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HOST MICROBE INTERACTIONS

Effect of the Nursing Mother on the Gut Microbiome of the Offspring During Early Mouse Development

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Abstract

The development of the gut microbiome is influenced by several factors. It is acquired during and after birth and involves both maternal and environmental factors as well as the genetic disposition of the offspring. However, it is unclear if the microbiome development is directly triggered by the mode of delivery and very early contact with the mother or mostly at later stages of initial development mainly by breast milk provided by the mother. To investigate to what extent the gut microbiome composition of the offspring is determined by the nursing mother, providing breast milk, compared to the birth mother during early development, a cross-fostering experiment involving two genetically different mouse lines was developed, being prone to be obese or lean, respectively. The microbiome of the colon was analyzed by high-throughput 16S rRNA gene sequencing, when the mice were 3 weeks old. The nursing mother affected both α- and β-diversity of the offspring's gut microbiome and shaped its composition. Especially bacterial families directly transferred by breast milk, like *Streptococcaceae*, or families which are strongly influenced by the quality of the breast milk like *Rikenellaceae*, showed a strong response. The core microbiome transferred from the obese nursing mother showed a higher robustness in comparison to the microbiome transferred from the lean nursing mother. Overall, the nursing mother impacts the gut microbial composition of the offspring during early development and might play an important role for health and disease of the animals at later stages of life.

Keywords Gut microbiome · Obesity · Cross-fostering · Mice · 16S rRNA sequencing · Birth mother · Nursing mother

Introduction

The gut microbiome contributes significantly to the metabolic phenotype of the host. It is involved in the development of metabolic syndrome, insulin resistance, and body weight, as the degradation and uptake of nutrients is catalyzed by the individual microbiome and its functional traits [1–3]. Vice versa, the composition and structure of the gut microbiome is influenced by factors like life style, age, gender, environment, and genetic disposition of the host [4–8]. The contribution of the genetic make-up of the host as driver for the composition of the gut microbiome has been studied in several settings and experimental conditions [6, 9, 10].

In addition, an important factor influencing the development of the gut microbiome of mammalians is the mode of delivery. Whereas vaginal birth brings about close similarities between the gut microbiome of the infants and the microbiome of the mother's vagina, the gut microbiome of infants delivered by C-section is more similar to the skin microbiome of the mother [11]. Besides these well-documented natal effects of

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the mother on the microbiome development of the offspring, also post-natal effects of the mother have to be considered, which may induce microbiome modulation. Breast milk is one of the first post-natal sources of microbiota for the offspring and has been shown to harbor beneficial bacteria for the infant's gut [12, 13]. In addition, a direct transfer of the skin microbiome from the mother to the offspring at post-natal phases is likely. Thus, pre- and post-natal effects of the mother are important drivers for the development of the offspring's microbiome. However, their concerted effects on the gut microbiome of the offspring are still not well characterized.

We conducted cross-fostering experiments to investigate the effect of the nursing mother for microbiome development in the offspring during early stages of life. Half of a mouse litter was exchanged between the mothers of two mouse lines, which had a genetic predisposition to be either lean or obese, directly after birth. Subsequently, we analyzed the effects of the nursing mother on the offspring's gut microbiome, focusing on microbiota from the colon. Analyses were performed at the age of 3 weeks, a time point where gut microbiome composition of mice was considered stable [14]. Microbial communities were analyzed using a molecular barcoding approach, based on DNA extracted from the colon, 16S rRNA gene PCR amplification, and high-throughput sequencing of amplicons.

Methods

Experimental Setup

All the procedures involving animals were performed according to local ethical and regulatory guidelines, which are in compliance with the EU regulations regarding research on experimental animals.

The polygenic mouse model used in this study was previously developed by divergent selective breeding to study consequences of obesity. The mouse lines originated from a three-way cross base (two inbred [CBA, JU] and one outbred line [CFLP]) and were selected for high-fat (fat line) or low-fat (lean line) content [15, 16]. During the first 20 generations, the selection of three replicate lines each was based on the ratio of gonadal fat pad weight to body weight in 10-week-old males. At generation 20, the replicate lines were merged to form a lean and an obese line, which were further selected by fat percentage. The resulting lines have been stable for more than 60 generations and differ more than fivefold in fat content having a body fat content of 4% (lean line) and 22% (obese line), respectively [17].

To separate the impact of the birth mother's microbiome from the post-natal influences of the nursing mother on the development of the gut microbiome of the offspring, we conducted a cross-fostering experiment by exchanging a part of the new-born mice to non-birth mothers who recently gave birth and were ready to nurse (Fig. 1). All new-borns were nursed until weaning. Our study included prenatal effects of the mother, including genetic make-up and microbial transfer during birth and first milk (BM), and post-natal effects of the nursing mother on the microbiome development of the offspring (NM). Mice were fed the same sterilized food and were provided with the same sterile wood chip bedding; thus, the main source of microbiota in the environment was the mouse mothers, e.g., microbiota from skin, gut, mouth, and milk. Consequently, the following settings were analyzed in this study: mice, switched to a genetically different mother for nursing (obeseBM/leanNM $n = 11$; leanBM/obeseNM $n = 10$; raised by four different nursing mothers per treatment); mice, which stayed with their birth mother, but got siblings from different birth mothers of the same genotype (leanBM/leanNM $n = 9$, obeseBM/obeseNM $n = 13$; raised by four different nursing mothers per treatment). A complete replacement of the litter was not possible as foster mothers do not accept a complete litter exchange. In addition, mice where the litter was not changed (obeseControl $n = 9$; raised by two different nursing mothers, leanControl $n = 4$; raised by one nursing mother) served as controls.

Mice were housed in individually ventilated polycarbonate cages (Techniplast Inc., VA, Italy) containing wood chip bedding (Mucedola, Italy). Standard chow (4RF21 standard diet for mice and rat reproduction, weaning and growth, Mucedola, Italy) and acidified water (pH range of 3 to 3.5) were accessible ad libitum. The environmental conditions of the facility were set to a temperature of 21 ± 2 °C, 40–70% humidity and 12:12-h light:dark cycle during the experiment.

Mice from the litter were sacrificed at 3 weeks of age and colon samples with content were immediately snap frozen and stored at − 80 °C. No significant differences in the weight of the offspring between the different groups were observed at the time point of sacrifice (data not shown).

DNA Extraction and Amplification of 16S rRNA Genes

Colon samples, including host tissue and digestive content, were treated using a tissue homogenizer (Precellys® PEQLAB GmbH, BY, Germany) at the speed of 5500 rpm for 30 s. The DNA was extracted by applying the PowerSoil® DNA Isolation Kit according to the manufacturer's protocol (MoBio, CA, USA). The DNA concentration was measured using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo Fisher Scientific Inc., MA, USA) according to the manufacturer's instructions. Buffer was used as a Blank extraction control to identify contaminating OTUs derived from the extraction kit.

For all PCRs, 50 ng of template DNA, 12.5 μL NEBNext® High-Fidelity 2X PCR Master Mix (New England Biolabs Inc., MA, USA), and 0.4 μL of the primer pair S-D-Bact-0008-a-S-16 S-D-Bact-0343-a-A-15 (10 μM) [18], which

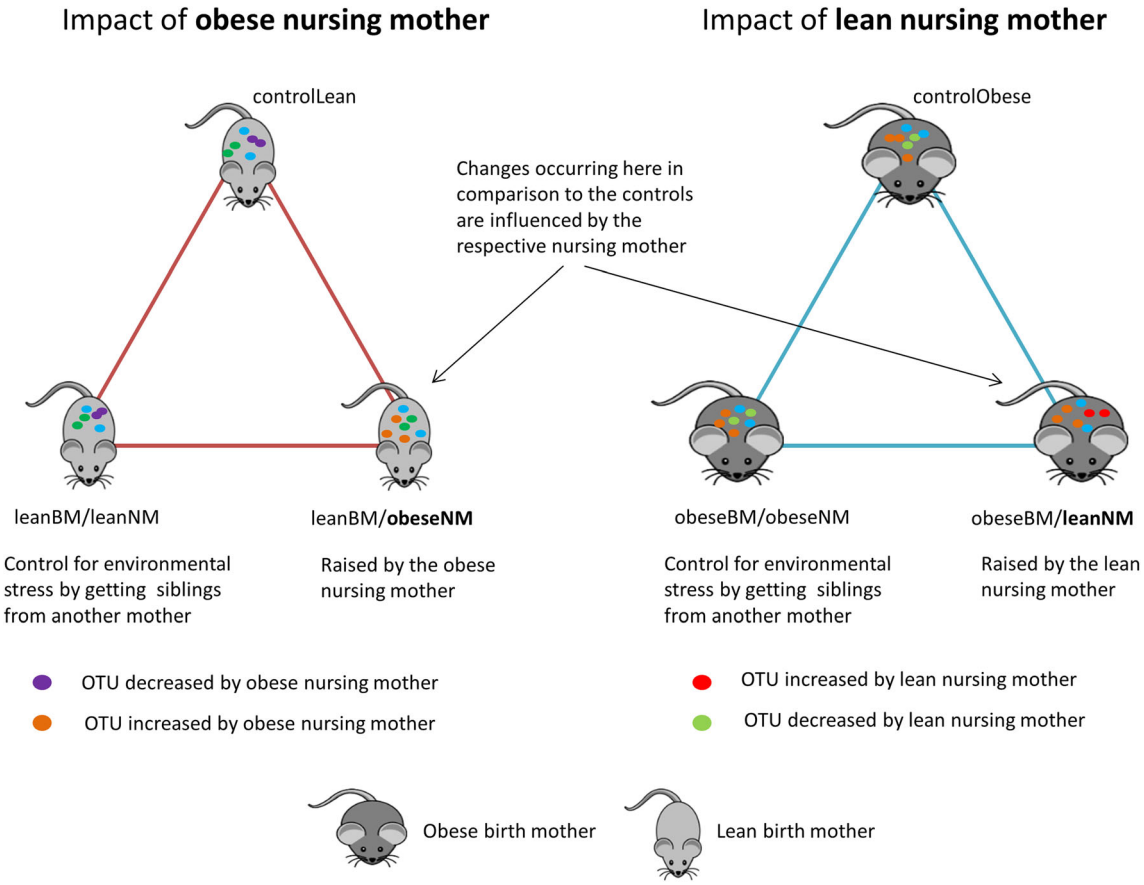

Fig. 1 Half of the litter was exchanged between genotypically lean and obese mouse mothers. Therefore, the study design included mice which were switched to a genetically different mother for nursing (obeseBM/leanNM $n = 11$; leanBM/obeseNM $n = 10$); mice which stayed with their birth mother, but got siblings from another birth mother (leanBM/leanNM $n = 9$, obeseBM/obeseNM $n = 13$) and controls where the litter was not changed (obeseControl $n = 9$, leanControl $n = 4$). To identify the impact of the respective nursing mother, the microbiota of the two controls was compared to the samples of mice which were switched to a different nursing mother

amplifies the V1/V2 region of the 16S rRNA gene, were used. The forward and reverse primers contained overhangs at their 5′ ends that were compatible with Nextera XT indices for multiplexing. The PCR was conducted as described in the Supplement.

Sequencing

The sequencing library preparation was conducted using 10 ng of template DNA, primers of the Nextera® XT Index Kit v2 Set A and Set C (Illumina, Inc., CA, USA), and the NEBNext® High-Fidelity 2X PCR Master Mix (New England Biolabs Inc., MA, USA). The indexing PCR was performed as described in the Supplement. The sequencing of the samples including blank extraction control and PCR negative controls was conducted on a MiSeq® System (Illumina, Inc., CA, USA) using the MiSeq® Reagent Kit v3 (600 cycle) for paired end sequencing according to the instructions in the "Preparing Libraries for Sequencing on the MiSeq®" protocol (Illumina, Inc., CA, USA). Three percent PhiX was used as a spike-in. The sequencing run was conducted according to the MiSeq® System User Guide (Illumina, Inc., CA, USA) using 13 pM of DNA. The obtained reads are available under the accession number SRP107967 of the Sequence Read Archive (SRA) of the NCBI.

Bioinformatics and Statistical Analysis

Demultiplexed raw data was processed using the open-source software package QIIME v. 1.9. (Boulder, CO, USA) (Python v. 2.7.6) [19]. Sequencing primers were identified and removed by the MiSeq® System software and the obtained reads were merged using FLASH v. 1.2.11 [20]. Contaminating reads of the phiX or mouse genome were removed with DeconSeq [21]. Quality (Phred score of 30) filtering and selection for fragments between 320- and 400-bp read length were conducted using QIIME and Biopieces [22], respectively. The filtered sequences were clustered at 97% identity by UCLUST (v. 1.2.22q) and taxonomically affiliated using the RDP classifier (release 2.11) [23] retrained with the Greengenes database (v. 13_5). Further statistical analysis was conducted with QIIME. Steps were parallelized

using GNU parallel [24]. Selected sequences were further analyzed with the Standard Nucleotide BLAST tool using the MegaBLAST program and the 16S ribosomal RNA sequences database [25, 26].

For statistical analysis and data visualization, the following packages of the open-source software R (v.3.1.1) were used [27]: vegan, gridExtra, gplots, ggplot2, reshape2, and plyr.

For evaluation of the α-diversity, the species richness was calculated as observed OTUs per sample and significant differences between treatments were determined by a Wilcoxon rank-sum test with the open-source software R (v.3.1.1). The β-diversity was calculated by unweighted UniFrac distance metric [28] using QIIME v. 1.9. Significant differences among the taxa were identified using unpaired *t* test statistics with Bonferroni correction using R (v.3.1.1) [27]. For the abundance values of the significantly changing taxa, log2 fold changes were calculated as

$$\log2\left(\frac{a+0.0001}{b+0.0001}\right) \quad (1)$$

where *a* and *b* are the average relative abundances of the taxa within the groups compared. To avoid a division by zero, pseudo-counts of 0.0001 were added to both abundance values.

For analysis of the impact of the nursing mother on OTU level, a serial group comparison, with pairwise Fisher's exact test and *p* value correction by Benjamini-Hochberg method using the modular R pipeline Rhea [29], was conducted.

The core microbiome was defined as OTUs that were shared among at least 50% of the samples [30]; Venn diagrams were created by an online tool provided by the University of Ghent [31].

Data Availability The sequence data was submitted to NCBI via the Sequence Read Archive (SRA) and is available under accession number SRP107967.

Results

Sequencing of the 56 samples resulted in a total of 5,873,538 reads, which were rarefied to 27,282 reads per sample (Table S1), and assigned to 864 OTUs at 97% sequence identity level. To exclude a potential bias introduced by contamination from the extraction kits, the presence of two OTUs with the highest abundance in the blank extraction control was analyzed in the samples. As these two OTUs were only found at a relatively low abundance in nine of the 56 samples, we concluded that the contamination effects in this study as a result of the presence of microbial residues in the DNA extraction kit were negligible. Sufficient sequencing depth was confirmed for the threshold of 27,282 reads through rarefaction curves which reached saturation for a level of 97% sequence identity (Fig. S1).

The effect of the nursing mother was analyzed by comparing colon samples from offspring exchanged between mothers (leanBM/obeseNM, obeseBM/leanNM) and the respective controls with the same birth mother (Fig. 1). Differences in the bacterial community structure between colon samples from the offspring obtained from leanBM/obeseNM samples and leanControl respectively leanBM/leanNM samples, as well as between obeseBM/leanNM samples and the obeseControl respectively obeseBM/obeseNM samples, indicated a strong influence of the nursing mother on the gut microbiome of the offspring.

Impact of the Nursing Mother on the α-Diversity on the Offspring's Gut Microbiome

The nursing mother had an impact on the OTU richness and evenness of the offspring's gut microbiome. Compared to the controls with the same type of birth mother (leanControl, leanBM/leanNM), the OTU richness in the leanBM/obeseNM samples was higher (average number of observed OTUs = 565) (Fig. 2a). The effect of the lean nursing mother showed the contrary effect, as the OTU richness was lower in obeseBM/leanNM samples (average number of observed OTUs = 464) compared to the controls with the same type of birth mother (obeseControl, obeseBM/obeseNM). The evenness of the offspring's gut microbial community was not affected by the change to an obese nursing mother, as the leanBM/obeseNM samples were in the same range as the controls (on average $J = 0.75$) (Fig. 2b). However, the shift of the offspring from an obese to a lean nursing mother (obeseBM/leanNM) lowered the evenness (on average $J = 0.68$) of the gut microbial community compared to the controls (obeseControl, obeseBM/obeseNM).

Impact of the Nursing Mother on the β-Diversity of the Offspring's Gut Microbiome

β-Diversity analysis showed a clear separation of samples according to the type of nursing mother (Fig. 3). The leanControl and obeseControl samples were clearly separated on the *x*-axis of the PCoA plot, explaining 20.8% of the difference in diversity. The obeseControl samples clustered with the obeseBM/obeseNM samples, while the leanBM/leanNM samples clustered marginally separate from the leanControl samples. The microbiome of the gut samples of the swapped offspring (obeseBM/leanNM, leanNM/obeseBM) clustered with the ones of their nursing mother, indicating close similarities with the gut microbiome of the respective nursing mother. This result was confirmed by a constrained analysis of principal coordinates (CCA), which showed 28.95% of the

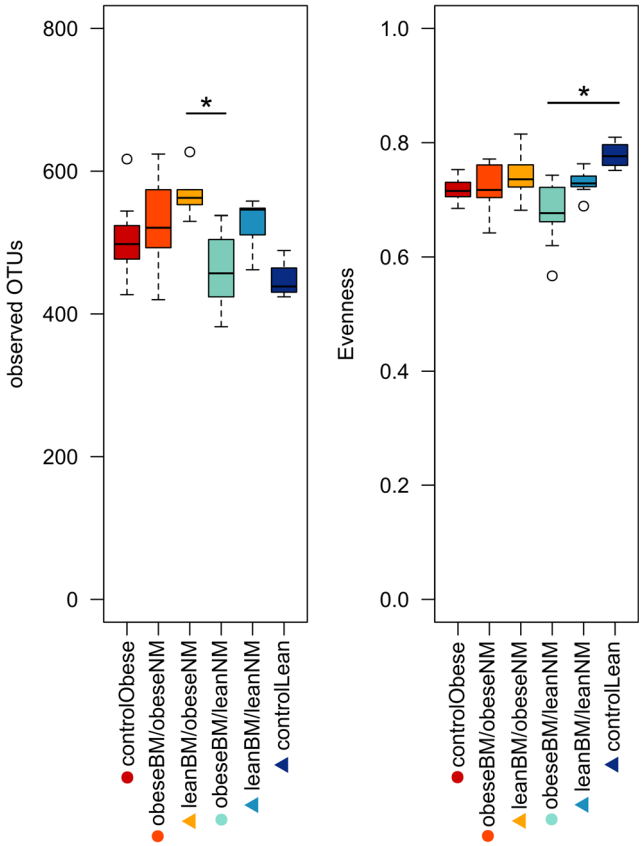

Fig. 2 α-Diversity measures of the gut microbiota. The boxplots are based on OTU table (subsampled to 27,282 reads per sample, 97% identity level). Depicted are the number of observed OTUs (**a**) and the evenness (**b**) of the six sample groups controlObese ($n = 9$), obeseBM/obeseNM ($n = 13$), leanBM/obeseNM ($n = 10$), obeseBM/leanNM ($n = 11$), leanBM/leanNM ($n = 9$), and controlLean ($n = 4$). Sample groups are additionally indicated by color (red/orange shade, obese nursing mother; blue shade, lean nursing mother) and shape (dot, obese birth mother; triangle, lean birth mother). An asterisk refers to statistically significant differences ($p < 0.05$). Significances were calculated by a Wilcoxon rank-sum test and were Bonferroni corrected

variation being explained by the type of nursing mother ($p = 0.001$).

Major Responders: Bacterial Families of the Offspring's Gut Microbiome Influenced by the Nursing Mother

To identify major responding families, significant differences in the abundance of bacterial families between groups were analyzed by Bonferroni-corrected pairwise *t* tests (significant = $p < 0.05$), of which the log2 fold changes were plotted as a heat map (Fig. 4).

To prove differences in bacterial community composition between the two genetically different types of mothers, control samples were compared. On family level, there was a significant difference in the abundance of 15 taxa between leanControl and obeseControl samples. While *Peptococcaceae*, *Veillonellaceae*, *Mycoplasmataceae*, *CW040 F16*, *Odoribacteraceae*, *Lactobacillaceae*, and OTUs which could not be further assigned than to the class *Clostridia* level were increased in abundance in the obeseControl samples, the families *Peptostreptococcaceae*, *Desulfovibrionaceae*, *Porphyromonadaceae*, *Anaeroplasmataceae*, *Turicibacteraceae*, *Clostridiaceae*, *Lachnospiraceae*, and OTUs which could not be further assigned than to the order *Clostridiales* level were higher abundant in the leanControl samples.

To control for the impact of stress on the gut microbiome of the offspring, induced by the exchange of siblings to a foreign mother, gut samples from the leanBM/leanBM and obeseBM/obeseNM samples were compared to the respective controls. The obeseControl samples showed a higher abundance of the family *Prevotellaceae*, while *Anaeroplasmataceae* were more abundant in the obeseBM/obeseNM samples. When the leanControl samples were compared to the leanBM/leanNM samples, differences were more pronounced as already indicated by the PCoA analysis and affected mainly *Desulfovibrionaceae*, *Coriobacteriaceae*, *Lachnospiraceae* (higher abundance in the leanControl samples), and *Peptococcaceae* (higher abundance in the leanBM/leanNM samples).

To analyze the influence of the nursing mother on the gut microbiota of the offspring, the leanBM/obeseNM and obeseBM/leanNM were compared to the respective controls. For obeseBM/leanNM, 12 and 7 taxa were observed which changed on the family level in comparison to the obeseControl respectively obeseBM/obeseNM samples (Fig. 4). Six of these taxa showed a significant change for both types of control. Mainly *CW040 F16*, *Coriobacteriaceae*, *Streptococcaceae*, *Mycoplasmataceae*, and not further classified *Bacteroidales* were decreased in the obeseBM/leanNM samples, while *Rikenellaceae* were increased by the swapping to a lean nursing mother. When analyzing the impact of the obese nursing mother in comparison to the respective controls, five taxa showed a significant change in abundance in comparison to the leanControl samples and six significant changes were found in comparison to the leanBM/leanNM samples. A group of not further classified *Firmicutes* were decreased compared to both types of controls, and were therefore considered as significantly influenced by the shift to an obese nursing mother.

For an in-depth analysis of major responders influenced by the nursing mother, a serial group comparison with pairwise Fisher's exact test and *p* value correction by Benjamini-Hochberg method was applied. Again, the leanBM/obeseNM samples were compared to the leanControl and the leanBM/leanNM samples for analysis of the effect of the obese nursing mother, and the obeseBM/leanNM to the obeseControl and obeseBM/obeseNM samples for analysis of the effect of the lean nursing mother. Representative

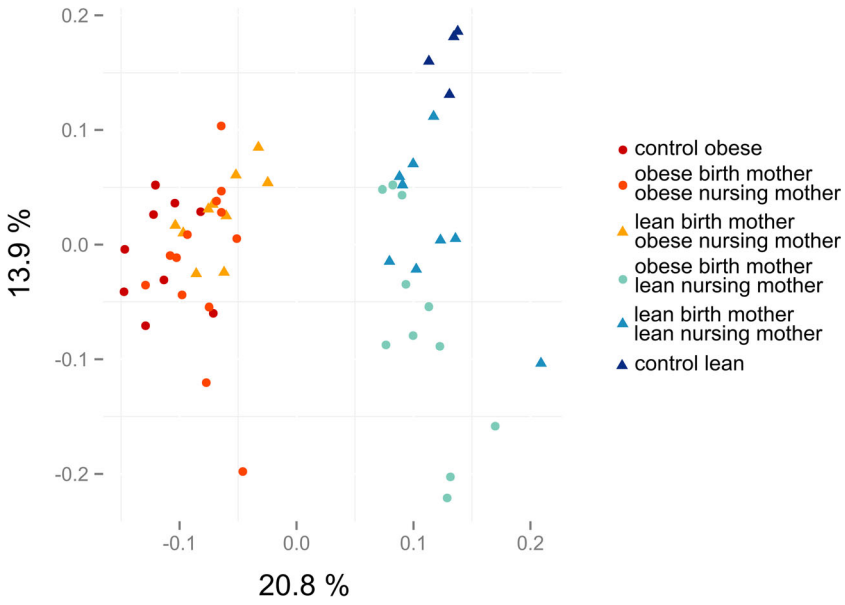

Fig. 3 Clustering of the gut microbiome (β-diversity) with respect to the type of nursing mother. The PCoA plot is based on unweighted UniFrac distances of the microbial communities of the colon. The six sample groups controlObese ($n = 9$), obeseBM/obeseNM ($n = 13$), obeseBM/leanNM ($n = 11$), leanNM/obeseBM ($n = 10$), leanBM/leanNM ($n = 9$), and controlLean ($n = 4$) are distinguished by color (red/orange shade, obese nursing mother; blue shade, lean nursing mother) and shape (dot, obese birth mother; triangle, lean birth mother)

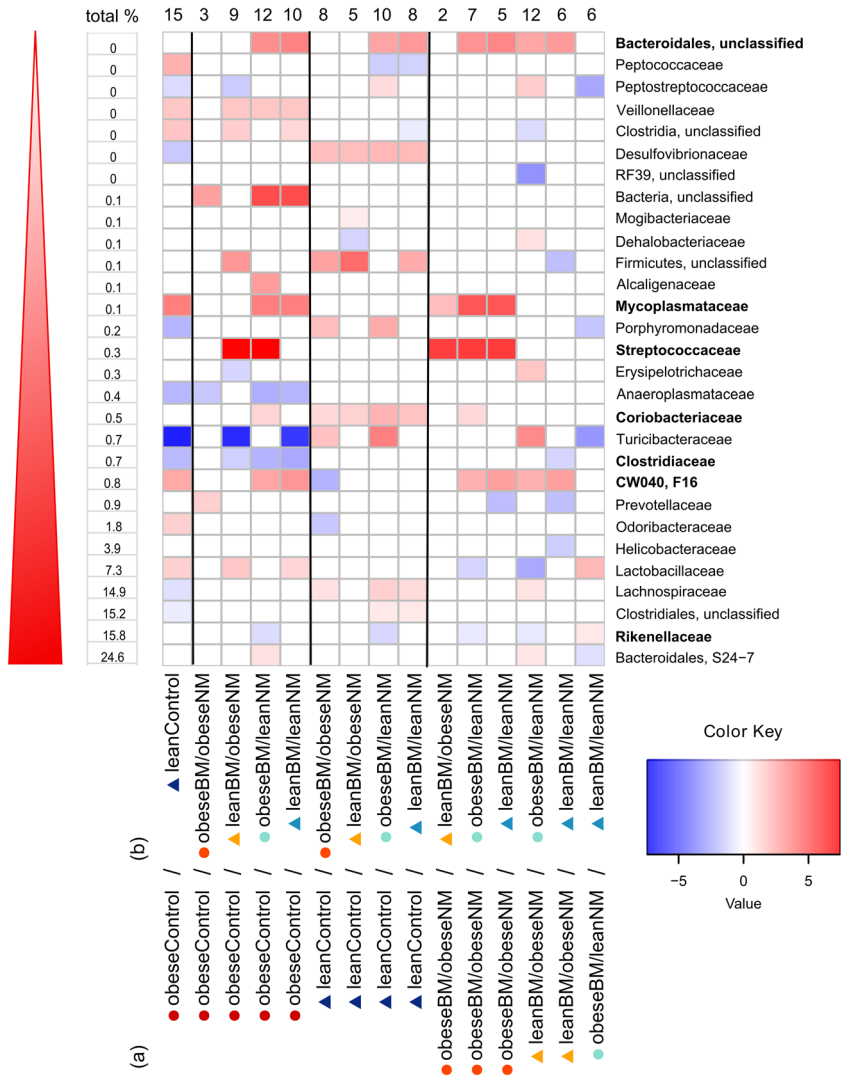

Fig. 4 Heat map of the log2 fold change of significant changes among the groups based on the relative abundance of the bacterial families shown. The log2 fold changes were calculated as $\log2\left(\frac{a+0.0001}{b+0.0001}\right)$. White color means the change was not significant. On the bottom, the groups compared are stated. The column to the left shows the relative abundance of the bacterial families described to the right. On top, the sum of observed significant changes for the groups compared is depicted. Sample groups are additionally indicated by color (red/orange shade, obese nursing mother; blue shade, lean nursing mother) and shape (dot, obese birth mother; triangle, lean birth mother). Significantly changing families influenced by the nursing mother are bold

sequences of the identified OTUs were annotated using the 16S ribosomal RNA sequences database of BLASTn.

Overall, the obese nursing mother had an influence on five OTUs and the lean nursing mother impacted 13 OTUs. The analysis confirmed the strong negative influence of the obese nursing mother on *Firmicutes* when the offspring was shifted from a lean birth mother, as relative abundance of OTUs annotated as *Roseburia intestinalis* (OTU 343630) and *Clostridium bolteae* were reduced in relative abundance. Furthermore, also OTUs assigned to *Bacteroidetes* including *Muribaculum intestinale* (OTU 276629) and *Alistipes senegalensis* (NCUR OTU885) were decreased in gut samples of the offspring of leanBM/obeseNM settings. This negative effect for *Bacteroidetes* was balanced out on the phylum level by *Butyricimonas faecihominis*, which was increased by shifting offspring from a lean birth to an obese nursing mother (Table S2 and S3).

Shifting the offspring from an obese to a lean nursing mother increased OTUs that could be annotated as *Alistipes senegalensis* (NCUR OTU885, OTU 336214) and *Alistipes putredini*. The related OTUs accounted for 17.05% of the total reads within the obeseBM/leanNM samples, therefore having a high impact on the overall abundance of the family of *Rikenellaceae*, which has been described above. Further, OTUs that were assigned to *Lactobacillus murinus*, *Anaeromassilibacillus senegalensis*, *Prevotella shahii*, and *Odoribacter splanchnicus* increased when shifting the offspring from an obese to a lean nursing mother. In contrast, OTUs assigned to *Muribaculum intestinale* (OTU 276509), *Gabonia massiliensis*, *Alistipes senegalensis* (NCUR OTU287), and *Butyricimonas faecihominis*, all members of the *Bacteroidetes* phylum, were reduced under these settings. The same was observed for the members of the *Firmicutes*, *Roseburia intestinalis* (OTU 275580) and *Eisenbergiella massiliensis* (Table S2 and S3).

Interestingly, two OTUs, OTU999 (*Butyricimonas faecihominis*) and NCUR OTU885 (*Alistipes senegalensis*), were influenced by both types of nursing mothers and showed an inverse behavior for the impact of the lean and the obese nursing mother, respectively.

Impact of the Nursing Mother on the Core OTUs of the Offspring's Gut Microbiome

The percentage of shared core OTUs between both controls and the samples of swapped offspring was considered a measure for the impact of the respective nursing mother (Fig. 5). Gut samples from the offspring derived from a lean birth mother had 453 OTUs shared, independent from the nursing mother. Depending on the nursing mother, in addition, 182 OTUs (obese nursing mother), respectively, 68 OTUs (lean nursing mother), were observed. Gut samples from the offspring derived from an obese birth mother shared 382 OTUs independent from the nursing mother. In addition for the obese nursing mother, 187 unique OTUs were found. Interestingly, these both in absolute and in relative numbers are higher in comparison to the lean birth and nursing mothers. In contrast, in the gut microbiome samples of the offspring that were shifted from an obese nursing mother to a lean nursing mother, only 93 OTUs in addition to the core could be detected. This is less than observed for the opposite shift from a lean birth mother to an obese nursing mother.

Discussion

The Role of the Nursing Mother in Shaping the Gut Microbiome of the Offspring

In this study, we investigated the influence of the nursing mother on the composition of the offspring's gut microbiome, using a cross-fostering experiment with genetically predisposed lean and obese mice and compared colon samples from offspring exchanged between mothers (leanBM/

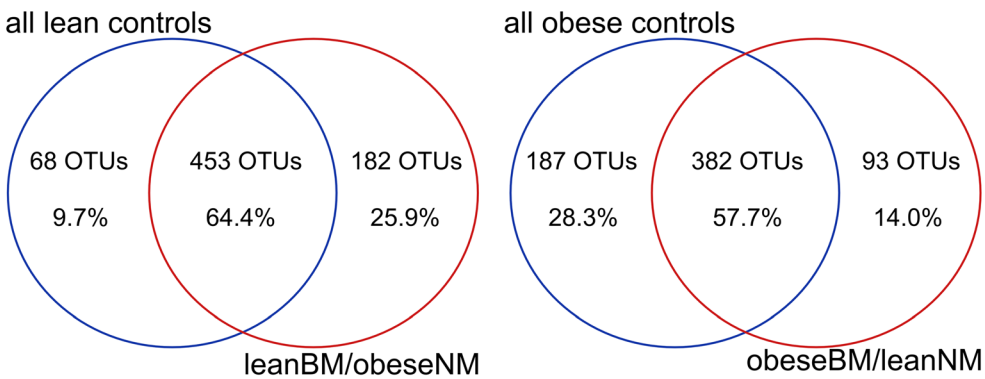

Fig. 5 The Venn diagrams show the percentage of OTUs present in 50% of the samples shared between the lean controls (controlLean, leanBM/leanNM) and the leanBM/obeseNM samples (left) and the obese controls (controlObese, obeseBM/leanNM) and the obeseBM/leanNM samples (right). The difference of the percentage of core OTUs shared is a measure for the impact of the nursing mother

obeseNM, obeseBM/leanNM) and the respective controls with the same birth mother to assess the changes in the gut microbiome (Fig. 1). The gut microbial composition of the offspring serving as controls (controlObese, controlLean) differed significantly, probably due to selection of a specific microbiome within the certain mouse strain over several generations by its genetics and behavior (food amount and choice), and therefore created a suitable frame for analyses of changes induced by the nursing mother. Still, caution has to be exercised, as the number of nursing mothers influencing the control samples was low. However, the addition of the second type of controls (leanBM/leanNM, obeseBM/obeseNM), with four different nursing mothers each, contributes to the robustness of the analysis.

Both the α- and β-diversity, of the gut microbiome, were influenced by the nursing mother. Despite the fact that obesity was shown to have a negative effect on the microbial diversity of the gut [32, 33], here we observed an increase in OTU richness shifting the offspring from a lean birth mother to an obese nursing mother, while a shifting from an obese birth mother to a lean nursing mother led to a decrease in OTU richness. This might be because in contrast to previous studies, obesity was not induced by a high-fat diet, but by a genetic predisposition and the murine litter did not differ in their weight irrespective of the genotype or the type of nursing mother. This might also explain why an increase of the *Firmicutes* to *Bacteroidetes* ratio, which is a common finding in obesity studies [34], could not be seen in our study. However, like in many other studies [34, 35], *Bacteroides* and *Firmicutes* were the most abundant phyla in the microbiome of the murine gut. From the 20 main genera described for the murine gut microbiome [36], 14 genera were also found in the present study. The lack of *Faecalibacterium*, *Anaerotruncus*, *Enterococcus*, *Pseudoflavonifractor*, *Butyrivibrio*, and *Blautia* might be a result of the differing workflow for 16S rRNA gene analysis compared to metagenome sequencing.

The pronounced effect of the nursing mother we observed is in accordance with a recently published cross-fostering study [37]. In contrast to Daft et al., this study used colon parts including tissue and content instead of fecal pellets in order to also cover bacteria adhering to the gut wall. Moreover in Daft et al., cross-fostering was conducted using a diabetic mouse line (NOD) and a non-diabetic mouse line (NOR) while our mouse model focused on the characteristic of obesity. The authors identified *Prevotella*, *Parabacteroides*, *Sutterella*, *Lysobacter*, *Anaeroplasma*, *Odoribacter*, *Bacteroides*, *Prevotella*, *Clostridium*, *Stenotrophomonas*, and *Akkermansia* as major responders to the nursing mother. Interestingly, despite the different settings in our study for three genera, we could confirm this pronounced effect of the nursing mother, namely for *Odoribacter*, *Prevotella*, and *Clostridium*. This is of high interest, as several studies have indicated health-beneficial properties of these genera [38–40].

Driving Factors for the Gut Microbiome Development of the Offspring

The difference in the offspring's gut microbial composition could have resulted from a differing gut microbiome of the nursing mothers. Previous studies indicated that the genetics of a host affects its microbiome [6, 32]. Subsequently, the genotype of the nursing mother could have shaped the microbiome before it was transferred to the offspring, e.g., by direct contact with the feces. This could for example explain the impact of the obese nursing mother for OTUs assigned to *Clostridium bolteae*, as *Clostridiaceae* have been linked to genetic traits of the host [41]. Also, the OTUs assigned to *Streptococcaceae* in our study which were phylogenetically related to the genus *Lactococcus* could be correlated with the differing genotypes, as a quantitative trait locus was associated with body weight in former studies [42]. In future studies, a higher taxonomic resolution could be obtained by using recently introduced amplicon sequence variant methods instead of OTU assignment [43]. Another important impact factor shaping the gut microbiome at early stages of development is breast milk. Thus, to a certain extent, the difference in the impact of the lean and the obese nursing mother might be explained by a different quality in their breast milk. Breast milk has an essential impact on the development of the gut microbiome and contains predominantly *Staphylococci*, *Streptococci*, lactic acid bacteria, and *Bifidobacteria* [12, 44, 45]. Offspring were sampled at an age of 3 weeks to cover the longest possible period of exposure to breast milk. Despite this coinciding with weaning and the start of intake of solid diet, breast milk-associated taxa show significant changes. The main genera reported to be transferred by breast-feeding are *Lactobacillus*, *Staphylococcus*, *Enterococcus*, and *Bifidobacterium* [12, 44]. This could explain the impact of the nursing mother on the family *Streptococcaceae* and the OTU belonging to *Lactobacillus murinus*. To verify this in future studies, it would be interesting to analyze the microbiome of the maternal milk, too. In addition, an indirect effect of the genera influenced by the breast milk could occur via lactic acid producing strains cross feeding butyrate producers like *B. faecihominis* and *Roseburia intestinalis* [45–47]. Further difference related to the nutritional composition of the milk could have an additional effect on the gut microbiome development of the offspring, as different substrates select for different bacteria. Human milk oligosaccharides for example have been shown to promote the growth of bifidobacteria and two species of the *Bacteroides* [46], while a high amount of fat correlated with an increase of *Clostridiaceae* and a decrease of *Bacteriodaceae*, *Prevotellaceae*, and

Rikenellaceae [47]. In accordance, the family *Rikenellaceae* correlated with the lean nursing mother in our experiment, implicating a relative decrease in the controls with obese nursing mothers, maybe because of a higher amount of fat in the maternal milk of obese nursing mothers. Furthermore, the maternal milk of mammals contains bioactive molecules, including immunocompetent cells, immunoglobulins, and antimicrobial peptides, which could select for different microbiota. As the family *F16* of the order *CW040*, from the candidate phylum *Saccharibacteria* were found to correlate with low IgA levels [48], a higher amount of these in the milk provided by the lean nursing mothers could explain the decrease found within mice raised by a lean nursing mother.

Exchanging Siblings Between Different Birth and Nursing Mothers Is Inducing Stress for the Offspring

Finally, our study highlights also the impact of stress on the gut microbiome and vice versa the importance of the gut microbiome to mitigate stress response. In our study, we induced stress by exchange of siblings to a foreign mother. By comparing lean and obese controls (where no exchange of siblings occurred) to leanBM/leanBM and obeseBM/obeseNM, we could show that the gut microbiome of lean mice was more susceptible to perturbation, although responding OTUs were also identified for the obese settings. One reason for this could be the decrease in OTU richness for the lean cases, as low microbial diversity has been associated with instability, reduced resilience, and less functional redundancy [49]. Still the stress effect was not large enough to exceed the impact of the nursing mother. Just the significant difference in abundance within the family *Coriobacteriaceae* could be considered to be caused by a stress effect, rather than being caused by the impact of the lean nursing mother, as there is an increase of this family within the controlLean samples.

Overall, our study demonstrates the importance of the nursing mother for modulating the gut microbiome of the offspring after birth. To investigate, if the described effects can be considered as important for the overall development of the mice and also trigger the health status of the animals at later stages of development, further studies must prove if the changes in the gut microbiome induced by the nursing mother at early development of the mice just reflect the moment of sampling or can be also followed at later stages of the development. It also remains to be clarified if the microbiome acquired from the respective nursing mother has a long-term effect on the body-weight status of the mice. Furthermore, also functional implications of shifts in the gut microbiome of the offspring induced by the nursing mother remain to be considered.

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Authors' Contributions ZP, BS, VM, AS, and MS designed the study. NST performed lab work and sequencing. GV and MK established the bioinformatics pipeline based on the open-source software package QIIME and helped with analysis. NST and AS generated and analyzed the sequence data. NST, MS, SP, BF, and AS conceptualized and wrote the manuscript. All authors contributed to revisions and approved the final manuscript.

Compliance with Ethical Standards

Ethics Approval and Consent to Participate All applicable national guidelines for the care and use of animals, which are all in compliance with the EU regulations, were followed.

Conflict of Interest The authors declare that they have no conflict of interest.

Abbreviations OTU, operational taxonomic unit; leanNM, lean nursing mother; obeseNM, obese nursing mother; leanBM, lean birth mother; obeseBM, obese birth mother

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