.

The genus-specific *tuf*-gene of *Enterococcus* spp. was detected by using PCR analysis. PCR amplification was performed according to the manufacturer's instructions of PCR Master Mix (HS-Taq, Biolabmix). The amplification reaction comprised DNA denaturation for 5 min at 95 °C, followed by 45 cycles of amplification of denaturation at 95 °C for 15 s and primer annealing and DNA synthesis at 62 °C for 50 s. The presence of PCR-amplified products and the minimum-detection cfu count for *E. faecalis* was confirmed by electrophoresis in 1.5% agarose gel (Figure 1).

Cytometry of blood and spleen cells. Blood samples were collected from the retroorbital sinus without the use of anesthesia, which can affect the immune cell count because of the glucocorticoid reaction.[38,56] For blood collection, the eyes of mice were treated with a drop of an ophthalmic anesthetic (0.5% proparacaine hydrochloride ophthalmic solution, Alcon Laboratories, Alcon-Couvreur, Brussels, Belgium). After blood collection, the mice were euthanized by using CO_2, and their spleens were placed in cold PBS and stored in plastic dishes (diameter, 50 mm). For flow cytometry, RBC were lysed by using ammonium chloride (0.15 M)–potassium carbonate (1 mM) buffer[20] and homogenized; the homogenate was filtered through 70-µm cell strainers (catalog no. CLS431751, Corning, Corning, NY). RBC were lysed, and splenocytes were washed twice.[7,20] Blood and spleen cells were stained with PE–antiCD3ε (hamster, clone 145-2C11), FITC–antiCD4 (rat IgG2b κ, clone GK1.5), PE/Cyanin7–antiCD8a (rat IgG2a κ, clone 53-6.7), PE–antiCD3ε (Armenian hamster IgG, clone 145 to 2C11), and FITC–antiCD19 (rat IgG2a κ, clone 6D5) antimouse antibodies (BioLegend, Dedham, MA) for 120 min at 4 °C in the dark and then analyzed by using a Guava easyCyte flow cytometer (Merck Millipore). For analysis of blood cells, singlets were gated on lymphocytes, and $CD3^+$ and $CD19^+$ lymphocytes were gated on lymphocyte singlets (Figure 2) or $CD4^+$ and $CD8^+$ cells were gated on $CD3^+$ lymphocyte singlets (Figure 2 C); 25,000 singlets were counted for each sample.[7] The gating strategy for the analysis of CD3+ and CD19+ splenocytes (Figure 2 C) was the same as for blood samples; 60,000 splenocytes singlets were counted for each sample.[31] The populations of $CD3^+$, $CD19^+$, $CD3^+CD4^+$, and $CD3^+CD8^+$ cells were determined in percent, and the numbers of cells were expressed relative to the total count of all leukocytes. For counting, cells aliquots of whole blood and spleen homogenates were stained with Türk solution (catalog no. 109277, Merck Millipore), and the number of cells was determined by using a counting chamber (catalog no. 12001711, Minimed, Suponevo, Russia).

Statistical analysis. To estimate the total sample size of mice, we used G*Power 3.1 software. The calculated total sample size for determining the main effect by using F tests–MANOVA was found to be 30 (Figure 3). The α probability of error was set to 0.05, f2(V) to 0.25, and power to 80%.

Statistical analysis was performed by SPSS Statistics software (version 23.0, IBM, Armonk, NY). The data were tested for normality of distribution by using the Kolmogorov–Smirnov test (Table 2). All data are presented as mean ± SE, except for bacterial abundance, where actual values for each sample are shown. Effect groups for all data were analyzed by using the Kruskal–Wallis test (Table 2). For the significance of immune cell analysis, the results of the bacterial real-time quantitative PCR analysis were evaluated between groups by using the Mann–Whitney *U* test. To compare the numbers of present and absent bands of the *tuf* gene of *Enterococcus* spp., a $\chi 2$ test was conducted between the 2 groups.

Results

Flow cytometry of blood cells revealed the immune cell blood phenotype of mice obtained by intrastrain breeding. Male mice born to dams of the 2 substrains (B6 > B6 and C > C groups) differed in blood leukocyte and B-cell ($CD19^+$) counts ($Z = 3.12$, $P = 0.002$ and $Z = 3.11$, $P = 0.002$). Specifically, the counts of leukocytes and B cells were significantly higher in male mice of the B6 > B6 group compared with C > C group (Figure 2 A) whereas the numbers of blood T-helper cells ($CD3^+CD4^+$) and B cells were lower in mice of the C > C group ($Z = 1.95$, $P = 0.052$). The number of cytotoxic T cells ($CD3^+CD8^+$) did not differ between offspring of the B6 > B6 and C > C groups ($Z = 0.62$, $P = 0.53$). Despite the differences in the blood immune cells, the numbers of splenic T and B cells did not differ between the male offspring of the B6 > B6 and C > C groups ($Z = 1.42$, $P = 0.16$ and $Z = 1.64$, $P = 0.10$; Figure 2 B).

The number of leukocytes in the B6 > C and C > B6 groups was different than in the B6 > B6 and C > C groups, respectively (Figure 2 A). The number of leukocytes was significantly decreased in the B6 > C group compared with that in the B6 > B6 group ($Z = 2.44$, $P = 0.015$). In contrast, mice of the C > B6 group had higher numbers of leukocytes than did those of the C > C group ($Z = 2.20$, $P = 0.028$). Moreover, the numbers of B cells and helper T cells were significantly higher in the C > B6 male mouse offspring compared with C > C group ($Z = 2.20$, $P = 0.028$; $Z = 2.05$, $P = 0.040$) and lower in B6 > C compared with B6 > B6 ($Z = 2.09$, $P = 0.036$; $Z = 2.16$, $P = 0.031$, respectively). In the C > B6 group, the number of cytotoxic T cells was higher than that of the C > C group ($Z = 2.13$, $P = 0.033$; Figure 2 A).

The percentage of blood immune cells in the male offspring of the B6 > C and C > B6 groups depended on their genotypes and not on the strain of the surrogate dam (Table 3). The number of splenocytes did not significantly differ between B6 > C and B6 > B6 ($Z = 0.85$, $P = 0.39$ for T-cell; $Z = 0.39$, $P = 0.69$ for B-cell) or C > B6 and C > C groups ($Z = 1.03$, $P = 0.31$; $Z = 1.46$, $P = 0.14$, respectively; Figure 2 B). Therefore, the percentage of blood

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Table 1. Primers used for the study

Primer	Sequence (5′ to 3′)	Annealing temperature (°C)	Target	Source
Efec_F	CGCTTCTTTCCTCCCGAGT	60	*Enterococcus faecalis*	reference [49]
Efec_R	GCCATGCGGCATAAACTG			
Etuf_F	TATCTTRGTAGTTTCTGCTGCTGA	62	*Tuf* gene, *Enterococcus* spp.	inhouse
Etuf_R	TCGTCAACTGCAGCCATYAATTC			
Lan_F	GGCAATGATGCGTAGCCGAAC	62	*Lactobacillus murinus*	inhouse
Lan_R	CGCACTTTCTTCTCTAACAACAGG			
16S_F	TCCTACGGGAGGCAGCAG	62	16S rRNA universal region	inhouse
16S_R	ATTACCGCGGCTGCTGG			

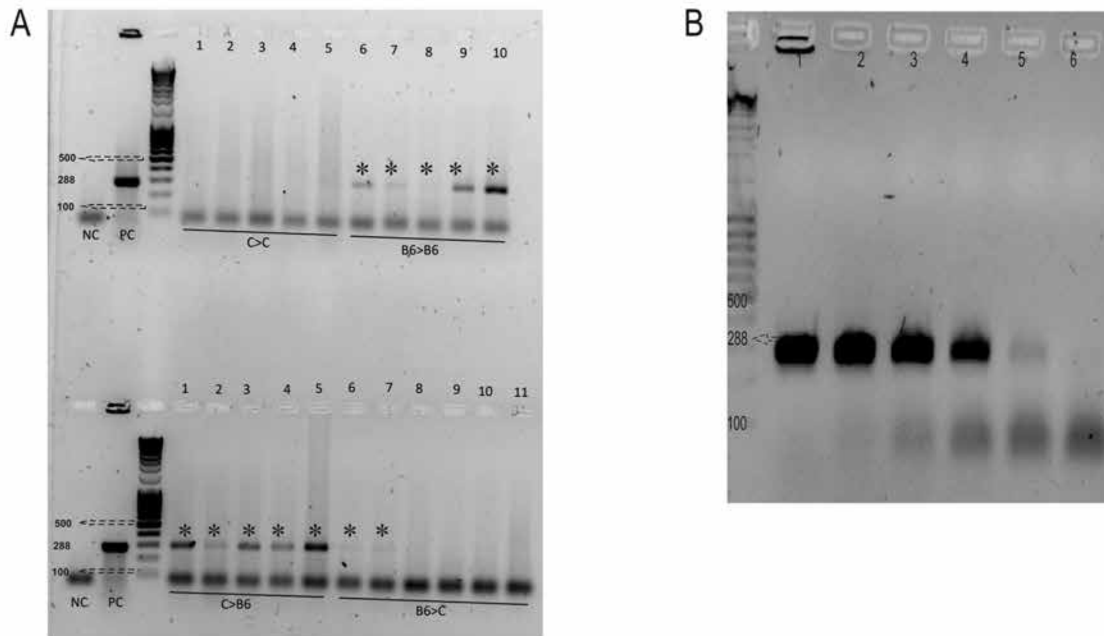

Figure 1. Gel electrophoresis of PCR products amplified by using primers for the *Enterococcus* spp. *tuf* gene. PC, positive control (amplified from *Enterococus fecalis* culture; product size, 288 bp). NC, negative control. (A) Gel of PCR products from fecal DNA samples. * indicates samples that have a positive band. (B) Gel illustrating *Enterococcus* spp. detection limit test of PCR products from *Enterococcus fecalis* culture. Lane 1: 532,000 cfu per reaction; 2: 50,640 cfu per reaction; 3: 5,320 cfu per reaction; 4: 532 cfu per reaction; 5: 50 cfu per reaction; 6: negative control. PCR assay detection limit is 50 CFU.

immune cells and number of splenocytes were determined by genetic strain of the embryo rather than by the strain of the surrogate dam.

A shift in microbiome is associated with changes in bacterial and host metabolite composition and immunity.[5] In male offspring from all the groups, we analyzed 5 bacterial genera (*Bacteroides* spp., *E. coli*, *Lactobacillus* spp., *Enterococcus* spp., and *Bifidobacteria* spp.), which are the most represented genera in the intestinal microflora of laboratory mice, according to the published literature and our preliminary data.[1] Analysis of the microbiota demonstrated that male offspring of the 2 substrains differed significantly in the number of *Enterococcus* spp. ($Z = 3.13$, $P = 0.002$). The amount of 16S gene of the *Enterococcus* spp. in the fecal content of male mice of the B6 > B6 group was significantly higher compared with that in the male mice of the C > C group (Figure 4 B). The amount of 16S gene of *Bacteroides* spp. ($Z = 0.45$, $P = 0.66$), *Lactobacillus* spp. ($Z = 0.57$, $P = 0.57$), and *E. coli* ($Z = 0.83$, $P = 0.41$) in the offspring did not differ between the 2 substrains (Figure 4 A, C, and D). The 16S gene of *Bifidobacteria* spp. was not detected in these mouse substrains. Therefore, carrying by surrogate dams caused changes in the amount of 16S gene in *Enterococcus* spp. and *Lactobacillus* spp. (Figure 4 A and B). In male mice of the B6 > C group, the amount of 16S gene of *Enterococcus* spp. was similar to that in the male mice of the C > C group ($Z = 1.86$, $P = 0.062$), whereas in the fecal content of male mice of the C > B6 group, the16S gene of *Enterococcus* spp. decreased until it was undetectable and similar to the level in male mice of the B6 > B6 group ($Z = 0.43$, $P = 0.67$; Figure 4 B). The 16S gene of *Lactobacillus* spp. decreased in the C57BL/6JNskrc male mice that were born to BALB/cJNskrc surrogate dams (B6 > C) compared with that in the B6 > B6 group ($Z = 2.74$, $P = 0.006$; Figure 4). In male offspring, differences were detected in the amount of the 16S gene of *Enterococcus* spp. between the B6 > C and C > B6 groups ($Z = 2.88$, $P = 0.004$; Figure 4 A and D).

To confirm the effect of carrying by surrogate dams on the change in the number of *Enterococcus* spp., we assayed the *tuf* gene of *Enterococcus* spp. in the fecal content of male mice from all the groups. In samples from male mice of the C > C group, no band corresponding to that in the positive control (288 bp) was obtained (Figure 1 A). In the B6> B6 group, the *tuf* gene of *Enterococcus* spp. was detected in all samples ($\chi^2 = 10$, $P < 0.01$). Therefore, carrying by surrogate dams reversed the presence of *tuf* gene of *Enterococcus* spp. In the male mice of the B6 > C group, the *tuf* gene of *Enterococcus* spp. was detected in only 2 samples ($\chi^2 = 5.24$, $P < 0.02$)., and the result was opposite in all

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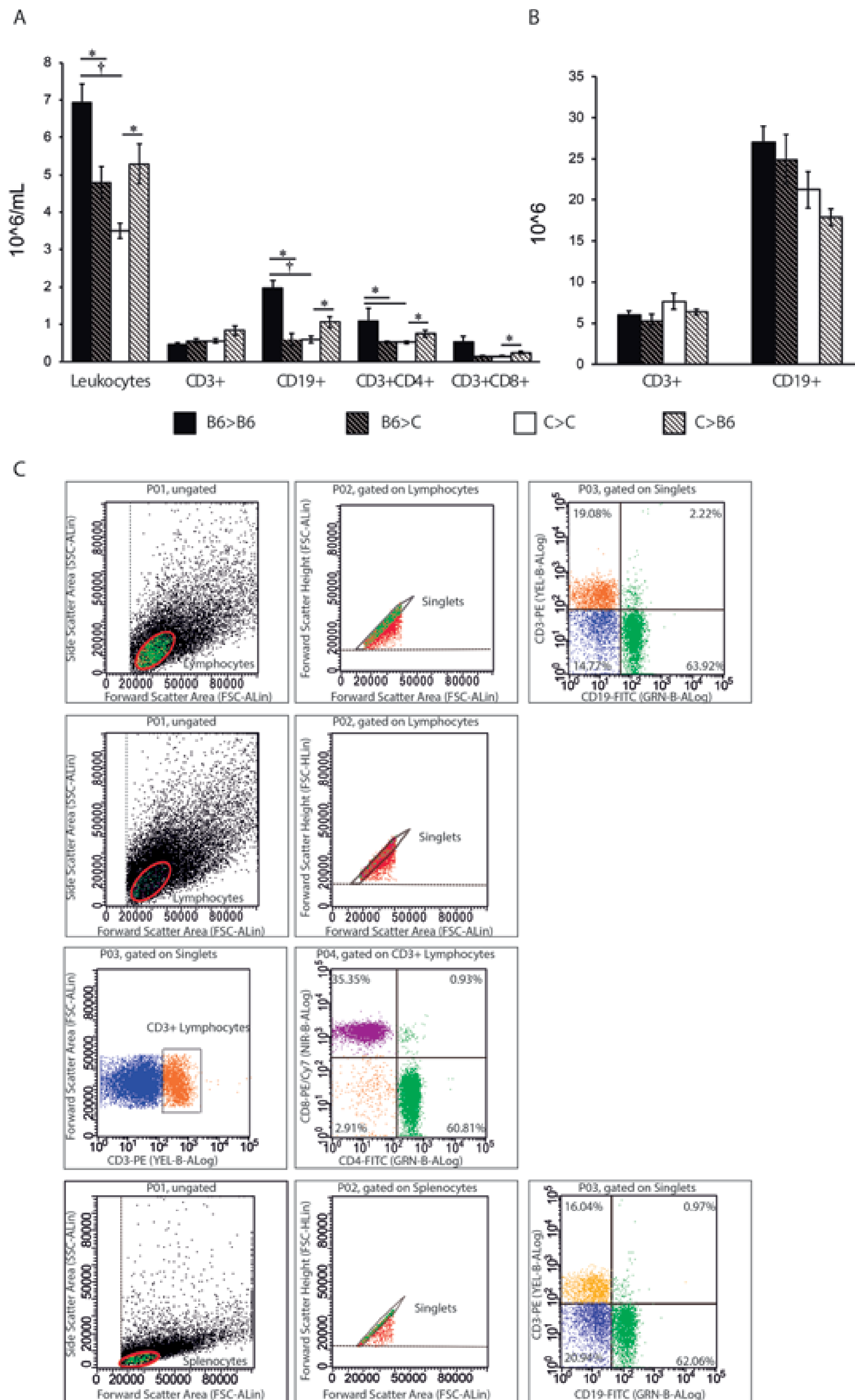

Figure 2. Numbers of (A) leukocytes, T cells ($CD3^+$), B cells ($CD19^+$), helper T cells ($CD3^+CD4^+$), and cytotoxic T cells ($CD3^+CD8^+$) in blood and (B) of splenic T cells ($CD3^+$) and B cells ($CD19^+$) in the male mouse offspring obtained by intrastrain breeding (open bars, B6 > B6, n = 11; black bars, C > C groups, n = 5) and interstrain embryo transfer (dark shading, B6 > C, n = 5; light shading, C > B6 groups, n = 8). Data are presented as (A) 10^6 cells/ mL blood and (B) 10^6 cells per spleen. *, $P < 0.05$; †, $P < 0.01$, Mann–Whitney U test. (C) Gating strategy for $CD3^+$ and $CD19^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ cells in blood, for $CD3^+$ and $CD19^+$ cells in spleen.

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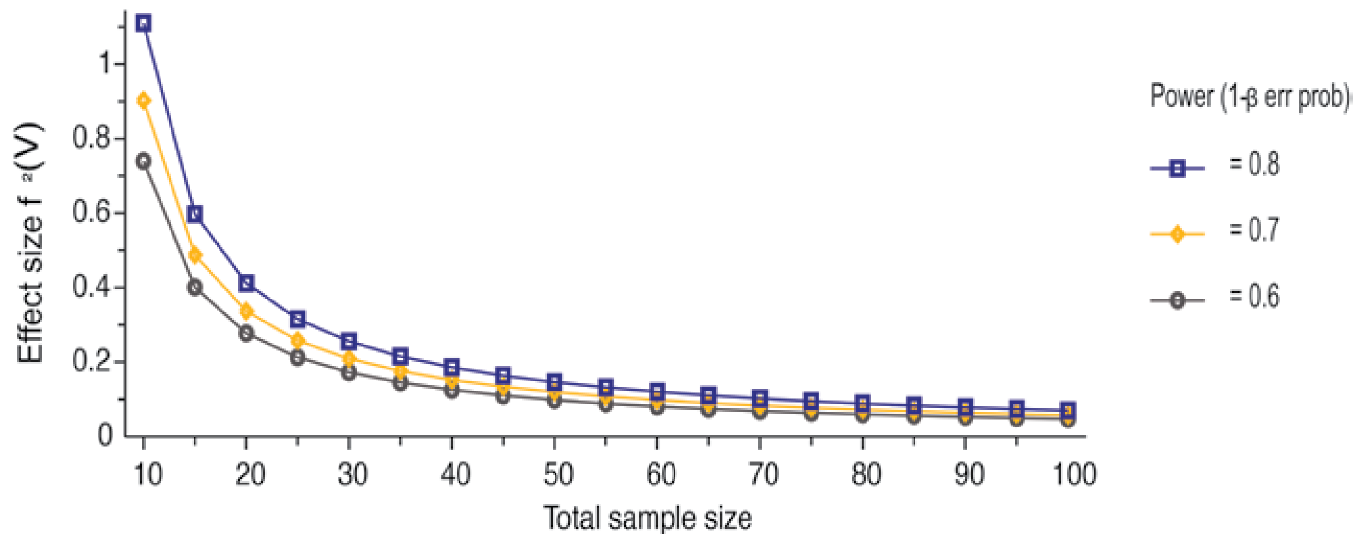

Figure 3. Dependence between effect size f2(V) and total sample size for F tests–MANOVA main effect under the condition when power (1 – β error probe) equals 0.6 (gray circle); 0.7 (yellow rhombus); 0.8 (blue square). Number of groups, 4; responsible variables, 2 (genotypes offspring and genotypes surrogate mother); α error probe *P* value, 0.05.

Table 2. Tests of normality of data and effect groups

Parameters	Kolmogorov–Smirnov test, *P*	Kruskal–Wallis, *P*	*n*
16S rRNA gene from *Bacteroides* spp.	0.134, *P* = 0.2*	1.65, *P* = 0.648	25
16S rRNA gene from *E.coli*	0.346, *P* < 0.01 NP	6.44, *P* = 0.092	25
16S rRNA gene from *Lactobacillus* spp.	0.166, *P* = 0.09 NP	**9.24, *P* = 0.026**	25
16S rRNA gene from *Enterococcus* spp.	0.230, *P* = 0.01 NP	**13.40, *P* = 0.004**	25
16S rRNA gene from *L murinus*	0.125, *P* = 0.2*	**13.44, *P* = 0.004**	25
16S rRNA gene from *E.faecalis*	0.166, *P* = 0.2*	5.74, *P* = 0.125	25
Leukocytes, $\times 10^6$/mL	0.127, *P* = 0.2*	**15.00, *P* = 0.002**	29
$CD3^+$ cells, $\times 10^6$/mL	0.102, *P* = 0.2*	**12.78, *P* = 0.005**	29
$CD19^+$ cells, $\times 10^6$/mL	0.147, *P* = 0.1 NP	**16.10, *P* = 0.001**	29
$CD3^+CD4^+$ cells, $\times 10^6$/mL	0.261, *P* < 0.01 NP	**10.24, *P* = 0.017**	29
$CD3^+CD8^+$ cells, $\times 10^6$/mL	0.341, *P* < 0.01 NP	**8.03, *P* = 0.045**	29
$CD3^+$ cells, $\times 10^6$/spleen	0.09, *P* = 0.2*	3.71, *P* = 0.295	29
$CD19^+$ cells, $\times 10^6$/spleen	0.149, *P* = 0.1 NP	**10.36, *P* = 0.016**	29

NP, nonparametric
Bold text indicates a significant statistical effect.
P = 0.2* is a lower bound of true significance of parametric data

of the mice of the C > B6 group ($\chi^2 = 10$, $P < 0.01$). The lowest detection level of the *tuf* gene of *Enterococcus* spp. corresponded to 50 cfu per PCR sample (Figure 1 B).

E. faecalis is the most represented *Enterococcus spp.* in the fecal contents of laboratory mice.[3] We evaluated the presence of *E. faecalis* DNA in male offspring from all groups, but found no significant differences between the B6 > B6 and C > C groups ($Z = 0.06$, $P = 0.95$; Figure 5 A). The amount of *E. faecalis* DNA in mice of the B6 > C group trended lower than in the B6 > B6 group ($Z = 1.81$, $P = 0.07$). This tendency could suggest that other members of *Enterococcus* genera likely contribute to the differences between the groups.

Among *Lactobacillus* spp., *L. murinus* is considered to be the most represented in the fecal content of laboratory mice.[48] The amount of *L. murinus* DNA in the fecal content of male offspring of the B6 > B6 group was significantly greater than that in males from the C > C group ($Z = 2.49$, $P = 0.013$; Figure 5 B). In the B6 > C group, the amount of *L. murinus* DNA was lower than that of male offspring from the B6 > B6 ($Z = 2.84$, $P = 0.004$) and C > B6 ($Z = 2.37$, $P = 0.018$) groups. These data suggest that the amount of *L. murinus* depends on the mouse genotype but can also be transferred from surrogate dams to the offspring.

Discussion

The present study used 2 different substrains of mice, C57BL/6JNskrc and BALB/cJNskrc, to show that microbiota inherited from the dam can persist in the progeny derived from the transferred embryos. A previous report showed that the fetus can inherit *Enterococcus* spp. from the dam before birth,[24] whereas *Lactobacillus spp.* colonize the intestine during the neonatal period.[33] We found that counts of *Enterococcus* spp.—but not of *E. faecalis*—and *L. murinus* were altered in the fecal contents of male offspring that were born to surrogate dams of different genotypes (B6 > C compared with B6 > B6 and C > B6 compared with C > C). These bacteria are classified as probiotic and are included in the altered Schaedler flora for mice and symbiotic drugs for humans.[16,55] The shifts in the normal gut microbiota can affect blood immune cells and possibly those

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Table 3. Percent of blood immune cells of the male offspring mice in B6>B6, C>C, B6>C and C>B6 groups.

Groups, (n)	T-cell (CD3+), %	B-cell (CD19+), %	T-helper(CD3+CD4+) gated by lymphocytes, %	T-cytotoxic (CD3+CD8+) gated by lymphocytes, %, %
B6>B6, (11)	14.03±2.85	62.38±3.49	12.33±1.78	6.77±1.37
B6>C, (5)	14.77±2.79	59.18±3.21	11.67±1.24	7.23±0.82
C>C, (5)	32.13±2.19**	34.04±3.82**	30.58±1.80**	8.60±0.51*
C>B6, (8)	29.81±2.56**,&	29.81±2.56**,&&	26.62±1.38**,&&	8.44±0.44*

*,** $P < 0.05$, $P < 0.01$ to compare with B6<B6 group, Mann–Whitney U test.
&,&& $P < 0.05$, $P < 0.01$ to compare with B6<C group, Mann–Whitney U test.
Date present as mean ± SE

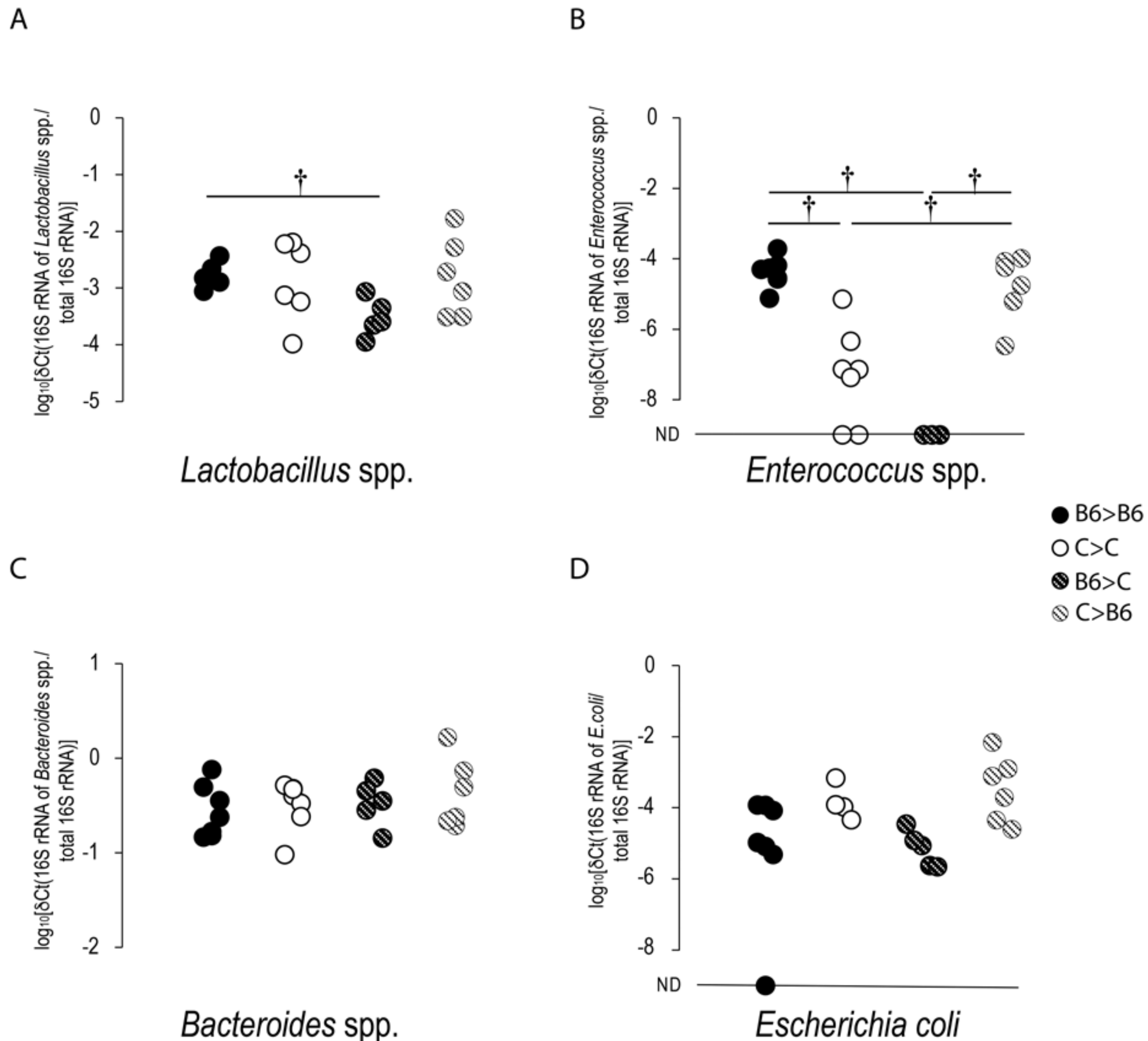

Figure 4. Abundance of the 16s rRNA of (A) *Lactobacillus* spp., (B) *Enterococcus* spp., (C) *Bacteroides* spp., and (D) *Escherichia coli* in the fecal content of male mouse offspring obtained by intrastrain breeding (open dots, B6 > B6, $n = 7$; filled dots, C > C groups, $n = 5$) and interstrain embryo transfer (dark shading, B6 > C, $n = 7$; light shading narrow black strip dots, C > B6 groups, $n = 6$). The abundance of intestinal bacteria in fecal content was measured through real-time quantitative PCR analysis and was normalized relative to the 16S rRNA gene. Data are presented as $\log_{10}$[δCt(16S rRNA of each bacterial group / total 16S rRNA)]. ND, not detected. †, $P < 0.01$, Mann–Whitney U test.

in the intestine. Both anesthesia and surgical procedures can also affect the immune parameters of surrogate dams and their offspring.[18,23,28,59] Changes in the immune parameters can lead to changes in the composition of the intestinal microbiome.[11]

The current study found changes in both the microbiome composition and the numbers of immune cells in blood. Specifically, the numbers of leukocytes, B cells, and T helper cells in male offspring of the B6 > C and C > B6 groups became similar to those in the male offspring of the C > C and B6 > B6 groups, respectively. These data indicate a change in the adaptive immune system in the blood. However, changes were not detected in the percentages of blood cells or the numbers of cells in the spleen. Numbers and percentages of immune cells are important indicators in many studies of the immune system.[53] However, the number and percentage of WBC in blood are also affected by various genetic, sex, and environ-

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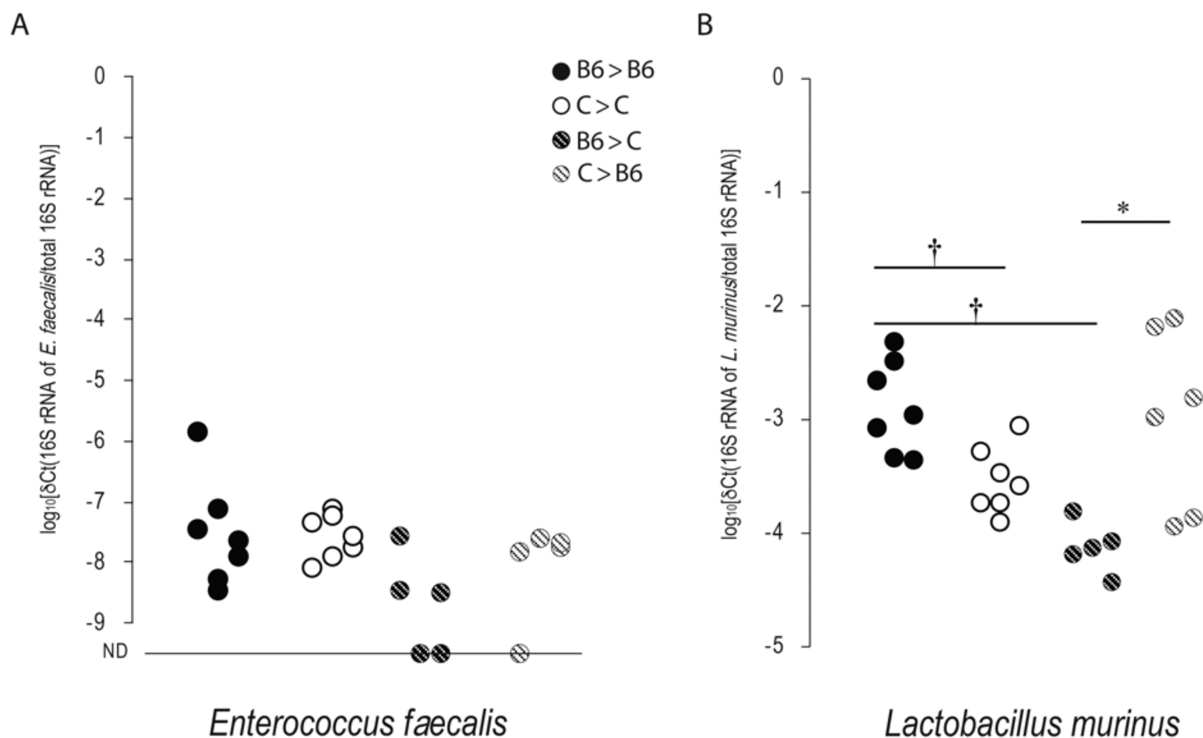

Figure 5. Abundance of the DNA of (A) *Enterococcus faecalis* and (B) *Lactobacillus murinus* in the fecal content of male mouse offspring obtained by intrastrain breeding (open dots, B6 > B6; filled dots, C > C groups) and interstrain embryo transfer (dark shading, B6 > C; light shading, C > B6 groups). The abundance of intestinal bacteria was measured by real-time PCR and was normalized with respect to the 16S rRNA gene. Data are presented as $\log_{10}$[δCt(16S rRNA of each bacterial group / total 16S rRNA)]. ND, not detected. *, $P < 0.05$, †, $P < 0.01$, Mann–Whitney *U* test.

mental factors. In mice of different strains, the percentages of various cell types differ.[39,40,41,44] Genetic differences are thought to underlie the differences in immunoreactivity of BALB/c and C57BL6 mice.[27,37,60] In addition, in some strains, the percentages and numbers of blood immune cells differs between males and females.[44] Environmental factors include housing conditions, microbiologic status, food and water quality.[37,53] Differences in the composition of the microflora can affect the immune cell composition not only in intestine but also in blood.[6,26] We suggest that despite the fact that the percentages of immune cells in blood are strictly genetically determined, environmental factors, including the genotype of carrying mother, can change the quantitative composition of blood leukocytes.

Many transgenic and inbred strains are stored as cryopreserved embryos for economic and technological reasons.[29] To maintain the purity of the genetic strain, the pedigree nucleus should be renewed at least every 5 generations, which is usually done by using frozen embryos stored when the strain was received.[13,46] Transportation of transgenic animal strains is also conveniently done by using frozen embryos, sperm, and oocytes.[57] To eliminate pathogens, frozen embryos are transferred to surrogate dams of the desired health status (SPF, SOPF).[29,43] For all of these procedures, the transfer of early-stage embryos or in-vitro–fertilized oocytes to surrogate dams is required. The strain of the surrogate dam used for embryo transfer can affect the microflora and immune cells of the offspring. The recovery of mouse strains from cryobanks may require the use of surrogate dams whose genotype is not C57BL/6.

Consequently, attention should be paid to the genotype and microbiome status of the surrogate dam because these parameters can have a significant effect on the reproducibility of the results obtained earlier from animals of the same strain but different rederivation. Results of various studies on the immune system and microbiota in mouse models can be significantly affected by the embryo transfer procedure itself. Therefore, careful documentation of all the manipulations performed for obtaining a specific strain is important, including whether frozen embryos, sperm, or oocytes are used. Because these manipulations can differ between facilities, they also can affect the reproducibility of experimental results.[14]

Avoiding the effect of surrogate dams on the reproducibility of the results requires a process of microflora standardization. A common approach involves obtaining F2 offspring by heterozygous crossing.[54] Sometimes cohousing is used; however, a recently published study demonstrated its low efficiency[45] because the formation of microbiome occurs at all of the different stages of pregnancy, delivery, and nursing. Therefore, one group has suggested the useful 'littermate method' of microbiota standardization for wildtype and mutant strains.[45]

The current study demonstrated that shifts in the microbiota and immune parameters occurred in offspring that were embryo-transferred to surrogate dams of a different genotype. However, the determination of causality in these events and the precise role of embryo transfer and mother–fetus genotype differences require further investigation. Although our data cannot be universally applied to all animal strains and embryo manipulations, they should be borne in mind when conducting animal research involving transgenic manipulations and embryo transfer in modern animal facilities.

We conclude that the microbiota introduced after weaning can persist in adult mice. Our results highlight the importance of using littermate controls to avoid confounding microbial influences on gene-driven immune cellular blood phenotypes and to prevent misinterpretation of findings in mouse models.

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